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Mitochondrial oxidative stress and epilepsy in SOD2 deficient mice: Attenuation by a lipophilic metalloporphyrin

Li-Ping Liang [a,1], Simon Waldbaum [a,1], Shane Rowley [a], Ting-Ting Huang [c,d], Brian J. Day [b], Manisha Patel [a,*]

[a] Department of Pharmaceutical Sciences, University of Colorado Denver, Aurora, CO 80045, USA
[b] Department of Medicine, National Jewish Health, Denver, CO 80201, USA
[c] Department of Neurology and Neurological Sciences, Stanford University, Stanford, CA 94305, USA
[d] Geriatric Research, Education, and Clinical Center, VA Palo Alto Health Care System, Palo Alto, CA 94304, USA

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ABSTRACT

Epileptic seizures are a common feature associated with inherited mitochondrial diseases. This study investigated the role of mitochondrial oxidative stress in epilepsy resulting from mitochondrial dysfunction using cross-bred mutant mice lacking mitochondrial manganese superoxide dismutase (MnSOD or SOD2) and a lipophilic metalloporphyrin catalytic antioxidant. Video-EEG monitoring revealed that in the second to third week of postnatal life (P14-P21) B6D2F2 *Sod2*$^{-/-}$ mice exhibited frequent spontaneous motor seizures providing evidence that oxidative stress-induced mitochondrial dysfunction may contribute to epileptic seizures. To confirm the role of mitochondrial oxidative stress in epilepsy a newly developed lipophilic metalloporphyrin, AEOL 11207, with high potency for catalytic removal of endogenously generated reactive oxygen species was utilized. AEOL 11207-treated *Sod2*$^{-/-}$ mice showed a significant decrease in both the frequency and duration of spontaneous seizures but no effect on seizure severity. A significant increase in the average lifespan of AEOL 11207-treated *Sod2*$^{-/-}$ mice compared to vehicle-treated *Sod2*$^{-/-}$ mice was also observed. Indices of mitochondrial oxidative stress and damage (aconitase inactivation, 3-nitrotyrosine formation, and depletion of reduced coenzyme A) and ATP levels affecting neuronal excitability were significantly attenuated in the brains of AEOL 11207-treated *Sod2*$^{-/-}$ mice compared to vehicle-treated *Sod2*$^{-/-}$ mice. The occurrence of epileptic seizures in *Sod2*$^{-/-}$ mice and the ability of catalytic antioxidant therapy to attenuate seizure activity, mitochondrial dysfunction, and ATP levels suggest that ongoing mitochondrial oxidative stress can contribute to epilepsy associated with mitochondrial dysfunction and disease.

Introduction

Epileptic seizures commonly occur in patients with inherited mitochondrial disease (Mecocci et al., 1993; Wallace et al., 1988) suggesting that mitochondrial dysfunction can contribute to seizures (Kunz, 2002; Patel, 2004). General and partial seizures with mitochondrial encephalopathy can be caused by mitochondrial dysfunction arising from mitochondrial mtDNA mutations (Shoffner et al., 1990; Wallace et al., 1988). Mitochondrial dysfunction is a consequence of many neurological insults such as neonatal or adult hypoxia, trauma and infections which are known risk factors for epilepsy development (Beal, 1998; Douglas et al., 2010; Jensen et al., 1991, 1992; Mustafa et al., 2010). These data strongly suggest that mitochondrial dysfunction *per se* may be a common pathway contributing to epilepsy development. Mitochondria have important functions that include cellular ATP production, control of apoptotic/necrotic cell death, reactive oxygen species (ROS) formation and calcium homeostasis. Which of these critical mitochondrial functions contributes to increased seizure susceptibility associated with inherited or acquired epilepsies remains unknown. Results from this and other laboratories suggest that mitochondrial oxidative stress and resultant dysfunction are not only a consequence of seizure activity but also render the brain more susceptible to age-related epileptic seizures (Jarrett et al., 2008; Kudin et al., 2002; Liang et al., 2000; Liang and Patel, 2004; Waldbaum et al., 2010).

To understand the role of oxidative stress in epilepsy associated with mitochondrial disease it is useful to utilize an animal model in which spontaneous epileptic seizures arise due to increased steady-state mitochondrial ROS. Mutant mice lacking manganese superoxide dismutase (MnSOD or SOD2), a critical mitochondrial antioxidant, provide such a model. Mitochondrial disease has been characterized in SOD2 deficient mice generated in several background strains with phenotypes characteristic of increased steady-state mitochondrial ROS. *Sod2*$^{-/-}$ mice bred from a C57B6 background (B6 *Sod2*$^{-/-}$) are neonatal lethal (Lebovitz et al., 1996), whereas CD-1 *Sod2*$^{-/-}$ mice develop and live approximately 8–10 days postnatal (Melov

* Corresponding author at: 12850 East Montview Boulevard, Aurora CO 80045, USA. Fax: +1 303 724 7266.
E-mail address: manisha.patel@ucdenver.edu (M. Patel).
[1] Indicates equal contribution.
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et al., 1999). Recently, *Sod2*$^{-/-}$ mutant mice from a mixed background (C57BL/6J X DBA/2J, B6D2) have been generated, which live approximately 3 weeks without pharmacological intervention (Huang et al., 2001). Behavioral tonic–clonic seizures have been anecdotally reported starting the second to third week of postnatal life in B6D2F1 *Sod2*$^{-/-}$ mice (Lynn et al., 2005). Therefore, increased lifespan of the cross-bred B6D2 *Sod2*$^{-/-}$ mice provides a model in which the underlying role of oxidative stress in mitochondrial disease epilepsy can be investigated.

Metalloporphyrin catalytic antioxidants are small molecule mimics of superoxide dismutase (SOD) and/or catalase (CAT), and are also potent detoxifiers of lipid peroxides and peroxynitrite ($ONOO^-$) (reviewed in (Day, 2004)). Because they are catalytic, and not merely bulk scavengers, these compounds are much more potent antioxidants than dietary additives such as vitamin E that act stoichiometrically. The manganese meso-porphyrin catalytic antioxidants combine the broad spectrum detoxification of reactive species like the stoichiometric antioxidants with the catalytic efficiency of the endogenous antioxidant enzymes. Additionally, these synthetic compounds can be chemically modified to increase their ability to cross the blood brain barrier (BBB), as well as their targeting to various subcellular compartments. Treatment of short-lived *Sod2*$^{-/-}$ mice in the CD-1 background with manganese tetrakis 5, 10, 15, 20-porphyrin (MnTBAP) ameliorated cardiomyopathy but not neurodegeneration (Melov et al., 1998) whereas EUK8 or EUK134 ameliorated spongiform encephalopathy and neurodegeneration (Melov et al., 2001). A major advancement in the field of catalytic antioxidants was the demonstration that AEOL 11207, a lipophilic metalloporphyrin, protected against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity *in vivo* following oral administration (Liang et al., 2007). This compound belongs to a series of metalloporphyrins which were designed to have greater lipid solubility, oral bioavailability, and cross the BBB. The objective of this study was to determine the role of mitochondrial oxidative stress in the development of epileptic seizures using B6D2 *Sod2*$^{-/-}$ mice. Seizure activity was monitored by video-EEG methods in conjunction with indices of mitochondrial oxidative stress. To confirm the role of mitochondrial oxidative stress in epilepsy in *Sod2*$^{-/-}$ mice we asked whether treatment with a lipophilic metalloporphyrin antioxidant could attenuate seizure activity.

Materials and methods

Animals

Animal studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). All procedures were approved by the Institute Animal Care and Use Committee (IACUC) of the University of Colorado Denver (UCD), which is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. Heterozygous MnSOD (*Sod2*$^{-/+}$) mutant mice on a C57BL/6J (B6) background were crossed with DBA/2J (D2) wild type mice to generate B6D2F1 *Sod2*$^{-/+}$ mice, both male and female from B6D2F1 *Sod2*$^{-/+}$ mice were used as breeding pairs to produce the F2 generation (B6D2F2) of *Sod2*$^{-/-}$ mice. The mutant mice were monitored on a daily basis to get an accurate birth and death data. Pups were not culled or handled before P5 to avoid maternal rejection. Pups were genotyped at P5 by PCR as previously described (Li et al., 1995).

Metalloporphyrin (AEOL 11207) administration

B6D2F2 *Sod2*$^{-/-}$ mice and their wild type littermates (*Sod2*$^{+/+}$) used and designated as control mice were treated with AEOL 11207 (5 mg/kg) or vehicle by subcutaneous (s.c.) injection daily starting at 5 days of age until death or being sacrificed. AEOL 11207 was dissolved in dimethyl sulfoxide (DMSO) and diluted with sterilized phosphate buffered saline (PBS) to achieve the desired final concentration (1% DMSO). The animals were divided into four different groups: 1) B6D2F2 *Sod2*$^{+/+}$ mice + vehicle; 2) B6D2F2 *Sod2*$^{-/-}$ mice + vehicle; 3) B6D2F2 *Sod2*$^{+/+}$ mice + AEOL 11207; 4) B6D2F2 *Sod2*$^{-/-}$ mice + AEOL 11207. Both male and female *Sod2*$^{+/+}$ and B6D2F2 *Sod2*$^{-/-}$ mice were used in this study. The ratios of the gender among the four groups were matched. The treated and untreated mice were sacrificed at P15-16 for pathology and biochemistry assays or until death for survival and seizure evaluation.

Behavioral seizure evaluation

Two groups of vehicle and AEOL 11207-treated *Sod2*$^{-/-}$ mice that were positively confirmed by genotyping aged P16-P20 were digitally recorded (Q-See QD14B, Anaheim, CA) daily in Plexiglass cages for quantification of age-related changes in seizure parameters. During the weaning period (P16-P18) mice were recorded in the presence of their mothers, and individually thereafter. Video was digitally recorded (Panasonic DMR-ES15) and stored on DVD-R's for observation and quantification of seizures.

Video-EEG recordings from Sod2$^{-/-}$ *mice*

Sod2$^{-/-}$ and *Sod2*$^{+/+}$ mice that were positively confirmed by genotyping aged P18 receiving either vehicle or AEOL 11207 treatment were anesthetized via isoflurane continuous inhalation and placed in a mouse stereotoxic unit (MyNeuroLab, Leica Microsystems, Richmond, IL). Bilateral stainless steel electrodes were placed on the skull over the motor cortices and secured with dental cement to monitor brain electrical activity. A screw electrode behind lambda served as a ground and reference. Video-Electroencephalograph (EEG) activity was recorded using Stellate systems (Natus Medical Incorporated, San Carlos, CA) for a minimum of 24 h and files analyzed and scored for seizure duration, severity, and frequency by an observer blind to genotype and treatment. Behavioral seizure severity was scored according to the following scale: 1 = immobilization and staring, 2 = head-nodding, shaking, 3 = forelimb tonic/clonic activity, 4 = continuous forelimb tonic/clonic activity with falling, running or jumping. In order to avoid overestimation and accurately define seizure events due to the occurrence of abnormal gait and posturing in *Sod2*$^{-/-}$ mice, only spontaneous motor seizures with scores > 3 were included for comparison between groups and all behavioral seizure activity correlated with EEG seizure events. Isolated electrographic seizures were differentiated from background noise and movement artifact by observing the corresponding video to verify motor seizures and by the appearance of rhythmic spike frequency activity that lasted a minimum of 5 s, had a clear beginning and resolution, and were separated from each other by a minimum of 30 s. Continuous seizure events were identified as rhythmic spike frequency activity that were separated from each other by less than 30 s and were confirmed with motor seizure behavior in the corresponding video.

Determination of metalloporphyrin levels

AEOL 11207 was measured by HPLC-UV following previously described methods (Liang et al., 2007).

Histochemical analyses

Mice were sacrificed at P15-16 and brain paraffin sections (10 μm) were cut coronally and stained with Hematoxylin and Eosin (H&E) following the company protocol (Sigma, St. Louis MO). Fluoro-Jade B (Histo-Chem Inc., Jefferson, AR) staining was performed following previously described methods (Hopkins et al., 2000; Liang et al.,