Increased Oxidative Damage Is Correlated to Altered Mitochondrial Function in Heterozygous Manganese Superoxide Dismutase Knockout Mice*

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This study characterizes mitochondria isolated from livers of Sod2<sup>−/−</sup> and Sod2<sup>+/+</sup> mice. A 50% decrease in manganese superoxide dismutase (MnSOD) activity was observed in mitochondria isolated from Sod2<sup>−/−</sup> mice compared with Sod2<sup>+/+</sup> mice, with no change in the activities of either glutathione peroxidase or copper/zinc superoxide dismutase. However, the level of total glutathione was 30% less in liver mitochondria of the Sod2<sup>−/−</sup> mice. The reduction in MnSOD activity in Sod2<sup>−/−</sup> mice was correlated to an increase in oxidative damage to mitochondria: decreased activities of the Fe-S proteins (aconitase and NADH oxidoreductase), increased carboxyl groups in proteins, and increased levels of 8-hydroxydeoxyguanosine in mitochondrial DNA. In contrast, there were no significant changes in oxidative damage in the cytosolic proteins or nuclear DNA. The increase in oxidative damage in mitochondria was correlated to altered mitochondrial function. A significant decrease in the respiratory control ratio was observed in mitochondria isolated from Sod2<sup>−/−</sup> mice compared with Sod2<sup>+/+</sup> mice for substrates metabolized by complexes I, II, and III. In addition, mitochondria isolated from Sod2<sup>−/−</sup> mice showed an increased rate of induction of the permeability transition. Therefore, this study provides direct evidence correlating reduced MnSOD activity in vivo to increased oxidative damage in mitochondria and alterations in mitochondrial function.

Under normal physiological conditions, metabolism of oxygen by aerobic organisms generates a wide variety of potentially deleterious reactive oxygen species. These reactive oxygen species initiate a large number of oxidative reactions in cellular systems that lead to the oxidation of macromolecules, e.g. DNA, proteins and lipids (1). Over the past decade, numerous investigators have argued that oxidative damage may contribute to a variety of chronic diseases (including emphysema, cardiovascular disease, cancer, and neurodegenerative diseases such as Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis) as well as aging (2). Although it is attractive to speculate that the accumulation of oxidative damage may be involved in the decline of physiological function and increased disease pathogenesis, there is no direct evidence for this concept.

Mitochondria are extremely susceptible to oxidative damage because 2–4% of the oxygen consumed by mitochondria is converted to superoxide anions by the electron transport chain (3) and because mitochondria have limited protection from oxidative stress (4). Therefore, mitochondria would be predicted to be one of the cellular components especially vulnerable to oxidative damage. Over the past 20 years, investigators have studied the effect of oxidative damage on mitochondrial function by subjecting isolated mitochondria to oxidative stress in vitro. Many functional properties of mitochondria are substantially impaired after exposure in vitro to a variety of reactive oxygen species. These include the inhibition of the complexes in the respiratory chain, especially complex I and ATP synthetase (5), decreased adenine nucleotide content, inhibition of the adenine nucleotide translocase (6), increased lipid peroxidation (7), and mitochondrial swelling (8). However, it is not clear that in vitro studies with isolated mitochondria are an accurate indication of what occurs in vivo. In other words, does the accumulation of oxidative damage under normal physiological conditions in vivo lead to mitochondrial dysfunction?

Transgenic or mutant animals with alterations in the antioxidant defense system provide investigators with a model system that can be used to test the effect of oxidative damage on cellular function in vivo. For example, two MnSOD<sup>−/−</sup> knockout mouse models have been produced by the disruption of different sections of the Sod2 gene (9, 10). In both knockout models, the homozygous (Sod2<sup>−/−</sup>) mutants exhibited no detectable MnSOD activity in any of the tissues studied. The Sod2<sup>−/−</sup> phenotype was lethal in both knockout models. The Sod2<sup>−/−</sup> mutants (Sod2<sup>−/−</sup>/Cje) produced by Li et al. (9) were smaller and paler and exhibited a hypotonic and hypothermic state compared with wild type mice and the heterozygous mice (Sod2<sup>−/+</sup>). In addition to their overall gross appearance, the homozygous null mutants appeared to fatigue more rapidly after any type of exertion; however, their behavior was otherwise normal. After 4–5 days, 37% of the Sod2<sup>−/−</sup> mice died, with nearly all the animals dead by day 10. At death, the Sod2<sup>−/−</sup> mice had enlarged hearts with a dilated left ventricular cavity and reduced left ventricular wall thickness, which...
suggested cardiomyopathy. Furthermore, the liver was increased in size and showed marked steatosis, and lipid deposits were observed in the skeletal muscle. Although these Sod2−/− mice exhibited extreme damage to the myocardium, these mutants did not show any motor disturbances, central nervous system injury, ultrastructural evidence of mitochondrial injury, or any gross mitochondrial DNA (mtDNA) rearrangements. The Sod2−/− mutants (Sod2−/−<tm1<BCM> produced by Lebovitz et al. (11) could not be distinguished from their littermate controls at birth. However, their diminished growth rate became apparent between day 2 and day 7 and continued until death, which usually occurred by 18 days after birth. Although no skeletal abnormalities were observed, they did exhibit reductions in both adipose tissue and skeletal muscle. Histological analysis revealed hypocellular bone marrow with marked anemia and atypical patterns of glycogen deposition in hepatocytes. Furthermore, analysis of the brain and spinal cord by electron microscopy revealed that these Sod2−/− mice demonstrated degenerative injury to large central nervous system neurons, particularly in the basal ganglia and brainstem, characterized by extensive mitochondrial damage, loss of polysomes and clearing of the cytoplasm. In addition, progressive motor disturbances were characterized by weakness, rapid fatigue, and circling behavior. Only 10% of these Sod2−/− mice died of cardiomyopathy. Electron micrographs of the myocardium revealed swollen and damaged mitochondria, which were not apparent in the mouse model developed by Li et al. (9).

The phenotypic differences between the two types of Sod2−/− mice have been shown to be attributable to differences in the strain background on which the mutant genes are expressed. For example, Huang et al. (12) showed that the phenotypes of the two mutations can be made to resemble one another by changing the genetic backgrounds of the mice.

Although the Sod2−/− mice have been characterized, there is relatively little information on the Sod2+/+ animals. The Sod2−/− mice produced by both Li et al. (9) and Lebovitz et al. (11) appear relatively normal, even though MnSOD activity is reduced in all tissues studied. However, the former animals have been found to develop larger cerebral infarcts when subjected to middle cerebral artery ischemia and reperfusion (13). In our present study, we show that the Sod2−/− mice, which have reduced MnSOD activity, exhibit increased oxidative damage to mitochondrial protein and DNA but no detectable changes in oxidative damage in the cytoplasmic/nuclear compartments of the cell. The increased oxidative damage in the mitochondria is correlated with altered mitochondrial function, including a reduction in mitochondrial respiration and an increase in the rate of induction of the permeability transition.

MATERIALS AND METHODS

Animals

The Sod2−/− mice, designated Sod2−/−<tm1>Cje, were originally produced in the CD1 strain of mice (9); however, the mice described in this study have been backcrossed to C57Bl/6 mice for 13 generations (B6−Sod2−/−<Cje>). The genotype of the Sod2−/− mice was determined by polymerase chain reaction analysis of DNA obtained from a 1-cm portion of tail (9). Female mice were fed ad libitum and maintained under barrier conditions on a 12-h dark/light cycle. At 2–4 months of age, the mice were sacrificed by cervical dislocation, and the livers were immediately excised and placed on ice. All procedures followed the guidelines approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center, San Antonio, TX.

Mitochondria were isolated according to the method of Johnson and Lardy (14). The mitochondrial pellet was resuspended in a buffer containing 250 mM mannitol, 75 mM sucrose, 500 μM EGTA, and 3 mM HEPES (pH 7.4), and this suspension was used to measure mitochondrial respiration, membrane potential, and the permeability transition.

Enzyme Assays

Mitochondrial Antioxidant Enzymes—Superoxide dismutase activity was measured using polycrylamide gels as described by Beauchamp and Fridovich (16). After staining, the gel was photographed, and the photograph was scanned and quantitated using ImageQuant software (Sunnyvale, CA). Glutathione peroxidase (GPX) activity was measured by the coupled reduction of cumene hydroperoxide and the oxidation of NADPH by glutathione reductase as described by Tappel (17). One unit of activity is defined as 1 μmol of NADPH oxidized/min.

Aconitase—The activity of aconitase in the mitochondrial and cytosolic fractions was measured by the conversion of citrate to α-ketoglutarate coupled to the reduction of NADP as described by Kennedy et al. (18) and Haasladen and Fridovich (15). Aconitase was reactivated by the addition of 0.5 mM diethiothreitol and 40 mM ferrous ammonium sulfate to the extract as described by Haasladen and Fridovich (15). One milliunit of aconitase activity is defined as the amount of enzyme necessary to catalyze the formation of 1 nmol of isocitrate/min at 37 °C.

Fumarase—Fumarase activity was measured by the conversion of fumarase to l-malate as described by Hill and Bradshaw (19). Changes in fumarate concentration were monitored at 250 nm for 5 min at 37 °C. One unit of activity is defined as the production of 1 μmol of fumarate/min.

NADH:CoQ Reductase and NADH:Ferricyanide Reductase—NADH:CoQ reductase activity was determined by measuring the reduction of coenzyme Q, an ubiquinone analog, by NADH as described by Estornell et al. (20). One unit of activity is defined as the oxidation of 1 μmol of NADH/min. NADH:ferricyanide reductase activity was determined by measuring the reduction of ferricyanide by NADH as described by Ragan (21). One unit of activity is defined as the oxidation of 0.5 μmol of NADH/min.

Glutamine Synthetase—Glutamine synthetase activity in the cytosol of the liver was measured by the conversion of l-glutamate to γ-glutamyl hydroxamate as described by Mariana and Relimpio (22). One unit of activity is defined as the formation of 1 μmol of γ-glutamyl hydroxamate/min.

Total Glutathione—The concentration of total glutathione in isolated mitochondria was determined by the rate of formation of 5-thio-2-nitrobenzoic acid as described by Anderson (23). The mitochondria were incubated with 25 mM succinate to maintain respiratory function for 10 min at 37 °C before the addition of perchloric acid to deproteinize the samples.

Mitochondrial Respiration

Oxygen consumption was measured using a Gilson oxygraph equipped with a Clark electrode as described by Estabrook (24). Reactions were conducted at 30 °C in a 2-ml chamber containing 1 mg of mitochondrial protein in a respiration buffer (250 mM sucrose, 10 mM KH2PO4, 1 mM EGTA, and 10 mM Tris-HCl, pH 7.4) containing the following three substrates: glutamate (1.7 mM) and malate (1.7 mM), succinate (2.5 mM) and rotenone (5 μM), and duroquinol (500 μM). State 3 respiration rates were determined by the addition ADP (200 nM final concentration), and state 4 respiration was measured in the presence of adequate substrate but without added ADP. The respiratory control ratio (RRC) was calculated as the ratio of state 3 to state 4 respiration rates.

RESULTS AND DISCUSSION

Antioxidant Status of Mitochondria—There were no discernible differences in mitochondrial yield between Sod2−/− and Sod2−/− mice: 6.2 ± 0.8 mg of mitochondrial protein/liver for the Sod2−/− mice compared with 6.3 ± 0.8 mg of mitochondrial protein/liver for the Sod2−/− mice (data expressed as mean ± S.E. for six animals). The antioxidant defense system in isolated liver mitochondria from Sod2−/− and Sod2−/− mice was characterized by measuring the activities of the two major antioxidant enzymes, MnSOD and GPX, which are localized in the mitochondrial matrix. Fig. 1 shows that MnSOD activity was reduced approximately 50% in liver mitochondria isolated...
from Sod2+/− compared with Sod2+/+ mice. In contrast, the activity of GPX remained unchanged (Fig. 1) and, therefore, did not compensate for the reduced activity of MnSOD in the liver mitochondria of the Sod2−/− mice. We also measured the CuZnSOD and catalase activities in these mitochondrial preparations. CuZnSOD has been reported to be localized not only in the cytosol, but also in the intermembrane space of the mitochondria (25). The activity of CuZnSOD was the same for the Sod2−/− and Sod2+/+ mice (Fig. 1). These data are in agreement with the previous report by Li et al. (9), in which no difference in CuZnSOD activity was observed in whole liver homogenates in these mice. There was no detectable catalase activity in the mitochondrial extracts from either the Sod2−/− or Sod2+/+ mice. This was not unexpected because catalase has been reported to be localized in the cytosol, specifically the peroxisomes (26).

We also measured total glutathione levels in isolated mitochondria because glutathione can act directly as an antioxidant or as a substrate for GPX. Only 10–15% of the total cellular glutathione is found inside the mitochondrial matrix (27); therefore, it was only possible to measure total glutathione levels in the mitochondrial extracts. The data in Fig. 1 show that total glutathione levels were decreased approximately 30% in mitochondria from the Sod2−/− mice compared with mitochondria from Sod2+/+ mice. This decrease could be of significance because the mitochondrial glutathione pool appears to be extremely important physiologically in protecting cells from oxidative stress. For example, Garcia-Ruiz et al. (28) recently showed that a decrease in the mitochondrial pool of glutathione (with the cytosolic glutathione pool intact) rendered cells more vulnerable to the endogenous oxidative stress induced by antimycin A compared with the situation when only the cytosolic glutathione pool was decreased.

Oxidative Damage—Because MnSOD is a key component of the antioxidant defense system, we determined if the reduction in MnSOD activity in the mitochondria of the Sod2−/− mice was correlated to a change in oxidative damage. We measured the activity of aconitase because it has been reported to be a sensitive measure of tissue/cellular levels of superoxide anions (29). Aconitase is an iron-sulfur protein that is inactivated by superoxide anions (29), which oxidizes the [4Fe-4S] cluster and leads to a loss of an iron ion (30). Aconitase inactivated by superoxide anions can be reactivated by the addition of a reducing agent and iron (15). Fig. 2 shows that the mitochondrial aconitase activity is reduced 30% in mitochondrial extracts from the livers of Sod2−/− mice compared with Sod2+/+ mice. However, the Western blot in Fig. 2 shows that levels of aconitase protein in the mitochondrial extracts from the Sod2−/− and Sod2+/+ were similar; therefore, the decrease in aconitase activity was not due to reduced levels of the protein. The data in Fig. 2 also show that reactivation of aconitase by the addition of iron and dithiothreitol resulted in an increase in the mitochondrial aconitase activity in the Sod2+/− mice to a level equal to that measured in the Sod2+/+ mice. Therefore, the decrease in aconitase activity in the liver mitochondria of the Sod2−/− mice appears to be the result of inactivation by superoxide anions. In other words, superoxide anion levels in the mitochondria of the Sod2−/− mice appear to be higher than the levels in the mitochondria of the Sod2+/+ mice. Previously, Li et al. (9) reported no difference in the total aconitase activity in the liver homogenates from Sod2+/− and Sod2+/+ mice; however, it is possible that they failed to detect changes in aconitase activity because they measured total rather than mitochondrial aconitase activity. There are approximately equal activities of the mitochondrial and cytosolic aconitase in liver (31).

We also measured the activity of NADH oxidoreductase (complex 1) in mitochondrial extracts because the [Fe-S] clusters in the complexes are sensitive to oxidative inactivation (21, 32). NADH oxidoreductase activity was measured by two assays that employ different terminal electron acceptors: ferricyanide and coenzyme Q (Table I). Coenzyme Q, a ubiquinone analog, accepts electrons from the [Fe-S] clusters of complex I; these clusters are sensitive to oxidative inactivation. The activity of NADH oxidoreductase using coenzyme Q as the substrate was significantly lower (30%) in the mitochondria isolated from the livers of the Sod2−/− mice compared with the Sod2+/+ mice (Table I). To determine if the loss in activity was due to inactivation of the [Fe-S] clusters, we also measured the NADH oxidoreductase activity using ferricyanide as the substrate. Ferricyanide is an artificial electron acceptor that accepts electrons directly from the reduced flavin mononucleotide and by-passes the [Fe-S] clusters of complex I (33). Using ferricyanide as the terminal electron acceptor, no difference in the activities of NADH oxidoreductase was observed for mitochondria isolated from the livers of Sod2−/− and Sod2+/+ mice (Table I). Therefore, the decrease in NADH oxidoreductase activity appears to be due to oxidative inactivation of the [Fe-S] clusters.

We also measured the activities of fumarase in the mitochondrial extracts as a control because fumarase is not iron-dependent (34) and it is insensitive to oxidative inactivation (35). The data in Table I show that fumarase activity was the same in the mitochondrial preparations from the Sod2−/− and Sod2+/+ mice even though aconitase and NADH oxidoreductase activities were significantly reduced in the Sod2−/− mice. We also measured the activities of aconitase and glutamine synthetase in the cytosolic extracts isolated from the livers of the Sod2−/− and Sod2+/+ mice. The mitochondrial and cytosolic aconitase are encoded by two different genes; however, the [4Fe-4S] structure is conserved, and cytosolic aconitase is sensitive to oxidative inactivation by superoxide anions (36). Glutamine synthetase is a cytosolic enzyme that has been shown to be sensitive to oxidative inactivation (37, 38) because a single histidine residue has been oxidized producing a carbonyl group.
Cytosolic enzymes and Methods. The values represent the mean ± S.E. of eight animals. *, p < 0.05 by paired Student’s t test. B shows the autoradiograph of a Western blot using a polyclonal antibody developed against aconitase. Mitochondrial proteins were separated electrophoretically under reducing conditions by SDS-polyacrylamide gel electrophoresis, and the blot was developed using a secondary antibody coupled to the horseradish peroxidase ECL system (Amersham, Les Ulis, France) as described by Heydari et al. (48).

![FIG. 2](image1)

**A.** Aconitase activities and protein levels in isolated mitochondria. A shows aconitase activities in liver mitochondria from Sod2<sup>+/+</sup> (open bars) and Sod2<sup>−/−</sup> (shaded bars) mice before and after reactivation by the addition of dithiothreitol and iron as described under “Materials and Methods.” The values represent the mean ± S.E. of eight animals. *, p < 0.05 by paired Student’s t test. B shows the autoradiograph of a Western blot using a polyclonal antibody developed against aconitase. Mitochondrial proteins were separated electrophoretically under reducing conditions by SDS-polyacrylamide gel electrophoresis, and the blot was developed using a secondary antibody coupled to the horseradish peroxidase ECL system (Amersham, Les Ulis, France) as described by Heydari et al. (48).

![FIG. 3](image2)

**B.** Oxidatively modified proteins containing carbonyl groups in mitochondria and cytosol. Mitochondrial and cytosolic extracts from livers of Sod2<sup>+/+</sup> (WT) and Sod2<sup>−/−</sup> mice were analyzed by SDS-polyacrylamide gel electrophoresia using the Oncor Oxyblot kit (Oncor, Gaithersburg, MD) as described by Keller et al. (40). A shows the autoradiograph of a Western blot using a polyclonal antibody developed against carbonyl groups. B shows the Coomassie Blue stain of the Western blot in A. To ensure that the antibody specifically detected carbonyl groups, a derivatization control was included in which no 2,4-dinitrophenyl hydrazine was added to the extracts. This resulted in a loss of binding of the antibody in both the cytosolic and mitochondrial extracts.

We also compared levels of oxidative damage in the livers of the Sod2<sup>−/−</sup> and Sod2<sup>−/−</sup> mice by measuring the levels of 8-hydroxydeoxyguanosine (8-OHdG) in mtDNA and nuclear DNA. The data in Fig. 4 show that the level of 8-OHdG in mtDNA from the livers of the Sod2<sup>−/−</sup> mice was significantly higher (30%) than that found in mtDNA from the Sod2<sup>+/+</sup> mice. In contrast, the levels of 8-OHdG in nuclear DNA were similar in the Sod2<sup>−/−</sup> and Sod2<sup>−/−</sup> mice. Mitochondrial Function—Because mitochondria from the Sod2<sup>−/−</sup> mice showed higher levels of oxidative damage, we compared the function of mitochondria isolated from the livers of Sod2<sup>−/−</sup> and Sod2<sup>−/−</sup> mice. The rates of state 3 and state 4 respiration and the RCR were measured because they are measurements of the efficiency of the movement of electrons along the electron transport chain and the coupling of this movement to the production of ATP by oxidative phosphorylation (24). Oxygen consumption of isolated mitochondria was measured using substrates that are metabolized through different complexes in the electron transport chain, and Table II shows the rates of state 3 and state 4 respiration with the three substrates. The RCR was significantly lower for mitochondria isolated from Sod2<sup>−/−</sup> mice for all three substrates. The decrease in RCR was greatest (29 and 33%) for the substrates glutamate/malate and duroquinol, which are metabolized...
Mitochondrial DNA were isolated from livers of mice. The concentrations of 8-OHdG and deoxyguanosine (dG) in the DNA hydrolysates were determined using high performance liquid chromatography and quantified by electrochemical detection as described by Floyd et al. (49). A CoulChem® electrochemical detection system (ESA model 5200, ESA, Inc., Chelmsford, MA) was used with a reverse phase, isocratic system as described by Beal et al. (50). The ratios of 8-OHdG/dG in liver nuclear and mitochondrial DNA from two livers were essentially identical in the mitochondria isolated from each experiment. *, **, (50). The ratios of 8-OHdG/dG in liver nuclear and mitochondrial DNA were determined using high performance liquid chromatography and quantified by electrochemical detection as described by Floyd et al. (49). A CoulChem® electrochemical detection system (ESA model 5200, ESA, Inc., Chelmsford, MA) was used with a reverse phase, isocratic system as described by Beal et al. (50). The ratios of 8-OHdG/dG in liver nuclear and mitochondrial DNA from two livers were essentially identical in the mitochondria isolated from each experiment. *, **, (50).

Through complexes I and III, respectively. This is not surprising because these two complexes have been reported to be more sensitive to oxidative damage for the following reasons: (a) they contain [Fe-S] clusters that are sensitive to oxidative stress (42), (b) they are the major sites for the production of reactive oxygen species (43), and (c) cardiolipin, which is an essential for the biological activities of these complexes, is sensitive to peroxidation by reactive oxygen species (44). The respiration data with glutamate/malate are also consistent with our data in Table II, which showed that the activity of NADH oxidoreductase with coenzyme Q as a substrate was reduced approximately 30%.

The decrease in the RCR appears to be due to a decrease in state 3 respiration. The data in Table II show a significant decrease (44%) in state 3 respiration with duroquinol as a substrate in the Sod2-/-/+ mice compared with the Sod2+/- mice. The substrates succinate and glutamate/malate did not show a statistical significant decrease in state 3 respiration in the Sod2+/-/+ mice; however, the decrease in state 3 respiration with glutamate/malate as a substrate approached statistical significance with a P value of 0.06. The rates of state 4 respiration are essentially identical in the mitochondria isolated from Sod2-/- and Sod2+/- mice, which suggests there was no disruption in membrane integrity in the inner mitochondrial membrane of the Sod2-/- mice (45). This observation was further substantiated when we measured the membrane potential of mitochondria isolated from Sod2-/- and Sod2+/- mice using safranine O as described by Akerman and Wikstrom (46). The membrane potentials for mitochondria isolated from the livers of Sod2-/- and Sod2+/- mice were -173.74 ± 15.61 mV and -167.30 ± 23.93 mV, respectively (data are expressed as the mean ± S.E. of six different experiments in each mitochondria were from two animals in each experiment. *, P < 0.05 by paired Student’s t test.)

Mitochondria isolated from the Sod2-/- mice also showed altered function with respect to the induction of the permeability transition. The permeability transition occurs through a proteinaceous pore, whose opening is induced by calcium and oxidative stress, such as t-butylhydroperoxide (47). The induction of the mitochondrial permeability transition pore is characterized by a sudden increase in the permeability of the mitochondrial inner membrane to small ions and molecules (calcium and glutathione) that can lead to a complete collapse of the membrane potential and swelling of the matrix. Fig. 5A shows that mitochondria isolated from the livers of Sod2-/- mice undergo more rapid swelling in the presence of calcium and t-butylhydroperoxide based upon the decrease in relative absorbance. To ensure that the increased rate of mitochondrial swelling was due to the induction of the permeability transition, cyclosporin A, an inhibitor of the permeability transition pore (42), was added to the reactions. As illustrated in Fig. 5, the addition of cyclosporin A inhibited the decrease in absorbance, indicating that the changes in absorbance were due to the induction of the permeability transition pore. Fig. 5 shows that the rate of induction (calculated as t1/2 in seconds) of the permeability transition by calcium was higher for mitochondria isolated from the livers of Sod2-/- mice. However, this increase was not statistically significant. Addition of both t-butylhydroperoxide and calcium increased the rate of induction of the permeability transition for mitochondria isolated from both the Sod2+/- and Sod2-/- mice. The data in Fig. 5 show that the rate of induction of the permeability transition was significantly higher (30%) for mitochondria isolated from the Sod2-/- mice compared with Sod2+/- mice in the presence of calcium and t-butylhydroperoxide. The more rapid induction of the mitochondrial transition pore in the Sod2-/- mice potentially could be serious physiologically because it could lead to the loss of mitochondrial membrane integrity. Once mitochondria can no longer maintain a proton-motive force, they are unable to generate ATP.

In summary, our data provides more direct and detailed evidence that the decrease in MnSOD activity in liver mitochondria of Sod2-/- mice is physiologically important. We found that superoxide anion levels were increased in the mitochondria from Sod2-/-/+ mice as measured by a loss of aconitate activity. The increased levels of superoxide anions would be predicted to lead to increased oxidative damage, and we have presented several lines of evidence showing that mitochondria from Sod2-/- mice experience greater oxidative stress/damage. Glutathione levels are reduced, NADH oxidoreductase activity is reduced, carbonyl groups in mitochondrial proteins are increased, 8-OHdG levels in mtDNA are increased, and the induction of the permeability transition is increased. Interestingly, we were unable to detect any evidence for changes in oxidative stress/damage in the cytosol and nuclei of liver from the Sod2-/- mice. Oxidation of cytosolic proteins (glutamine synthetase activity and carbonyl groups) and nuclear DNA (8-OHdG) are similar in the Sod2-/- and Sod2+/- mice. Thus, it appears that the physiological impact of the mutation in the Sod2-/- mice is limited to the mitochondria, which is further evidence for MnSOD playing a critical role in the first line of defense against superoxide anions that are produced during normal aerobic respiration in mitochondria. One of the major observations from our study was that the function of mitochondria from the Sod2-/- mice was compromised. Mitochondria

### Table II

| Substance    | State 3 | State 4 | RCR      |
|--------------|---------|---------|----------|
| Glutamate/malate | 30.7 ± 3.0 | 6.5 ± 0.8 | 5.1 ± 0.5 |
| Sod2+/-      | 38.9 ± 3.9 | 14.8 ± 2.0 | 2.6 ± 0.1 |
| Sod2-/-      | 39.2 ± 5.7 | 14.2 ± 1.9 | 2.2 ± 0.1** |

** nmol O2/min/mg protein.
isolated from the livers of Sod2−/− mice showed reduced respiratory control ratios with substrates that are metabolized by complex I, II, and III. In other words, it appears that the coupling of oxygen consumption to ATP production is less efficient for liver mitochondria from Sod2−/− mice. Thus, we have strong in vivo evidence that increased oxidative damage leads to reduced mitochondrial function. Our data in vivo are consistent with the data that has been obtained in vitro over the past 20 years showing that mitochondrial function is substantially impaired when mitochondria are exposed to oxidative stress (5).

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