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Original Contribution

Impaired spare respiratory capacity in cortical synaptosomes from *Sod2* null mice

James M. Flynn [1], Sung W. Choi [1], Nicholas U. Day, Akos A. Gerencser, Alan Hubbard, Simon Melov *

Buck Institute for Age Research, 8001 Redwood Boulevard, Novato, CA 94945, USA

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ABSTRACT

Presynaptic nerve terminals require high levels of ATP for the maintenance of synaptic function. Failure of synaptic mitochondria to generate adequate ATP has been implicated as a causative event preceding the loss of synaptic networks in neurodegenerative disease. Endogenous oxidative stress has often been postulated as an etiological basis for this pathology, but has been difficult to test *in vivo*. Inactivation of the superoxide dismutase gene (*Sod2*) encoding the chief defense enzyme against mitochondrial superoxide radicals results in neonatal lethality. However, intervention with an SOD mimetic extends the life span of this model and uncovers a neurodegenerative phenotype providing a unique model for the examination of *in vivo* oxidative stress. We present here studies on synaptic termini isolated from the frontal cortex of *Sod2* null mice demonstrating impaired bioenergetic function as a result of mitochondrial oxidative stress. Cortical synaptosomes from *Sod2* null mice demonstrate a severe decline in mitochondrial spare respiratory capacity in response to physiological demand induced by mitochondrial respiratory chain uncoupling with FCCP or by plasma membrane depolarization induced by 4-aminopyridine treatment. However, *Sod2* null animals compensate for impaired oxidative metabolism in part by the Pasteur effect allowing for normal neurotransmitter release at the synapse, setting up a potentially detrimental energetic paradigm. The results of this study demonstrate that high-throughput respirometry is a facile method for analyzing specific regions of the brain in transgenic models and can uncover bioenergetic deficits in subcellular regions due to endogenous oxidative stress.

Presynaptic nerve terminals require a constant supply of ATP for the proper maintenance of active physiological events such as neurotransmitter release and reuptake. To meet these ATP demands at the synaptic bouton, mitochondria are physically located at the terminus. There has been a growing body of evidence that synaptic dysfunction is a critical event in a number of neurodegenerative diseases, including Alzheimer and Parkinson disease [1,2], dementia [3,4], and Huntington disease [5,6]. It has been postulated that one possible mechanism underlying such synaptic dysfunction is a depletion of ATP at the terminus, mediated by an "ATP crisis." This concept has been referred to as the "spare respiratory capacity" hypothesis and proposes that when the bioenergetic reserve present in a neuron is exceeded by energetic demand outpacing supply, a lack of ATP results, with potential neurodegenerative consequences [7]. This may result in an escalating sequela of pathologies as the synaptic network breaks down [2]. Most evidence for impaired synaptic function due to mitochondrial abnormalities has been provided by *in vitro* tissue culture studies, which rely on impairing mitochondrial function through the addition of mitochondrial toxins such as rotenone to directly impair the respiratory chain, thereby mimicking an endogenous respiratory defect [8,9]. However, the use of a genetic model of mitochondrial deficiency affords the opportunity to test bioenergetic hypotheses in the brain in a more direct fashion. The *Sod2* null mouse is one such model of endogenous mitochondrial dysfunction.

Superoxide dismutase 2 (SOD2) is arguably one of the most important oxidative stress defense enzymes in the cell. SOD2 is exclusively located in the mitochondrial matrix and scavenges superoxide radicals, a by-product of mitochondrial oxidative phosphorylation. Constitutive lack of SOD2 in mice on multiple genetic backgrounds mediated via homologous recombination results in an embryonic (C57BL/6 J) or neonatal lethal (CD1, DBA/2 J, and B6D2F1) phenotype [10,11]. *Sod2* null mice on a CD1 background treated with antioxidants from an early age present with pronounced neurodegeneration [12,13]. This demonstrates the importance of controlling endogenous levels of superoxide anion in the mitochondria and facilitates study of the consequences of endogenous mitochondrial oxidative stress on synaptic function. Neurological phenotypes that have been reported in constitutive *Sod2* null mice include seizures, spongiform encephalopathy, tremor, and neurodegeneration [12–15]. Biochemical characterization of mitochondria isolated from the forebrains of *Sod2* null mice show profound respiratory chain defects including reductions in the catalytic activity of complexes I, II, III, and IV, as well as tricarboxylic acid (TCA) cycle enzymes [16]. Further, gene expression profiling studies have also revealed a transcriptional signature indicative of neurodegeneration involving abnormal glutamate neurotransmitter

* Corresponding author. Fax: +1 415 209 2231.
E-mail address: smelov@buckinstitute.org (S. Melov).
[1] These authors contributed equally to this study.

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metabolism [17]. The neurodegeneration and spongiform encephalopathy reported in *Sod2* null mice were uncovered after therapeutic treatment with catalytic antioxidants, which have the ability to extend the life span of these mice by up to 4-fold [12–14]. Treatment of *Sod2* null mice with such an antioxidant, EUK189, at either "high" or "low" doses, prevents or allows a spongiform encephalopathy, with accompanying neurodegeneration. Without any antioxidant treatment, *Sod2* null mice expire in the first week of life [11,12,14]. Therefore, the coupling of *Sod2* null mice with antioxidant treatment encapsulates a system that can be exploited to investigate the consequences of endogenous mitochondrial oxidative stress on the central nervous system (CNS) and, in the case of our study, subregions within the brain. Mitochondrial function is best evaluated using respiration rate, as quantitating the steady-state concentration of ATP alone is not informative with regard to how the mitochondria perform work under energetic load, because of the dynamic nature of ATP supply and demand [18]. The recent introduction of a high-throughput respirometer, which in the context of studying synaptosomes requires as much as 10-fold less biological material than a classical Clarke oxygen electrode [19–21], allows detailed bioenergetic information to be obtained from synaptosomes prepared from specific brain regions of individual mice as opposed to whole-brain homogenates. Other advantages in studying synaptosomes via this method are a closer approximation of mitochondrial function to their *in vivo* physiology, as mitochondria are evaluated in the synaptic bouton, more closely approximating their cellular environment, whereas traditional bioenergetic measurements (oxygen electrode) often use mitochondria purified from total tissue homogenates. We present here direct evidence demonstrating that endogenous mitochondrial oxidative stress prevents an appropriate bioenergetic response to increased ATP demand within the frontal cortex of *Sod2* null mice, which has mechanistic implications for neurodegenerative diseases in which mitochondrial dysfunction is implicated.

Methods

Reagents

All chemicals used in these experiments were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Animals

Constitutive *Sod2* null homozygous mice on a CD1 background ranging in age from 17 to 21 days and age-matched wild-type siblings were used for all experiments as previously described [12,14]. Genotype determination was performed within 3 days of birth, as previously described [14], and either 1 or 30 mg/kg of the synthetic antioxidant EUK189 (Dalton Pharma Services, Toronto, ON, Canada) was injected daily via intraperitoneal injection from the fourth day after birth until the day before the animals were euthanized. All mouse procedures were carried out under approved Buck Institutional IACUC protocols.

Preparation of cortical synaptosomes

Mice were euthanized by CO_2 overdose and decapitation, and cortices were removed and subjected to a synaptosome isolation method as previously described [22], with slight modifications. Briefly, the frontal cortex was rapidly isolated (about 100 mg/brain) and rinsed with ice-cold sucrose medium (320 mM sucrose, 1 mM EDTA, 0.25 mM dithiothreitol, pH 7.4) to remove excess blood, transferred to a prechilled Dounce glass homogenizer containing 3 ml sucrose medium, and homogenized gently by 10 strokes. The homogenate was then centrifuged at 1000 *g* for 10 min at 4 °C. The supernatant was carefully layered on top of a discontinuous Percoll gradient (3, 10, and 23% Percoll in sucrose medium) in a 15-ml centrifuge tube and centrifuged at 32,500 *g* for 10 min at 4 °C in a JA-25.50 fixed-angle rotor in a Beckman Avanti J-26 XPI centrifuge. Synaptosomes were isolated and recovered from the band between the 10 and the 23% Percoll layers. The synaptosomes isolated from the 10–23% interface were diluted into ionic medium (20 mM Hepes, 10 mM D-glucose, 1.2 mM Na_2HPO_4, 1 mM $MgCl_2$, 5 mM $NaHCO_3$, 5 mM KCl, 140 mM NaCl, pH 7.4) for measurement of respiration. The final synaptosome pellet was then resuspended in ionic medium, and protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA, USA).

Respirometry

For monitoring respiration, 10 μg synaptosomal protein per well was aliquotted into a 24-well cell culture microplate (Seahorse Bioscience, North Billerica, MA, USA) coated with 1:15,000 diluted polyethyleneimine. The plate was centrifuged at 3400 *g* for 1 h at 4 °C in an A-4-62 rotor in an Eppendorf 5810R centrifuge. Then, the medium was replaced with 700 μl of Seahorse incubation medium (3.5 mM KCl, 120 mM NaCl, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 1.2 mM Na_2SO_4, 15 mM glucose, 10 mM pyruvate, 2 mM $MgSO_4$, and 0.4% (w/v) fatty-acid-free bovine serum albumin). The cell culture microplate was incubated and loaded into the Seahorse XF24 extracellular flux analyzer following the manufacturer's instructions. All experiments were performed at 37 °C. All reagents were added at appropriate dilutions in 75 μl of Seahorse medium. The protocol for the measurement of oxygen consumption and extracellular acidification rate was as previously described [20,21]. Two biological replicates per plate were typical, and experiments ($N>50$ animals total) were carried out over a 6-month period. A minimum of five technical replicates were performed in each measurement, greatly increasing the reliability of oxygen consumption measures, and a minimum of four mice per group per experiment were used as biological replicates for each measurement to determine statistical difference per treatment. Statistical significance was obtained when the p value was lower than 0.05 by two-tailed nonparametric t test.

Measurement of mitochondrial enzyme activities

Synaptosomes were lysed by repetitive freeze-and-thaw, and protein concentration was determined using the Bradford method. Five micrograms of lysed synaptosomes was used for colorimetric enzyme activity assays of complex I, complex II, and citrate synthase as previously reported [23].

Glutamate release assay

Calcium-dependent and -independent glutamate release from synaptosomes was measured using an enzyme-linked fluorimetric assay [24,25]. A Fluoroskan Ascent microplate fluorimeter (Thermo Scientific, Waltham, MA, USA) was set to kinetic measurement mode using an excitation of 355 nm and an emission of 460 nm. Synaptosomes (100 μg/well) were placed into a final volume of 200 μl of ionic medium used in the respirometry experiments without the addition of calcium (see above). The buffer was then supplemented with 1 mM $NADP^+$ and either 1.2 mM $CaCl_2$ or 0.5 mM EGTA to determine the calcium-dependent release. Last, 8 units per reaction of glutamate dehydrogenase enzyme was added to start the reaction. After a period of baseline measurement, the depolarizing agent 4-aminopyridine (4-AP; 1 mM) was injected into each well. Each curve generated was normalized to the baseline reading before the injection of depolarizing agents. Three synaptosome samples per animal were assayed ($N=7$), and the individual curves from each well were normalized and presented as the mean of all curves. Statistical significance was determined by two-tailed nonparametric t test applied to each time point to determine significant differences between the curves of wild-type and of *Sod2* null animals at each time point.