Mitochondrial Reactive Oxygen Species in Mice Lacking Superoxide Dismutase 2

ATTENUATION VIA ANTIOXIDANT TREATMENT *

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Mice that lack the mitochondrial form of superoxide dismutase (SOD2) incur severe pathologies and mitochondrial deficiencies, including major depletion of complex II, as a consequence of buildup of endogenous reactive oxygen species (Melov, S., Coskun, P., Patel, M., Tuinstra, R., Cottrell, B., Jun, A. S., Zastawny, T. H., Dizdaroglu, M., Goodman, S. I., Huang, T. T., Miziorio, H., Epstein, C. J., and Wallace, D. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 846–851 and Li, Y., Huang, T. T., Carlson, E. J., Melov, S., Ursell, P. C., Olson, J. L., Noble, L. J., Yoshimura, M. P., Berger, C., Chan, P. H., Wallace, D. C., and Epstein, C. J. (1995) Nat. Genet. 11, 376–381). These problems can be greatly attenuated or rescued by synthetic antioxidant treatment, such as with the catalytic antioxidant EUK189 (Hinerfeld, D., Traini, M. D., Weinberger, R. P., Cochran, B., Doctrow, S. R., Harry, J., and Melov, S. (2004) J. Neurochem. 88, 657–667). We have used heart mitochondria from sod2 null mice to better understand mitochondrial reactive oxygen species production both in the absence of SOD2 and following in vivo antioxidant treatment. Isolated heart mitochondria from 5-day-old sod2 null animals respiring on the complex II substrate succinate exhibited statistically significant higher levels of mitochondrial \( \text{O}_2^- \) (157%, \( p < 0.01 \)) but significantly less \( \text{H}_2\text{O}_2 \) (33%, \( p < 0.001 \)) than wild type littersmates. Treatment of sod2 nullizygous mice with EUK189 proportionately increased the levels of complex II and \( \text{H}_2\text{O}_2 \). Increased production of \( \text{O}_2^- \) resulting from complex II normalization had no effect on steady state levels due to the rapid conversion to \( \text{H}_2\text{O}_2 \), a process presumably aided by the presence of the EUK189, an SOD mimetic.

Age-related diseases, including cancer, type 2 diabetes, and Alzheimer and Parkinson diseases, show a clear correlation with mitochondrial dysfunction and reactive oxygen species (ROS)³ (5–12). Tissues with high energy expenditure such as brain, heart, muscle, and pancreas have significant ATP requirements. The vast majority of such ATP is derived from mitochondrial oxidative phosphorylation, a series of sequential electron transfers along the respiratory chain resulting in the reduction of \( \text{O}_2 \) to water concomitant with the production of ATP. During the course of normal respiration under state IV conditions, as a result of endogenous oxidative stress (3–5, 38). Further, treatment with sod2 null mice with the synthetic antioxidant EUK8 completely rescued the deficiencies in complexes I, III, and IV caused by endogenous oxidative stress and greatly increased the catalytic activity of complex II being severely affected and complex IV the least affected.

The synthetic antioxidant EUK189 (4) used in the present studies is an SOD and catalase mimetic having broad biological efficacy in numerous oxidative stress paradigms (38). Our prior studies showed that treatment of the sod2 null mouse with such synthetic antioxidants could prolong lifespan and rescue or attenuate many of the pathologies arising as a result of endogenous oxidative stress (3–5, 38). Further, treatment of sod2 null mice with the synthetic antioxidant EUK8 completely rescued the deficiencies in complexes I, III, and IV caused by endogenous oxidative stress and greatly increased the catalytic activity of complex II. This demonstrated that antioxidant treatments of mitochondrial disor-

³ The abbreviations used are: ROS, reactive oxygen species; SOD, superoxide dismutase; MOPS, 4-morpholinepropanesulfonic acid; LD, low dose; HD, high dose.

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We have used sod2 mice to evaluate a number of antioxidants in this context and have identified compounds that are effective at preventing mitochondrial-mediated ROS pathologies such as cardiomyopathy, hepatic lipid accumulation, and neurodegeneration (3, 4, 37). Such antioxidants may be similarly useful in the treatment of aging and its diseases where ROS may have a significant pathological role (39, 40). Accordingly, in the present work, we have examined in more detail how the synthetic antioxidant EUK189 (3, 4) modulates ROS levels in mitochondria isolated from hearts of the sod2 null mouse.

MATERIALS AND METHODS

Chemicals—Unless otherwise stated, all chemicals used in this study were obtained from Sigma.

Animals and EUK189 Treatment—Mouse genotyping and EUK189 treatment of sod2 null mice were carried out as previously described (37). Briefly, mice were genotyped between 2–3 days of age, and sod2 null mice were injected intraperitoneally every 24 h (±3 h) with the indicated dose of EUK189 (Dalton Chemical Laboratories Inc., Toronto, Ontario, Canada). Mice received their last treatment of EUK189 24 h before tissue harvest. Wild type sod2 mice were left untreated except in one experiment to show that EUK189 treatment had negligible detrimental effects in wild type mitochondria. Mice were killed between 5–27 days of age. All animal procedures were carried out under approved Institutional Animal Care and Use Committee animal protocols at the Buck Institute, which is American Association for Accreditation of Laboratory Animal Care accredited.

Preparation of Heart Mitochondria—Individual hearts were finely chopped in H buffer (0.21 M mannitol, 70 mM sucrose, 1 mM EGTA, and 5 mM HEPES, pH 7.2), homogenized in a tight fitting glass pestle, and subjected to standard differential centrifugation to obtain a crude mitochondrial preparation. Once large non-mitochondrial debris had been removed by centrifugation at 1,000 × g the mitochondrial supernatant fraction was further clarified four-five times by centrifugation at 1,000 × g. This improved the consistencies of mitochondrial preparations produced on different days (data not shown). The supernatant was transferred to a new tube and mitochondria pelleted by spinning at 8,500 × g for 10 min at 4 °C. Mitochondria were resuspended in 100–500 μl of oxygen electrode buffer (OEB) (0.25 M sucrose, 5 mM MOPS (pH 7.4), 5 mM KH2PO4 (pH 7.4), 5 mM MgCl2) and mitochondrial protein concentration determined using a BCA protein assay (Pierce).

For the Amplex Red and dihydroethidium assays, heart mitochondria were further diluted in OEB containing 0.1% bovine serum albumin to give a final concentration of 80 μg/ml and kept on ice until required.

Mitochondrial Quality Assessment—Preparations of purified mitochondria routinely exhibited a respiratory control ratio of >4.0 and no measurable NADH oxidase activity, an indication of intactness of the mitochondria (data not shown).

Amplex Red and Dihydroethidium Assays—Assays for measuring mitochondrial ROS fluorometrically were modified from previous studies in order to work on smaller amounts of material (24). Levels of ROS were corrected for background fluorescence associated with reagents and inhibitors.

Amplex Red Measurement of Mitochondrial Hydrogen Peroxide Levels—Heart mitochondria (4 μg of mitochondrial protein/well) were incubated with respiratory chain substrates, 1 μM Amplex Red (Molecular Probes), 0.2 units/ml horseradish peroxidase in standard OEB (+0.1% bovine serum albumin). Reactions were set up in triplicate in 100-μl volumes in a 96-well plate format. The rate of hydrogen peroxide produced was measured fluorometrically (excitation 544, emission 590 nm) over 30 min at 37 °C in a Spectra Max Gemini fluorescent plate reader (Molecular Devices). H2O2 detection by Amplex red was shown to be catalase sensitive, consistent with previously published studies (data not shown and Ref. 19). All Amplex Red hydrogen peroxide data are expressed as the change in fluorescence excitation 544 nm and emission 590 nm/μg mitochondria/min.

Dihydroethidium Measurement of Mitochondrial Superoxide Levels—Mitochondrial superoxide production was determined on the same mitochondrial preparations used in the Amplex Red assays. Superoxide assays were carried out by incubating 4 μg of heart mitochondria with mitochondrial substrates and 5 μM dihydroethidium in a 96-well plate format at 37 °C for 60 min. The reaction was stopped by the addition of 0.6% Triton and 10 μg of sonicated salmon sperm DNA and the ethidium bromide/DNA fluorescence measured by excitation at 544 nm and emission at 612 nm. Previous studies suggest that the reaction of dihydroethidium with superoxide generates a range of products (termed “Et” products) that when combined with DNA generate products with different excitation and emission characteristics (41–43). Our studies measured fluorescence at excitation 544/emission 612 nm based on previous microscopy studies in which dihydroethidium had been used to measure superoxide production in a number of stroke models (44).

Mitochondrial Enzymology and Immunoblotting—Mitochondria used for ROS assays were frozen at −70 °C and used for assessment of complex II activity and complex II subunit levels. The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyterazolium bromide assay allows a rapid assessment of complex II activity on multiple heart preparations (45). The presence of the specific inhibitor malonate enabled complex II activity to be distinguished from those of the other mitochondrial dehydrogenases. Frozen mitochondria (5 μg of mitochondrial protein) were incubated with 2.5 mM succinate and 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterazolium in OCB (+0.1% bovine serum albumin) with and without malonate (2.5 mM) for 30 min at 37 °C in 96-well plates. Plates were centrifuged at 4,000 rpm for 10 min. The supernatant was removed and the water-insoluble formazan crystals dissolved in 100 μl of Me2SO. The resulting purple color was measured at 570 nm.

Changes in complex II subunit levels following treatment of animals with EUK189 (low dose, 1 mg/kg; high dose, 30 mg/kg) treatment were determined by Western blot analysis. Equal amounts of mitochondrial protein (1 μg) were subjected to SDS-PAGE on 4–20% Nupage gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane for Western blot analysis. Western blot analysis was performed using the ECF detection system as recommended by the manufacturer (Amersham Biosciences), probing with rabbit polyclonal antibodies raised against the bovine ip (30 kDa) subunit of complex II. The same mitochondrial preparations were used for Western blot analysis as were used in the ROS and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterazolium assays.

RESULTS

Mitochondria isolated from either sod2 null mouse brain (3) or heart exhibit lower complex II activity, stemming at least in part from decreased assembly of the complex based on abundance of the 70- and 30-kDa subunits of the enzyme. As opposed to enzyme depletion, the level of ROS is expected to be increased by the lack of SOD2. Heart

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mitochondria isolated from 5–6-day-old sod2 null mice showed elevated superoxide levels (157%, $p < 0.01$) relative to those from wild type animals when respiring on succinate (Fig. 1B). In contrast, mitochondrial hydrogen peroxide production with the same substrate (Fig. 1C) was significantly decreased (37%, $p < 0.001$). Mitochondrial hydrogen peroxide produced from complex I substrates (pyruvate/malate) was not above background levels (data not shown).

Treatment of sod2 null mice with the synthetic antioxidant EUK189 did not alter the levels of superoxide whether the mitochondria were oxidizing complex I substrates or succinate (Fig. 1, A and B). In contrast, EUK189 treatment altered hydrogen peroxide production from complex II substrates relative to wild type mitochondria in a dose-dependent fashion (untreated sod2 null 37%, sod2 null treated with EUK189 (LD) 55%, and sod2 null treated with EUK189 (HD) 73.3%) (Fig. 1C). EUK189 treatment of wild type control animals had no effect on heart mitochondrial superoxide or hydrogen peroxide production (data not shown).

Increased $\text{H}_2\text{O}_2$ production from sod2 null heart mitochondria following EUK189 treatment correlated with increased complex II activity (Fig. 2A) and increased complex II 30-kDa subunit levels (Fig. 2, B and C). A linear relationship between the abundance of the 30-kDa subunit of complex II and the activity of succinate dehydrogenase was observed for mitochondria isolated from the hearts of all genotypes with and without EUK189 treatment (Fig. 2D).

Measurements of superoxide levels in heart mitochondria isolated from wild type mice covering the initial life span between 4 and 26 days of age showed them to be both age and substrate dependent. Although similar levels of superoxide were generated from pyruvate/malate and succinate up to 10 days of age, those with succinate increased thereafter and those with pyruvate/malate decreased (Fig. 3A). The trends become more obvious if the scatter of data points due to interexperimental variation is minimized by plotting the ratio of superoxide levels generated by pyruvate/malate and succinate for each animal (Fig. 3C, $r^2$ is 0.68, $p < 0.0001$). The respective hydrogen peroxide levels obtained with these substrates showed no significant differences over the same timespan (Fig. 3B). As shown in Fig. 3D, mouse heart mitochondria show a significant increase in complex II activity up to 26 days of age ($r^2$ is 0.91, $p < 0.0001$), which likely accounts for much of the observed increase in ROS production when oxidizing succinate (Fig. 3A).

**DISCUSSION**

The major objective of this study was to investigate ROS production in mitochondria isolated from mice that lack SOD2 and determine whether therapeutically beneficial antioxidant treatment modulates or attenuates ROS production directly. This question is complicated by the fact that mitochondria isolated from sod2 nullizygous mice show a range of severe mitochondrial biochemical defects (1). Complex II activity (succinate dehydrogenase) has been reported to be severely impaired in brain and heart mitochondria of 5–6-day-old sod2 null mice with levels reduced to <15% that of controls (1, 3), and the activities of $\alpha$-ketoglutarate dehydrogenase and aconitase are also severely compromised in these mitochondria. Restricted tricarboxylic acid cycle function would be expected to impair the supply of reducing equivalents to the respiratory chain and negatively influence both energy and superoxide production. Our experiments showed that heart mitochondria from sod2 null mice, where the complex II activity was depleted by at least 70% (Fig. 3), exhibited a statistically significant (50%) increase in $\text{O}_{2}^-$ levels when respiring on succinate as compared with those of wild type (Fig. 1B). This was associated with a 3-fold decrease in the levels of $\text{H}_2\text{O}_2$ (Fig. 1C), consistent with a lowered overall production of ROS compared with wild type. Clearly the content of complex II in sod2 null mitochondria was limiting for superoxide production in this assay, because both enzyme content and ROS were coordinately shifted toward wild type levels following treatment of the mice with the synthetic antioxidant EUK 189 (see below). In contrast to respiration on succinate, oxidation of complex I substrates produced a general increase in $\text{O}_{2}^-$ level as compared with wild type that failed to reach statistical significance (Fig. 1A). Of interest, therefore, is our demonstration that heart mitochondria from sod2 null mice do not suffer from an over-
whelming flood of superoxide but contain steady state levels akin to those in wild type animals because there is restricted entry of reducing equivalents into the respiratory chain as a result of lowered tricarboxylic acid cycle activity. That complex II content would limit superoxide production sod2 null mitochondria is taken as evidence that energy production is similarly limited.

As these mitochondria lack the matrix SOD2, a question remaining concerns the mechanism by which any superoxide released into the matrix during the oxidation of substrate might contribute to the H$_2$O$_2$ detected extramitochondrially. Matrix O$_2^-$ does not cross the inner membrane even in its lipid-permeant, protonated form (19), and aqueous dismutation of O$_2^-$ is some three to four orders of magnitude less than that catalyzed by SOD. The bulk of detectable H$_2$O$_2$ would thus seem to derive from superoxide entering the intermembrane space from complex III. Lack of oxidation of exogenous NADH by our mitochondrial preparations (see “Materials and Methods”) argues against the participation of SOD in the dismutation of enzyme flavins exposed to solution. A detailed investigation of how the lack of SOD2 might influence mitochondrial bioenergetics is currently underway.

Previous studies on mitochondrial function in developing tissues have shown that there are tissue- and species-specific differences in the activities of enzymes involved in supplying energy from birth to adulthood (46–48). A number of studies have shown that there is a sharp transition from the essentially glycolytic state of the newborn neonate to a high reliance on oxidative phosphorylation for supplying energy demands in the first few weeks after birth (46–48). Accompanying this switch in energy metabolism in the heart is an increase in mitochondrial volume and number (49), as well as enzymatic activities of mitochondrial enzymes such as citrate synthase and 3-hydroxyacyl-CoA dehydrogenase. Of direct relevance to our studies, Bass et al. (50) found an approximate doubling of the activities of citrate synthase and 3-hydroxyacyl-CoA dehydrogenase between 7 and 30 days of age in mitochondria isolated from left ventricular myocardium of developing rats. Our data in Fig. 3D show a similar trend, in that complex II activity increases sharply between 5 and 25 days of age in wild type animals consistent with the greater requirement for energy for growth and development. It seems unlikely that the sod2 null animal would be able to make this transition due to its severely depleted complex II content.

Treatment with EUK189 did not statistically significantly alter O$_2^-$ levels from those observed in control mitochondria from sod2 null animals oxidizing succinate (Fig. 1B) or complex I substrates (Fig. 1A), respectively. In contrast, antioxidant treatment clearly increased the level of H$_2$O$_2$, measured with succinate as substrate and in a dose-dependent fashion, reaching 3-fold at the highest concentration of EUK189 used in these experiments (Fig. 1C). The effect is consistent with the previously reported superoxide dismutase/catalase mimetic behavior of EUK189 (30), in that a higher dose would be predicted to convert more O$_2^-$ to H$_2$O$_2$, and eventually to water, thereby reducing the cumulative load of ROS in mitochondria lacking SOD2. This decreased burden of total mitochondrial ROS directly correlates with increased complex II activity. We previously reported in brain (1), and now confirm, that complex II activity in heart mitochondria from sod2 null mice is dramatically lowered by as much as 75% compared with that in wild type concomitantly with depletion of enzyme subunits in the membrane (1, 3). Antioxidant treatment improved both content (Fig. 2B) and activity of complex II (Fig. 2A) in a complementary and dose-dependent manner. Our prior results in brain also demonstrated that other complexes such as respiratory chain complexes I, III, and IV were also amenable to antioxidant treatment (3). Although not achieving wild type levels of protein or activity, treatment with the highest dose of EUK189 produced an approximate doubling of the levels of the enzyme. A deleterious effect of oxidative stress on complex II levels is manifest in the decreasing activities and abundance of the 30-kDa subunit of the enzyme between mitochondria obtained from wild type, nullizygous animals treated with high and low doses of EUK189, and sod2 nullizy-
The effect of age on heart mitochondrial superoxide and hydrogen peroxide levels and complex II activity. Superoxide (A) and hydrogen peroxide (B) levels were determined in isolated wild type heart mitochondria respiring on pyruvate/malate and succinate. Each point represents the mean value for one heart mitochondrial preparation. The non-malonate-sensitive activity for the mitochondrial preparations is shown as the non-complex II component. Regression analysis shows a highly significant trend ($r^2 = 0.91$, $p < 0.0001$, 95% confidence limits are shown). D, the increase in activity of complex II with age. Each point represents the mean complex II activity of a single wild type heart preparation. The non-malonate-sensitive activity for the mitochondrial preparations is shown as the non-complex II component. Regression analysis shows a highly significant trend ($r^2 = 0.91$, $p < 0.0001$, 95% confidence limits are shown).

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