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Reactive oxygen species mediate cognitive deficits in experimental temporal lobe epilepsy

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ABSTRACT

Cognitive dysfunction is an important comorbidity of temporal lobe epilepsy (TLE). However, no targeted therapies are available and the mechanisms underlying cognitive impairment, specifically deficits in learning and memory associated with TLE remain unknown. Oxidative stress is known to occur in the pathogenesis of TLE but its functional role remains to be determined. Here, we demonstrate that oxidative stress and resultant processes contribute to cognitive decline associated with epileptogenesis. Using a synthetic catalytic antioxidant, we show that pharmacological removal of reactive oxygen species (ROS) prevents 1) oxidative stress, 2) deficits in mitochondrial oxygen consumption rates, 3) hippocampal neuronal loss and 4) cognitive dysfunction without altering the intensity of the initial status epilepticus (SE) or epilepsy development in a rat model of SE-induced TLE. Moreover, the effects of the catalytic antioxidant on cognition persisted beyond the treatment period suggestive of disease-modification. The data implicate oxidative stress as a novel mechanism by which cognitive dysfunction can arise during epileptogenesis and suggest a potential disease-modifying therapeutic approach to target it.

1. Introduction

Epilepsy is increasingly recognized as a spectrum disorder characterized by chronic, spontaneous seizures (SRS). Temporal Lobe Epilepsy (TLE) is the most prevalent form of acquired epilepsy and is defined by the appearance of SRS after a precipitating brain event such as trauma, ischemic stroke or status epilepticus (SE). In addition to experiencing unpredictable seizures, patients with TLE and indeed all epilepsies, tend to suffer from debilitating comorbidities. Up to half of the people with epilepsy experience one or more psychiatric or cognitive comorbidities (Wiebe and Hesdorffer, 2007). These comorbid conditions occur with epilepsy and impair quality of life. In fact, the burden of comorbidities can surpass those of the seizures themselves as quality of life correlates more with the comorbidities of epilepsy as opposed to the seizures alone (Gilliam et al., 2003). Prominent among these comorbidities of epilepsy is cognitive impairment (Institute of Medicine, 2012). Currently available anti-seizure drugs control seizures in roughly 60% of patients; however, they often exacerbate comorbidities such as cognitive impairment, particularly deficits in memory (Helmstaedter and Kurthen, 2001; Helmstaedter et al., 2003). Whereas the cognitive comorbidities of epilepsy are widely recognized for their frequent occurrence and debilitating nature, currently there are no targeted therapies to treat them or sufficient knowledge regarding their underlying mechanisms.

A growing body of research has implicated oxidative stress and mitochondrial dysfunction as contributing factors to numerous conditions with comorbid cognitive dysfunction including Alzheimer's disease (AD), Parkinson's disease (PD) and even normal aging (Butterfield, 2006; Lin and Beal, 2006). Excessive production of reactive oxygen species (ROS) leads to elevated levels of cellular macromolecule oxidation contributing to cell dysfunction and cell death (Hensley et al., 1995). ROS-induced neuronal death or processes underlying neuronal death could have a detrimental effect on areas of the brain controlling learning and memory processes such as the hippocampus, a brain region intimately associated with memory function. Samples obtained from patients with epilepsy show increased levels of oxidized proteins and lipids in serum, implicating oxidative stress as an ongoing process in these patients (Menon et al., 2012). Data from our laboratory have demonstrated increased steady-state levels of reactive species and impaired glutathione redox status in the hippocampus in two separate TLE models including the pilocarpine model and the kainic acid model (Liang et al., 2000, 2008, 2012; Patel et al., 2001, 2008; Ryan et al., 2014; Rowley et al., 2015). Collectively, this work shows that oxidative

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damage occurs during epileptogenesis and contributes to acute injury-induced neuronal damage (Liang et al., 2000; Fujikawa, 2005). Indeed, recent work from our laboratory in the kainic acid model of TLE has shown that attenuation of oxidative stress by a synthetic catalytic antioxidant porphyrin, Mn^{III}TDE-2-ImP^{5+}, is sufficient to protect mitochondrial function (Rowley et al., 2015). However, the functional role of oxidative stress in TLE or associated cognitive comorbidities is unknown. Furthermore, the role of neuronal damage per se on any functional deficits such as cognitive dysfunction associated with TLE is yet to be determined.

Here, we tested the hypothesis that injury-mediated oxidative stress induces mitochondrial dysfunction and neuronal death leading to learning and memory deficits associated with epileptogenesis. Using Mn^{III}TDE-2-ImP^{5+}, we determined if pharmacological scavenging of ROS prevents 1) oxidative stress, 2) deficits in mitochondrial oxygen consumption rates (OCR), 3) hippocampal neuronal loss and 4) cognitive dysfunction in a rat model of TLE.

2. Materials and methods

2.1. Reagents

All reagents were purchased from Sigma-Aldrich or Fisher Scientific. Manganese (III) meso-tetrakis (di-N-ethylimidazole) porphyrin or Mn^{III}TDE-2-ImP^{5+} (also known in the literature as AEOL 10150) was pharmaceutical grade and obtained from Aeolus Pharmaceuticals.

2.2. Pilocarpine treatment

Animals were treated in accordance with NIH guidelines and all experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver. Adult, male Sprague-Dawley rats (250–300 g), purchased from Harlan Laboratories (Indianapolis, Indiana) were used in all experiments. Upon arrival, rats were individually housed on a 14/10 light/dark cycle (lights on at 6:00 am and off at 8:00 pm) with ad libitum access to both food (Harlan rat chow) and filtered water. After one week of acclimation, rats were randomly assigned to groups treated with saline or pilocarpine hydrochloride, to induce SE. Control rats were given scopolamine and saline instead of pilocarpine. Experimental rats were injected with scopolamine (1 mg/kg) 30 min prior to pilocarpine (340 mg/kg) to limit peripheral cholinergic effects and diazepam (10 mg/kg) 90 min after pilocarpine to terminate SE. All rats were visually monitored during SE and behavioral seizures were scored using a modified Racine scale (Liang et al., 2000). Briefly, seizures were scored on the following scale: P1—freezing behavior, P2—head nodding, P3—unilateral forelimb clonus, P4—bilateral forelimb clonus with rearing, and P5—bilateral forelimb clonus with rearing, falling, and/or hind limb clonus. Only rats experiencing SE defined as: at least five stage P3 or higher seizures followed by a period of continuous seizure activity for at least two hours, were included in further analyses as SE is the best predictor for the development of chronic epilepsy. Rats given pilocarpine but not developing SE as described above were deemed “non-responders”. Non-responders provide an ideal control group to differentiate the effects of pilocarpine versus the effects of SE and as such, non-responders were tested for indices of oxidative stress, mitochondrial function and deficits in memory.

2.3. Catalytic antioxidant treatment

Rats were treated with the metalloporphyrin catalytic antioxidant, Mn^{III}TDE-2-ImP^{5+} (5 mg/kg s.c.) or vehicle (saline for controls) starting 60 min after pilocarpine and continuing every 4 h until sacrifice (for most endpoints — 24 h). For behavior testing, treatment with saline (controls) or Mn^{III}TDE-2-ImP^{5+} started 60 min after pilocarpine and continued every 4 h for the first 48 h followed by a gradual tapering of doses (q4 for 48 h followed by q6, q12 and q24) ending two days before acclimation to behavioral testing parameters and five days before NOR learning and memory was tested. Animals were divided into 4 groups: 1) Control (saline–saline), 2) Control + Mn^{III}TDE-2-ImP^{5+}, 3) Pilo + saline, and 4) Pilo + Mn^{III}TDE-2-ImP^{5+}. It was determined that for most end-points (indices of oxidative stress, oxygen consumption rates and cell death) the Mn^{III}TDE-2-ImP^{5+} alone group displayed no discernible difference compared to control animals and were therefore not included in further analyses.

2.4. Assessment of brain penetration of Mn^{III}TDE-2-ImP^{5+}

We used the known plasma half-life and dosing paradigm of Mn^{III}TDE-2-ImP^{5+} (5 mg/kg, s.c. q4h) as a guide to determine its brain penetration (O'Neill et al., 2010). Trunk blood was obtained and hippocampal brain regions were rapidly dissected 24 h following dosing of Mn^{III}TDE-2-ImP^{5+} (5 mg/kg, s.c. q4h). Samples were randomized and blinded before detection of Mn^{III}TDE-2-ImP^{5+} was performed using HPLC (as described in O'Neill et al. (2010)).

2.5. Measurement of redox biomarkers by HPLC

Glutathione (GSH), glutathione disulfide (GSSG), tyrosine and 3-nitrotyrosine (3-NT) assays were performed 24 h after SE, blinded with an ESA (Chelmsford, MA) 5600 CoulArray HPLC equipped with eight electrochemical cells following the company instruction (ESA Application Note 70-3993) and previously described in the literature with small modifications (Lakritz et al., 1997; Liang and Patel, 2006). The potentials of the electrochemical cells were set at 400/450/500/570/630/690/830/860 mV vs. Pd. Analyte separation was performed using a TOSOHAAS (Montgomeryville, PA) reverse-phase ODS 80-TM C-18 analytical column (4.6 mm × 250 cm; 5 μm particle size). A two-component gradient elution system was used with component A of the mobile phase composed of 50 mM NaH_2PO_4 pH 3.0, and component B composed of 50 mM NaH_2PO_4 and 50% methanol pH 3.0. An aliquot (20 μl) of the supernatant was injected into the HPLC. The level of 3-NT was expressed as a ratio of 3-NT to tyrosine. Samples were randomized and blinded before assessment.

2.6. Assessment of mitochondrial function

Synaptosomes were isolated from the hippocampus of control and treated animals and mitochondrial oxygen consumption ratios (OCR) 24 h after SE and assessed using an Extracellular Flux Analyzer (Seahorse) according to methods previously described (Rowley et al., 2015). All samples were blinded and randomized before assessment.

2.7. Fluoro-Jade B analysis

Frozen sections (20 μm) were blinded, cut coronally through hippocampus and stained with Fluoro-Jade B (Histo-Chem) as previously described (Schmued and Hopkins, 2000). Briefly, the sections were hydrated and immersed in a solution of alcohol and 1% sodium hydroxide for 5 min. After a wash, the sections were placed into a solution of 0.06% potassium permanganate for 10 min and washed again for 2 min. The staining solution (0.004% Fluoro-Jade B in 0.1% acetic acid vehicle) was prepared fresh. After a 15 min incubation in the staining solution, the sections were rinsed and dried. The sections were cleared by xylene for at least a minute before coverslipping with DPX. Imaging was performed using a Nikon Eclipse TE2000-U microscope. Using Image-J, the Fluoro-Jade B positive signal of areas of interest (Hippocampal areas CA1, CA3 and hilus) was measured. The average of the fluorescent relative density was quantified for each animal and the group average was expressed as percentage of the control.