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Scavenging reactive oxygen species inhibits status epilepticus-induced neuroinflammation

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Abstract

Inflammation has been identified as an important mediator of seizures and epileptogenesis. Understanding the mechanisms underlying seizure-induced neuroinflammation could lead to the development of novel therapies for the epilepsies. Reactive oxygen species (ROS) are recognized as mediators of seizure-induced neuronal damage and are known to increase in models of epilepsies. ROS are also known to contribute to inflammation in several disease states. We hypothesized that ROS are key modulators of neuroinflammation i.e. pro-inflammatory cytokine production and microglial activation in acquired epilepsy. The role of ROS in modulating seizure-induced neuroinflammation was investigated in the pilocarpine model of temporal lobe epilepsy (TLE). Pilocarpine-induced status epilepticus (SE) resulted in a time-dependent increase in pro-inflammatory cytokine production in the hippocampus and piriform cortex. Scavenging ROS with a small-molecule catalytic antioxidant decreased SE-induced pro-inflammatory cytokine production and microglial activation, suggesting that ROS contribute to SE-induced neuroinflammation. Scavenging ROS also attenuated phosphorylation of ribosomal protein S6, the downstream target of the mammalian target of rapamycin (mTOR) pathway indicating that this pathway might provide one mechanistic link between SE-induced ROS production and inflammation. Together, these results demonstrate that ROS contribute to SE-induced cytokine production and antioxidant treatment may offer a novel approach to control neuroinflammation in epilepsy.

Keywords

epilepsy; reactive oxygen species; neuroinflammation; antioxidant; cytokine; mammalian target of rapamycin; microglial activation; pilocarpine

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INTRODUCTION

Neuroinflammation is a hallmark of several neurological disorders such as epilepsy, Parkinson's disease, Alzheimer's disease and stroke (Amor et al., 2010; Floyd, 1999). The role of inflammatory processes in the etiology of epilepsy has gained considerable attention in the past decade (Aronica and Crino, 2011; Vezzani et al., 2011). Epilepsy is one of the most common neuronal disorders affecting approximately 1% of the population in the United States. Status epilepticus (SE) is a medical emergency arising due to systemic factors or chemical exposures. SE, like traumatic brain injury, hypoxia-ischemia or infection can result in the development of chronic epilepsy. Various biochemical, physiological and structural changes such as neuronal death, axonal sprouting, neurogenesis, and gliosis are initiated following SE leading to network excitability and spontaneous, recurrent seizures by a process known as epileptogenesis (Loscher and Brandt, 2010). Neuroinflammation occurs in human and experimental TLE and is primarily characterized by robust astrogliosis, microglial activation and the production of cytokines and chemokines (Vezzani, 2014; Vezzani et al., 2012). Key evidence supporting the potential role of inflammatory processes in the epilepsies comes from the demonstration that (1) anti-inflammatory therapies have been shown to exert anticonvulsant effects in drug-resistant human epilepsies (Granata et al., 2003; Riikonen, 2003), (2) several mediators have been found in tissue surgically resected from patients with refractory TLE (Baranzini et al., 2002; Hulkkonen et al., 2004), (3) seizure activity *per se* can cause the activation of the resident immune cells of the brain or microglia, leading to the production of inflammatory cytokines (Riazi et al., 2010) and (4), spontaneous seizures can also perpetuate chronic inflammation (Vezzani et al., 2012). For example, chemically and electrically-induced seizures have shown increased levels of cytokines in the rodent brain (De Simoni et al., 2000; Lehtimäki et al., 2003; Minami et al., 1991). Additional evidence supporting the role of inflammation in epilepsy comes from studies involving transgenic mice overexpressing interleukin-6 (IL-6) or tumor necrosis factor- α (TNF- α), revealing that chronic inflammation can result in several pathological changes including seizures (Akassoglou et al., 1997; Campbell et al., 1993). Therefore, inflammation is not only a consequence of seizure activity but has also been shown to contribute to epileptogenesis. Given that inflammation plays a key role in epilepsy, it is important to understand the detailed mechanisms underlying SE-induced neuroinflammation and identify novel therapeutic avenues for controlling it. One novel mechanism that has recently been linked to inflammation is the production of reactive species and consequent redox alterations.

Pro-inflammatory processes such as cytokine production can be modulated by ROS and redox status (Bulua et al., 2011; Turens, 2003). ROS have been examined in the etiology of seizures and epileptogenesis (Rowley and Patel, 2013). ROS play a physiological role in cellular processes via redox signaling as well as a deleterious role via oxidation of cellular macromolecules, leading to cellular dysfunction and death (Andersen, 2004). The importance of ROS and redox status in epilepsy comes from several lines of evidence. First, studies have demonstrated that prolonged seizure activity can cause an increase in superoxide ($O_2^{\cdot-}$) and hydrogen peroxide from both mitochondria and extracellular sources such as NADPH oxidases (Nox2), leading to oxidative damage to cellular macromolecules

and ultimately neuronal damage (Liang et al., 2000; Liang and Patel, 2006; Patel et al., 2005; Williams et al., 2015). Secondly, oxidative stress and alterations to the glutathione redox status have been shown to occur throughout the course of epileptogenesis in experimental models of TLE (Rowley et al., 2015; Ryan et al., 2014; Waldbaum et al., 2010). Additionally, pharmacological scavenging of seizure-induced ROS inhibits neuronal death, improves mitochondrial function and cognitive function in chemoconvulsant models of TLE (Pearson et al., 2015; Rowley et al., 2015). Finally, we have recently demonstrated the occurrence of oxidative and nitrative stress in a mouse model of virus-induced TLE by Theiler's murine encephalomyelitis virus (TMEV) infection, where seizures arise because of inflammation (Bhuyan et al., 2015). Therefore, both inflammation and oxidative stress occur in various animal models of epilepsy. However, whether SE-induced ROS and redox alterations contribute to neuroinflammation is unknown. We hypothesized that SE-induced ROS contribute to pro-inflammatory cytokine production. The following observations support the hypothesis. (1) ROS production is critical to the development of several diseases with a major inflammatory component such as chronic obstructive pulmonary disease (COPD), inflammatory bowel disease, acute pancreatitis and hypertension (Escobar et al., 2012; Rahman and Adcock, 2006; Vaziri, 2008; Zhu and Li, 2012). (2) $O_2^{\cdot-}$ derived from NADPH oxidases as well as the mitochondrial electron transport chain (ETC) is sufficient to increase the production of pro-inflammatory cytokines (Bulua et al., 2011; Turrens, 2003). (3) Changes in the redox potential of tissues has also been demonstrated to modulate the release of pro-inflammatory cytokines (Iyer et al., 2009).

The goals of this study were to determine 1) the time-course of inflammatory cytokine production following pilocarpine-induced SE, 2) if pharmacological removal of ROS mitigates SE-induced cytokine production and microglial activation and 3) which redox-sensitive signaling pathway that is also involved in inflammation, is affected by removal of seizure-induced ROS. SE sufficient to cause epilepsy resulted in a time- and seizure-dependent upregulation of pro-inflammatory cytokines. Catalytic removal of ROS utilizing a small-molecule antioxidant inhibited SE-induced cytokine production, activation of microglial cells and activation of the mammalian target of rapamycin (mTOR) pathway.

METHODS

Reagents

All materials were purchased from Sigma or Fisher Scientific unless otherwise mentioned. Manganese (III) meso-tetrakis (di-N-ethylimidazole) porphyrin or $Mn^{III}TDE-2-ImP^{5+}$ (known as AEOL 10150 in the literature) was obtained from Aeolus Pharmaceuticals.

Induction of status epilepticus (SE)

All animal housing and treatments were conducted in compliance with NIH guidelines and following protocols approved by the Institutional Animal Care and Use Committee at the University of Colorado Anschutz Medical Campus. Adult male Sprague Dawley rats (~250–300 grams) were purchased from Harlan Laboratories (Indianapolis, Indiana). After a week of acclimation, rats were randomly assigned to saline or pilocarpine group. Pilocarpine rats were treated with methylscopolamine (1 mg/kg) intraperitoneally (i.p.) to mitigate the

peripheral cholinergic effects of pilocarpine, thirty minutes prior to subcutaneous (s.c.) injection with pilocarpine hydrochloride (340 mg/kg) to induce SE. Diazepam (10 mg/kg) was administered i.p., 90 mins after pilocarpine to attenuate SE. Age-matched control rats received scopolamine and saline instead of pilocarpine. Rats were sacrificed by CO₂ inhalation followed by immediate decapitation using a guillotine suitable for adult rats at 6 hours (h), 24 h, 48 h, 1 week (wk) and 6 wks following pilocarpine treatment and hippocampi and piriform cortices were dissected out for biochemical analyses.

Monitoring of behavioral seizures

Behavioral seizures were evaluated by direct observation for 6 h after initial injection and scored according to a modified Racine scale (Racine et al., 1972). Briefly, motor seizure severity was classified as follows: Class III animals displayed forelimb clonus with lordotic posture; class IV animals reared with concomitant forelimb clonus; and class V animals, in addition to having a class IV seizure, fell over. Only rats having at least 3 class III convulsive seizures every hour up to 3 h were included in the study. Direct observation confirmed that animals were having very few seizures 48 h post pilocarpine and no convulsive activity 1 wk post pilocarpine. Rats were also video monitored for 48 h prior to euthanasia in order to confirm epilepsy at the 6 wk time-point, by assessing behavioral seizures. Only animals with at least two class IV seizures according to the Racine scale were included in the study as responders. To determine the role of SE in neuroinflammation, cytokines were analyzed in the brains of rats which did not undergo SE (termed as non-responders).

Metalloporphyrin administration

Pilocarpine treated rats exhibiting SE (according to the above mentioned criteria), were treated with Mn^{III}TDE-2-ImP⁵⁺ (5 mg/kg, s.c.) also denoted in the literature as AEOL 10150 or saline for controls, starting 60 minutes after pilocarpine and every 4 hours until sacrifice (approximately 6 injections over a 24 hour time period). Mn^{III}TDE-2-ImP⁵⁺ was dissolved in phosphate buffered saline to achieve a final concentration of 5mg/ml. Animals were divided into 4 groups: 1) Saline-Saline 2) Pilocarpine-saline 3) Pilocarpine-Mn^{III}TDE-2-ImP⁵⁺ and 4) Saline-Mn^{III}TDE-2-ImP⁵⁺. Saline- Mn^{III}TDE-2-ImP⁵⁺ group had no difference compared to saline-saline animals (data not shown). Animals were sacrificed at 24 and 48 h for biochemistry and pathology.

Measurement of oxidative stress markers by HPLC

Glutathione (GSH), glutathione disulfide (GSSG), tyrosine (Tyr) and 3-nitrotyrosine (3NT) assays were performed with an ESA (Chelmsford, MA) 5600 CoulArray HPLC equipped with eight electrochemical cells as previously described in the literature (Hensley et al., 1998; Lakritz et al., 1997; Liang et al., 2007). One hippocampus from each rat was sonicated in 0.1N ice cold perchloric acid (PCA) and centrifuged at 13,000rpm for 10 minutes and supernatants collected. The potentials of the electrochemical cells were set at 400/450/500/570/630/690/830/860 mV vs. Pd. Analyte separation was conducted on a TOSOHAAS (Montgomeryville, PA) reverse-phase ODS 80-TM C-18 analytical column (4.6 mm × 250 cm; 5 µm particle size). The mobile phase was composed of a two-component gradient elution system with component A of the mobile phase consisting of 50

mM NaH₂PO₄ pH 3.0, and component B consisting of 50 mM NaH₂PO₄ and 50% methanol pH 3.0. Aliquots (20 µl) of the supernatant were injected into HPLC. The level of 3-NT was expressed as a ratio of 3-NT to Tyr.

Multiplex pro-inflammatory cytokine measurement

Levels of the pro-inflammatory cytokines, TNF- α , IL-1 β , IL-6 and KC/GRO (keratinocyte chemoattractant/growth-related oncogene), were measured using a rat multiplex pro-inflammatory cytokine array from Mesoscale Discovery (MSD) according to manufacturer's instructions. Briefly, the assay was run as follows. One hippocampus from each rat was lysed in MSD Tris Lysis buffer supplemented with protease and phosphatase inhibitors in a 1:10 ratio. The lysates were then centrifuged at 13,000rpm for 10 minutes and supernatants collected. Protein concentrations were determined in the supernatants using a Bradford protein assay and 250µg of protein were loaded per well for each sample in duplicate. Calibration curves were prepared in the supplied diluent with a range of 40,000 pg/ml to 2.45 pg/ml. Prior to incubation with samples, wells were blocked for 30 minutes, following which samples were added to the wells and incubated with shaking (300–1000rpm) for 2 hours. The plate was then washed with PBS + 0.05% Tween 20 and 25µl of detection antibody was added and incubated for an additional 2 hours. Finally, the plate was washed, MSD read buffer was added to the wells and the plate was read using Sector Imager 2400. Concentrations of analytes were determined by the measuring the intensity of light emitted at 620nm and Softmax Pro software using curve-fit models.

Immunohistochemical staining for microglial activation

Whole brains were fixed in 10% formalin for 48 hours, dehydrated and embedded in paraffin wax before cutting coronally into 12µm thick sections. Sections were deparaffined with Hemo D (xylene) and rehydrated using 100% ethanol for 5 minutes, 70% ethanol for 2 minutes and dH₂O for 1 minute. They were then incubated with the primary Iba1 antibody (ionized calcium binding adaptor molecule 1; Wako, Code No. 019–1971) at a dilution of 1:500 in 1% goat serum PBS-T (phosphate buffered saline with tween 20) overnight, following which sections were rinsed in PBS and incubated with the secondary antibody (Rhodamine Red goat anti-rabbit conjugated, 1:100 in PBS-T) for 60 minutes. Sections were rinsed again, excess water drained, air dried, mounted on cover slips with DPX (Fluka) non-fluorescent mounting media and image on a fluorescent microscope.

Immunoblotting

Hippocampal homogenates from rats treated with saline and pilocarpine, with or without Mn^{III}TDE-2-ImP⁵⁺ were utilized to blot for the expression of the phospho- and total forms of S6, ERK and JNK. 25µg of protein was run on a 4–20% gradient gel before transferring on to a polyvinylidene difluoride membrane (PVDF) membrane of 0.2µm pore size (Bio-Rad). The membrane was then blocked in 5% nonfat dry milk (Bio-Rad) and probed at 4°C overnight with rabbit polyclonal antibodies against p-S6, p-JNK and p-ERK (Cell Signaling) at a 1:1000 dilution. This was followed by washing in TBS-T prior to incubating with horse-radish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:10,000) for 1 h and developed using ECL reagent (Pierce) on a Bio-Rad Chemidoc Imaging system. Bands were stripped using Restore Western Blot Stripping Buffer (Thermo

Scientific), reprobed with antibodies against total forms of S6, JNK and ERK (1:1000; Cell Signaling) and developed in the same manner as the phospho proteins. Quantification of the bands was performed by using Image Lab software (Bio-Rad). *Statistical Analysis* All data are expressed as mean $\pm$ SEM. Statistical differences were analyzed by two-tailed t test or one-way ANOVA with post hoc Tukey or Dunnett's tests. P values less than 0.05 were considered statistically significant. All analyses were performed using Prism 5 software (Prism 5. GraphPad Software, San Diego, CA).

RESULTS

Status Epilepticus (SE) induced pro-inflammatory cytokine production

Neuroinflammation is a well-known consequence of seizure activity. Studies from several animal models have demonstrated that pro-inflammatory cytokine expression is increased after SE (Ravizza et al., 2008; Vezzani and Granata, 2005). We determined the time-course of changes in proinflammatory cytokines (TNF- α , IL-1 β , IL-6 and KC/GRO) in the hippocampi and piriform cortices, two regions of the brain most affected in the pilocarpine model, using a multiplex proinflammatory cytokine array from Mesoscale Discovery in a model of pilocarpine-induced epilepsy. We observed significant increases in the levels of all the analytes at 6 and 24 h with a return to control levels at the latent time point (1 wk), in the hippocampus, as shown in figure 1A–D. Additionally, levels of IL-6 and KC/GRO were still upregulated significantly at the 48 h time point in the hippocampus. Control values (mean $\pm$ SEM) of TNF- α , IL-1 β , IL-6 and KC/GRO were 21.69 ± 6.07 , 3.99 ± 1.157 , 38.06 ± 9.437 and 5.254 ± 1.613 pg/ml (per 250 μ g protein), respectively.

In the piriform cortex, we observed similar changes as hippocampus with the levels of all the analytes being upregulated at 6 and 24 h and those of IL-6 and KC/GRO at 48 h (Fig. 1E–H). IL-1 β levels were also significantly elevated at 48 h in the piriform cortex after pilocarpine injection. The control values (mean $\pm$ SEM) in the piriform cortex for TNF- α , IL-1 β , IL-6 and KC/GRO were 54 ± 14.01 , 3.966 ± 1.092 , 138.7 ± 25.29 and 5.764 ± 0.926 pg/ml (per 250 μ g protein), respectively. Interestingly, the peak induction of TNF- α , IL-1 β and KC/GRO in the piriform cortex appeared to occur at the 6 h time-point whereas peak induction in the hippocampus appeared to occur at the 24 h time point. These findings suggest a differential temporal expression of the cytokines in two different brain regions affected by pilocarpine-induced SE, with the piriform cortex showing an earlier induction of SE-induced proinflammatory cytokines compared to the hippocampus. To determine the role of seizure activity in neuroinflammation, we analyzed cytokines in the piriform cortices of rats injected with pilocarpine that did not undergo SE i.e. non-responders. No statistically significant increases in cytokine levels were observed in vehicle controls vs pilocarpine-treated non-responders (piriform cortex cytokine levels in control and pilocarpine-injected non-responder rats expressed as mean $\pm$ SEM, per 250 μ g protein, n=6 control rats and n=4 non-responder rats were as follows: TNF- α , 54 ± 14.01 and 25.34 ± 2.09 , IL-1 β , 3.966 ± 1.092 and 4.42 ± 0.19 , IL-6, 138.7 ± 25.29 and $137.42.7 \pm 5.24$ and KC/GRO, 5.764 ± 0.926 and 7.03 ± 1.04 pg/ml). Since pilocarpine primarily induces seizures via M1 receptors (Hamilton et al., 1997) but activates multiple muscarinic receptors including M3 receptors which have been linked to proinflammatory signaling (Xu et al., 2013), we

assessed pilocarpine-induced cytokine induction in the presence of atropine, a centrally-active, non-specific muscarinic receptor antagonist instead of scopolamine which only targets peripheral muscarinic receptors. The data demonstrate that AEOL10150 inhibits pilocarpine-induced cytokine induction in the piriform cortex in the presence of atropine sulfate (data not shown).

Inflammatory cytokine production in the hippocampi of chronically epileptic rats

We measured the levels of TNF- α , IL-1 β , IL-6 and KC/GRO in the hippocampus of epileptic rats 6 wks after injection with saline or pilocarpine. Only rats that had at least two class IV seizures were included in this study. There was an increase by ~80%, 67% and 193% over controls in TNF- α , IL-1 β and KC/GRO levels respectively, whereas IL-6 levels remained unchanged (Fig. 2). This data demonstrates differential upregulation of certain cytokines in the hippocampus in epileptic rats. However, these increases were not as high in magnitude as the ones observed shortly after SE. This could be attributed to the difference in the intensity of seizures in the acute and chronic phase, where the former is characterized by more intense seizure activity. Additionally, spontaneous, epileptic seizures are variable in severity as well as number amongst the rats injected with pilocarpine. Taken together, these data suggest that seizure activity may be a key driver of pro-inflammatory cytokines in the pilocarpine model of epilepsy.

Catalytic antioxidant treatment attenuates SE-induced oxidative stress and induction of proinflammatory cytokines

Oxidative stress and tissue redox balance have been implicated in controlling the release of pro-inflammatory molecules in several disease states (Haddad, 2002; Yao et al., 2007; Zhu and Li, 2012). Previous work in our laboratory has demonstrated an increase in oxidative stress in chemoconvulsant animal models. To test the hypothesis that ROS mediate SE-induced neuroinflammation, a catalytic antioxidant Mn(III)tetrakis-(*N,N*'diethylimidazolium-2-yl)porphyrin (Mn^{III}TDE-2-ImP⁵⁺) was used to pharmacologically scavenge seizure-induced ROS. Mn^{III}TDE-2-ImP⁵⁺ is a water-soluble manganese metalloporphyrin, with high superoxide dismutase (SOD) and catalase activities. This compound possesses the capacity to catalytically scavenge O₂⁻, hydrogen peroxide, peroxynitrite and lipid peroxides. We have previously shown that 5mg/kg, s.c. injection of Mn^{III}TDE-2-ImP⁵⁺ 60 min after pilocarpine SE did not interfere with SE (Pearson et al., 2015). Using this dosing paradigm, we first confirmed the ability of this compound to inhibit SE-induced oxidative stress by measuring the levels of the most abundant non-protein thiol glutathione (GSH) and its oxidized form GSSG. Compared to saline-injected controls, rats subjected to pilocarpine-induced SE showed significant decreases in the levels of GSH by 43.47% in the hippocampus at 24 h. This effect was significantly reversed by treatment with 5mg/kg of Mn^{III}TDE-2-ImP⁵⁺ (increased by 33.33% compared to pilocarpine alone), as illustrated in Fig. 3A. Similarly, in the piriform cortex, there was a 37.55% reduction in GSH levels (Fig. 3C) which was rescued with antioxidant treatment (25.1%). GSSG was significantly increased by 67.5% in the pilocarpine alone group (Fig. 3D) and decreased by 22.71% with antioxidant treatment (not significant). Pilocarpine treatment resulted in a 59.8% decrease in the GSH/GSSG ratio compared to saline injected control rats, which was significantly reversed with Mn^{III}TDE-2-ImP⁵⁺ (38.9% decrease compared to pilocarpine

alone), demonstrated in figure 3E. Furthermore, we measured levels of 3-Nitrotyrosine (3NT), which is a marker of protein nitration, a posttranslational modification specific to tyrosine residues of amino acids that can lead to protein dysfunction. A major source of protein nitration is the formation of peroxynitrite by a reaction between nitric oxide and O_2^- . The levels of 3NT/Tyr were significantly increased in the hippocampi of pilocarpine treated rats by 86.1% compared to saline controls at the 24h time point (figure 3B) and attenuated by the catalytic antioxidant treatment by 51.8%. In the piriform cortex, pilocarpine caused a substantial increase in 3NT/Tyr ratio by 250.86% compared to saline injected controls, which was significantly attenuated by 39.79% treatment with the catalytic antioxidant at 24 h (Fig. 3F).

Catalytic antioxidant treatment attenuates SE-induced pro-inflammatory cytokine production

To address the hypothesis that seizure-induced ROS contribute to neuroinflammation in the pilocarpine model, the effect of $Mn^{III}TDE-2-ImP^{5+}$ was assessed 24 h after pilocarpine-induced SE when both oxidative stress and cytokine production have the highest induction in the hippocampus at this time point. Pilocarpine-induced SE caused significant increases in TNF- α , IL-1 β , IL-6 and KC/GRO levels at 24 h in the hippocampus, as observed in the prior experiment. The production of TNF- α (Fig. 4A) and IL-1 β (Fig. 4B) in the hippocampus was reduced by approximately 54.4% and 50% respectively upon treatment with the antioxidant, compared to the pilocarpine group. However, there was no effect of the drug treatment on IL-6 (Fig. 4C) and KC/GRO (Fig. 4D) levels. In the piriform cortex, antioxidant treatment resulted in significant decreases in levels of TNF- α (56.31%), IL-1 β (62.13%), IL-6 (64.9%) and KC/GRO (69.12%) at the 24 h (Fig. 4E–H), compared to pilocarpine alone group. Therefore, antioxidant treatment significantly attenuated SE-induced pro-inflammatory cytokines.

Catalytic antioxidant treatment attenuates SE-induced microglial activation

Microglial cells, the resident macrophages in the brain, are one of the major sources of pro-inflammatory cytokines. Microglial activation or microgliosis is a well-described feature of TLE and studies have suggested that it might contribute to epileptogenesis (Shapiro et al., 2008; Vezzani et al., 1999). In addition to cytokines, activated microglia can also produce ROS and RNS as well as other cytotoxic factors (Burguillos et al., 2011; Choi and Koh, 2008; Purisai et al., 2007). Rats injected with pilocarpine showed significant Iba1 staining indicating microglial activation in the CA3 and hilar regions of the hippocampus at 24 h which was inhibited by $Mn^{III}TDE-2-ImP^{5+}$ (Fig. 5). Pilocarpine-induced SE resulted in a 141.79% increase in Iba1 immunofluorescence in the CA3 region compared to saline injected animals which was attenuated by 25.7% in the pilocarpine+ $Mn^{III}TDE-2-ImP^{5+}$. In the hilus, there was an approximate 200% increase in Iba1 fluorescence intensity in rats injected with pilocarpine, which was attenuated by ~39% upon antioxidant administration (Fig. 5A, 5B).

Catalytic antioxidant treatment attenuates phosphorylation of S6 ribosomal protein

There are multiple cellular signaling pathways that link ROS production with neuroinflammation. We first determined which redox-sensitive signaling pathways known to

play a role in inflammation were activated following pilocarpine-induced SE. The activation of the NF- κ B and MAPK pathways were assessed at the 24 h time point. There was no significant upregulation of p-p65, p-p38 (data not shown) or p-JNK (Fig. 6A) 24 h after pilocarpine in the hippocampus. By contrast, p-ERK was significantly upregulated 24 h after pilocarpine (Fig. 6B).

An attractive candidate signaling pathway which has been shown to be altered by redox, metabolic and inflammatory changes is the mammalian target of rapamycin (mTOR) pathway (Russo et al., 2012). In fact, mTOR pathway has also been implicated to play a role in SE-induced epileptogenesis (Huang et al., 2010; Zeng et al., 2009). To determine if the mTOR pathway is activated in the pilocarpine model, we performed western blotting to assess phosphorylation of S6 ribosomal protein, a downstream target of mTOR. We found ~1000% increase in p-S6 levels in the hippocampus of pilocarpine treated rats compared to saline injected controls at 24 h (Fig. 7), as previously published data in the pilocarpine model (Huang et al., 2010). Furthermore, treatment with Mn^{III}TDE-2-ImP⁵⁺ significantly attenuated pilocarpine-induced phosphorylation of S6. However, the levels of p-ERK another redox sensitive pathway were not significantly inhibited by Mn^{III}TDE-2-ImP⁵⁺.

DISCUSSION

This study demonstrates for the first time, that ROS play a crucial role in controlling SE-induced inflammatory responses in experimental models of TLE. The studies show that (1) pilocarpine-induced SE leads to increased production of pro-inflammatory cytokines acutely and (2), treatment with a catalytic antioxidant Mn^{III}TDE-2-ImP⁵⁺ after the onset of SE inhibits oxidative stress, inflammatory cytokine production, microglial activation, and mTOR activation. The findings of this study and the proposed mechanism by which ROS contribute to SE-induced neuroinflammation have been summarized in figure 7.

There is ample evidence in the literature that animal models of chemoconvulsant-induced TLE display increased markers of neuroinflammation. Ravizza et al., demonstrated that IL-1 β and its receptor IL-1R1 were upregulated acutely in microglia and astrocytes in the lithium-pilocarpine (Li-pilo) model of TLE (Ravizza et al., 2008). In a separate study also in the Li-pilo model, the immunohistochemical expression of IL-1 β , COX-2 and NF- κ B increased during the acute phase (Voutsinos-Porche et al., 2004). Additionally, in a pilocarpine model similar to ours, increased mRNA expression of several inflammatory factors such as toll-like receptor 2 (TLR2), I κ B- α , TNF- α and COX-2 were observed (Turrin and Rivest, 2004). Similar to these literature reports, we observed an acute upregulation of several pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6 and KC/GRO) in the hippocampus and the piriform cortex, two regions of the brain most affected in the pilocarpine model. The temporal pattern of cytokine production, correlates with recent seizure activity in that animals showed highest cytokine induction shortly after SE with return to control values during the latent period when behavioral seizures are minimal and returning to above baseline values when chronic epilepsy was observed. Furthermore, pilocarpine-treated rats that did not undergo full SE did not show increases in cytokine production in the piriform cortex, at the early time points which suggest a role of SE in the mechanism. These results are in accordance with previously published papers where seizure

activity *per se* can cause the increase in cytokine release and levels of cytokines return to basal levels during the latent period (Vezzani et al., 2012; Vezzani et al., 2011; Voutsinos-Porche et al., 2004). However, these previous publications utilized immunohistochemical staining techniques and did not provide quantification for the observations. To the best of our knowledge, the results described in this study provide for the first time, a quantifiable measure of the protein levels of pro-inflammatory cytokines in the pilocarpine model using a sensitive and reliable multiplex assay.

The increases in pro-inflammatory cytokines follows a similar pattern as the production of ROS, mitochondrial complex I inactivation, post-translational modifications and occurrence of mitochondrial respiration deficits in chemoconvulsant models of TLE (Jarrett et al., 2008; Pearson et al., 2015; Rowley et al., 2015; Ryan et al., 2012). It is also somewhat reminiscent of the time course of alterations in tissue redox status following kainic acid or pilocarpine-induced SE (Ryan et al., 2014; Waldbaum et al., 2010) suggesting a relationship between ROS production, redox state and inflammation. Chronic induction of pro-inflammatory cytokines has been previously shown to occur in the lithium-pilocarpine model (Ravizza et al., 2008). However, this was also achieved by immunohistochemical methods without providing quantification of the staining, making it difficult to ascertain the extent of chronic inflammation in this study. Therefore, our findings provide a quantitative measure of pro-inflammatory cytokine induction during the chronic stage of epileptogenesis.

In this study we utilized a pharmacological approach i.e. treatment with MnIIIITDE-2-ImP5⁺, small molecule catalytic antioxidant to demonstrate that ROS contribute to the release of pro-inflammatory cytokines resulting from SE. This compound has high SOD and catalase activities and therefore can scavenge a wide range of reactive species including superoxide (O₂⁻), hydrogen peroxide (H₂O₂), peroxynitrite (ONOO⁻) and lipid peroxides (Day, 2004). Treatment with this compound after the onset of SE ameliorated inflammatory cytokine production as well as microgliosis in pilocarpine injected rats. Recent publication from our laboratory has demonstrated that Mn^{III}TDE-2-ImP5⁺ penetrates the BBB and attains effective concentrations in the brain, without interfering with pilocarpine-induced SE (Pearson et al., 2015). It is important to emphasize that MnIIIITDE-2-ImP5⁺ does not directly interfere with SE when administered 60 after pilocarpine injection (Pearson et al., 2015), suggesting that its effects on inflammatory cytokine production was not due to its effect on ongoing SE. Our results are also in agreement with previous demonstration of efficacy of this compound against expression of inflammatory markers in a rat model of 2-chloroethyl ethyl sulfide (CEES)- induced lung injury (O'Neill et al., 2010), a mouse model of stroke (Bowler et al., 2002) and mouse model of chlorine-induced lung injury (McGovern et al., 2011). Additionally, these studies also demonstrated the efficacy of the antioxidant in inhibiting oxidative stress (Castello et al., 2008; O'Neill et al., 2010; Tewari-Singh et al., 2014). We confirmed its antioxidant action in the pilocarpine model using the tissue samples from the same rats that were utilized for the multiplex cytokine analysis, indicating that the effect of the compound on inflammation is most likely due to its effect on oxidative stress, confirming target engagement by the drug. To date, there is no evidence that the compound has any direct anti-inflammatory effects. Together, this data suggests that inhibition of oxidative stress results in the reduction of inflammatory cytokine production and microglial activation.

Microglial activation can lead to the production of cytotoxic factors in addition to inflammatory mediators (Block and Hong, 2005; Boje and Arora, 1992) and their combined effects could contribute to neuronal death in animal models of TLE (Devinsky et al., 2013; Ravizza et al., 2005). We have previously shown that Mn^{III}TDE-2-ImP⁵⁺ attenuates pilocarpine-induced cell death in the CA3 and hilar regions of the hippocampus, in a pattern similar to microglial activation in the present study (Pearson et al., 2015). Since inflammatory mechanisms can play a crucial role in seizure-induced neuronal death, our findings suggest that SE-induced oxidative stress contributes to microglial activation and inflammatory cytokine production which could lead to consequent neuronal damage and associated comorbidities, shedding more light on the biochemical events underlying the pathogenesis of seizures.

There are two potential sources of ROS that are produced from SE which could be contributing to the inflammatory responses – mitochondrial ROS and extracellular ROS from activation of NADPH oxidase (Nox2) (Liang et al., 2000; Patel et al., 2005; Williams et al., 2015). Mitochondria have been shown to be a source of seizure-induced ROS formation based on evidence that mitochondrial, but not whole tissue, cytosolic or nuclear targets are disproportionately oxidized following SE (Liang et al., 2000; Jarrett et al., 2008; Liang et al., 2006). Utilizing mice either overexpressing or deficient in Sod2, we have previously shown that mitochondrial ROS contribute to seizure-induced neuronal damage (Liang et al., 2000). Moreover, heterozygous Sod2^{-/+} mice showed exacerbation of neuronal death and lower seizure threshold due to an increase in steady-state mitochondrial ROS (Liang et al., 2012). The role of extracellular ROS in SE-induced neuronal damage is highlighted by studies in the kainate model of TLE which demonstrated that activation of Nox2 occurred in parallel to microglial activation, lending support to the hypothesis that microglia might be a source of Nox-derived O₂⁻ (Patel et al., 2005). Several groups have also shown Nox2's involvement in NMDAR-induced ROS production (Brennan et al., 2009; Girouard et al., 2009; Kovac et al., 2014) which partially contribute to SE. However, whether mitochondrial- or Nox2-driven ROS contribute to SE-induced neuroinflammation remained to be further studied. Since, Mn^{III}TDE-2-ImP⁵⁺ is a broad spectrum antioxidant and targets ROS from multiple sources, it is difficult to delineate the exact source of ROS that is affecting inflammation in our studies. Nevertheless, there is evidence of cross-talk between NADPH oxidases and the mitochondria in mediating inflammatory responses (Dikalov, 2011) and therefore, should be taken into consideration while defining the redox events controlling seizure-induced neuroinflammation.

There are multiple mechanisms linking ROS production to the release of proinflammatory molecules. These can include but are not limited to activation of redox-sensitive transcription factors such as nuclear factor kappa b (NF-κB) or by increasing the phosphorylation of redox-sensitive proteins such as the MAP kinases, both of which can lead to the production of inflammatory mediators (Flohe et al., 1997; Kaminska, 2005; Son et al., 2011). Our data suggest that the mTOR pathway may be the common mediator of SE-induced oxidative stress based on the finding that treatment with the catalytic antioxidant inhibited SE-induced phosphorylation of S6 but not p-ERK. Ample evidence in the literature suggests that a redox-sensitive mechanism can regulate mTOR signaling whereby treatment with oxidizing agents can increase S6 phosphorylation and that with a reducing

agent inhibits the process (Sarbasov and Sabatini, 2005). Chen et al have also demonstrated the role of ROS in activating the mTOR pathway in an *in-vitro* model of cadmium toxicity (Chen et al., 2011). Interestingly, the mTOR pathway has also been implicated in regulating innate immune responses. Activation of mTOR can trigger the release of cytokines from mast cells and neutrophils. Moreover, rapamycin has potent immunosuppressive properties (Weichhart et al., 2015) and the mTOR pathway is a target of several immunosuppressive therapies. The PI-3 kinase-AKT-mTOR pathway has also been implicated in IL-1 β mediated NF- κ B activation (Jung et al., 2003). Taken together, this suggests that oxidative stress might contribute to the activation of this pathway in the pilocarpine model and provide us with a potential signaling pathway that might be linking the redox events and immune responses observed in this model. The mTOR signaling pathway has gained considerable attention with regards to both acquired and genetic epilepsies. It is involved in several disorders associated with epilepsy such as, tuberous sclerosis (TSC), focal cortical dysplasia and ganglioma (Wong, 2008). Additionally, inhibition of the mTOR pathway with rapamycin has shown beneficial effects in the kainate and pilocarpine models of chemoconvulsant-induced TLE (Huang et al., 2010; Zeng et al., 2009). Our results highlight the importance of redox mechanisms in driving SE-induced neuroinflammation and suggest a novel approach to mitigate the harmful inflammatory responses associated with it.

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Highlights

- Status epilepticus increases proinflammatory cytokines in a time- and seizure-dependent manner.
- Proinflammatory cytokines do not increase during seizure-free latent period.
- A catalytic antioxidant inhibits status epilepticus-induced oxidative stress and neuroinflammation.
- Redox modulation is a novel approach for controlling neuroinflammation in epilepsy.

Fig. 1. Production of pro-inflammatory cytokines in the acute and latent stages of epileptogenesis
Levels of TNF- α , IL-1 β , IL-6 and KC/GRO were measured in the hippocampus (A–D) and piriform cortex (E–H) using a multiplex array. Animals were injected with saline or pilocarpine (pilo) and sacrificed at indicated times. Values are expressed as percent of control. Data are represented as mean $\pm$ SEM. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared to saline injected controls by one-way ANOVA with Dunnett's test. n=5–9 rats per group.

Fig. 2. Production of pro-inflammatory cytokines in the chronic phase of epileptogenesis
Levels of (A) TNF- α , (B) IL-1 β , (C) IL-6 and (D) KC/GRO were measured in the hippocampus 6 wks after pilo. Values are expressed as percent of control. Data are represented as mean $\pm$ SEM. * p <0.05, ** p <0.01 and *** p <0.001 compared to saline injected controls by unpaired t-test. n =13–15 rats per group.

Fig. 3. Pharmacological removal of ROS decreases oxidative stress

Reduced glutathione (GSH) levels (A) and 3-nitrotyrosine (3-NT) to tyrosine (Tyr) ratio (B) in the hippocampi, GSH (C), oxidized glutathione (GSSG) levels (D), GSH/GSSG ratio (E), and 3-NT/Tyr ratio (F) in the piriform cortices of rats injected with saline (sal) alone, pilo+sal or pilo+Mn^{III}TDE-2-ImP⁵⁺ were measured by HPLC with electrochemical detection 24 h following pilocarpine injection. Data are represented as mean $\pm$ SEM.

* $p < 0.05$ compared to saline alone and # $p < 0.05$ compared to pilo+sal group by one-way ANOVA. $n = 5-8$ rats per group for hippocampus and 6-11 for piriform cortex.

Fig. 4. Pharmacological removal of ROS attenuates pro-inflammatory cytokine production

Levels of TNF- α , IL-1 β , IL-6 and KC/GRO were measured in 250 μ g protein in the hippocampus (A–D) and piriform cortices (E–H) in rats injected with saline alone, pilo+sal or pilo+MnTDE-2-ImP5+. Data are represented as mean $\pm$ SEM. *p<0.05 compared to saline alone and #<0.05 compared to pilo+sal group by one-way ANOVA. n=5–10 rats per group for hippocampus and 4–8 for piriform cortex.

Fig. 5. Pharmacological removal of ROS attenuates microglial activation in the hippocampus
Microglial activation was assessed by Iba-1 staining in the hippocampi of rats injected with saline alone, pilo+sal or pilo+Mn^{III}TDE-2-ImP⁵⁺ (A). Fluorescence density in the CA3 region and hilus is quantified in (B). Values are expressed as percent of saline-injected controls. * $p < 0.0001$ compared to saline alone and # < 0.01 compared to pilo+sal group by one-way ANOVA. $n = 6$ rats per group.

Fig. 6. MAPK pathway in the pilocarpine model

(A) Phosphorylation of ribosomal S6 was detected by western blotting in the hippocampi of rats injected with saline alone, pilo+sal or pilo+Mn^{III}TDE-2-ImP⁵⁺. (B) Quantification of the blot by expressing the ratio of phospho-S6/total S6 expressed as a percent of saline controls. * $p < 0.0001$ compared to saline alone and # $p < 0.01$ compared to pilo+sal group by one-way ANOVA. $n = 5-9$ rats per group.

Fig. 7. Schematic of proposed role of ROS in modulating neuroinflammation

Following an inciting injury i.e. status epilepticus (SE), there is increased production of reactive oxygen species (ROS), which can in turn activate microglial cells resulting in pro-inflammatory cytokine production. Cytokine production can result in further activation of the microglia and ROS production. Inflammatory cytokines can activate downstream receptors, leading to activation of certain kinases, which can result in network reorganization, neuronal injury and eventual development of epilepsy. i.e. epileptogenesis. Blocking ROS production by a catalytic antioxidant results in attenuation of microglial

activation and inflammatory cytokine production, potentially by attenuation of SE-induced activation of the redox-sensitive mTOR pathway.

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