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Persistent impairment of mitochondrial and tissue redox status during lithium-pilocarpine-induced epileptogenesis

Simon Waldbaum, Li-Ping Liang, and Manisha Patel

Department of Pharmaceutical Sciences, University of Colorado Denver Anschutz Medical Campus, Aurora, Colorado, USA

Abstract

Mitochondrial dysfunction and oxidative stress are known to occur following acute seizure activity but their contribution during epileptogenesis is largely unknown. The goal of this study was to determine the extent of mitochondrial oxidative stress, changes to redox status, and mitochondrial DNA (mtDNA) damage during epileptogenesis in the lithium-pilocarpine model of temporal lobe epilepsy. Mitochondrial oxidative stress, changes in tissue and mitochondrial redox status, and mtDNA damage were assessed in the hippocampus and neocortex of Sprague–Dawley rats at time points (24 h to 3 months) following lithium-pilocarpine administration. A time-dependent increase in mitochondrial hydrogen peroxide (H₂O₂) production coincident with increased mtDNA lesion frequency in the hippocampus was observed during epileptogenesis. Acute increases (24–48 h) in H₂O₂ production and mtDNA lesion frequency were dependent on the severity of convulsive seizure activity during initial status epilepticus. Tissue levels of GSH, GSH/GSSG, coenzyme A (CoASH), and CoASH/CoASSG were persistently impaired at all measured time points throughout epileptogenesis, that is, acutely (24–48 h), during the ‘latent period’ (48 h to 7 days), and chronic epilepsy (21 days to 3 months). Together with our previous work, these results demonstrate the model independence of mitochondrial oxidative stress, genomic instability, and persistent impairment of mitochondrial specific redox status during epileptogenesis. Lasting impairment of mitochondrial and tissue redox status during the latent period, in addition to the acute and chronic phases of epileptogenesis, suggests that redox-dependent processes may contribute to the progression of epileptogenesis in experimental temporal lobe epilepsy.

Keywords

mitochondrial dysfunction; mitochondrial oxidative stress; temporal lobe epilepsy

Temporal lobe epilepsy (TLE) is a recent addition to the neurological disorders in which mitochondrial oxidative stress and dysfunction have been suggested to be contributing factors (Schapira *et al.* 1990; Kunz *et al.* 2000; Cock *et al.* 2002; Patel 2004). The mechanisms by which mitochondria control neuronal injury and seizure susceptibility associated with TLE have not been fully elucidated, but the role of oxidative stress appears to be critical (Cock *et al.* 2002; Liang and Patel 2004). Mitochondria are a significant source

of reactive oxygen species (ROS), uniquely vulnerable to oxidative damage, and have been suggested to be an important contributor to mechanisms of neurodegenerative disorders and seizure-induced brain damage, for example, excitotoxicity (Dugan *et al.* 1995; Patel *et al.* 1996) and inflammation (Roberts *et al.* 2009; Victor *et al.* 2009). Catalytic removal of ROS has been demonstrated to prevent status epilepticus (SE)-induced cell loss (Rong *et al.* 1999; Liang *et al.* 2000), and patients with TLE have demonstrated dramatic metabolic and bioenergetic changes as well as mitochondrial electron transport chain (ETC) complex I deficiency which could lead to decreased ATP production and neuronal damage (Kunz *et al.* 2000; Pan *et al.* 2008). SE has been demonstrated to produce acute increases in mitochondrial oxidative stress and subsequent damage to sensitive targets such as excessive ROS production, increased oxidation of cellular macromolecules, lipid per-oxidation, impaired mitochondrial Ca^{2+} sequestering, mitochondrial DNA (mtDNA) damage, decreased activity of ETC complexes I and III, and increased nitric oxide (NO) and peroxynitrite (ONOO^-) generation at time points preceding neuronal death in susceptible brain regions (Griffiths *et al.* 1984; Cheng and Sun 1994; Bruce and Baudry 1995; Kunz *et al.* 1999; Erakovic *et al.* 2000; Frantseva *et al.* 2000; Liang *et al.* 2000; Patel *et al.* 2001, 2008; Milatovic *et al.* 2002; Chuang *et al.* 2004; Slevin *et al.* 2006; Jarrett *et al.* 2008). Evidence of mitochondrial dysfunction, that is, decreased ETC complex I and IV activity, subunit expression, and ultrastructural damage to mitochondria during chronic epilepsy has recently emerged from animal studies (Kudin *et al.* 2002; Chuang *et al.* 2004; Gao *et al.* 2007; Jarrett *et al.* 2008). However, the question of whether mitochondrial oxidative stress and consequent damage contribute to epileptogenesis and TLE remains to be fully explored.

Recent work from our laboratory has demonstrated a time-dependent increase in mitochondrial oxidative stress, oxidative damage to mtDNA, and decreased mtDNA repair capacity (Jarrett *et al.* 2008) prior to the onset of spontaneous seizures following kainic acid (KA)-induced epileptogenesis. A key finding of this study was that failure of adaptive responses to ongoing oxidative stress in the brain during epileptogenesis, such as mtDNA repair, could lead to an increase in seizure susceptibility. Two important questions, however, remain to be answered. First, these observations raise the question of whether mitochondrial oxidative stress and dysfunction occur during epileptogenesis across animal models. The KA model has been used to demonstrate mostly acute oxidative alterations associated with seizure activity (Bruce and Baudry 1995; Gluck *et al.* 2000; Liang *et al.* 2000, 2007; Patel *et al.* 2001; Chuang *et al.* 2004; Liang and Patel 2006), but the model specificity and chronic occurrence of these changes is largely unknown. The systemic injection of the muscarinic cholinergic agonist, pilocarpine (Pilo), with lithium-chloride (Li) pre-treatment has been used to induce SE and as a chronic model of TLE in rodents (Honchar *et al.* 1983; Williams and Jope 1994; Marinho *et al.* 1998) which replicates key features of the human condition such as spontaneous seizures, specific patterns of neuronal cell loss in the hippocampus, astrogliosis, and axonal reorganization (Hinterkeuser *et al.* 2000; Ben-Ari 2001; Lehmann *et al.* 2001). The lithium-pilocarpine (Li-Pilo) model is particularly valuable to understanding the mechanisms of oxidative stress-induced epileptogenesis because its mechanism lacks direct activation of glutamate receptor-induced neuronal excitotoxicity.

A second important question that emerges from our previous work is whether mitochondrial and/or cellular redox status is impaired during the latent period so as to facilitate the

progression of chronic epilepsy. In our previous study, we observed extramitochondrial hydrogen peroxide (H_2O_2) production, an index of oxidative stress, during acute and chronic phases of epileptogenesis but not during the latent period (Jarrett *et al.* 2008). The latter is most likely because extramitochondrial release of H_2O_2 requires robust increases in intramitochondrial superoxide ($O_2^{\bullet-}$) and H_2O_2 that exceeds the capacity of mitochondrial antioxidant defenses. Therefore, a more sensitive index of intracellular oxidative stress is required to detect redox changes that may be occurring during the latent period of epileptogenesis. Redox couples such as GSH, the most abundant intracellular non-enzymatic oxidant defense in the body catalyzing cellular H_2O_2 removal (Meister and Anderson 1983), and its disulfide (GSSG) serve as biomarkers of oxidative stress and damage (Reed and Savage 1995; Liang and Patel 2006). Coenzyme A (CoASH) and its disulfide with GSH (CoASSG), which are primarily compartmentalized within mitochondria, can be measured as a marker of mitochondrial specific redox status (Liang and Patel 2006). Following KA-induced SE, we have shown a significant decrease in mitochondrial GSH/GSSG and tissue CoASH/CoASSG up to 7 days in hippocampal tissue (Liang and Patel 2006), but it is unknown whether chronic alterations in tissue and mitochondrial redox status persist throughout epileptogenesis. The goal of this study was to determine whether mitochondrial oxidative stress, mitochondrial specific changes in redox couples (GSH/GSSG and CoASH/CoASSG), and mtDNA damage occurred during Li-Pilo induced epileptogenesis.

Methods

Lithium-pilocarpine administration

Animal housing was conducted in compliance with the University of Colorado Denver Anschutz Medical Campus procedures and protocols. Intraperitoneal (i.p.) injections were performed on adult male Sprague–Dawley rats with lithium-chloride (Sigma 310468; Sigma, St Louis, MO, USA) (50.8 mg/mL; 127 mg/kg) followed by methyl-scopolamine (Sigma S8502) (0.8 mg/mL; 1 mg/kg) 19–24 h later to attenuate the peripheral effects of pilocarpine treatment. Thirty minutes later rats were injected with either saline or pilocarpine-hydrochloride (Sigma P6503) (50 mg/mL; 30 mg/kg) to induce status epilepticus (SE) which was attenuated after 90 min by injecting diazepam (Hospira Inc., Lake Forest, IL, USA) (5 mg/mL; 10 mg/kg). Age-matched control rats received all treatments (lithium, methyl-scopolamine, diazepam), but saline instead of pilocarpine. A subset of rats were killed at 24–48 h that did not receive diazepam treatment, allowing SE in these animals to continue unabated. Rats treated with diazepam were killed 24 h, 48 h, 96 h, 7 days, 21 days, and 3 months following SE.

Monitoring of behavioral seizures

Behavioral seizure severity during SE was evaluated by direct observation for 6 h after the initial treatment and scored based on a modified Racine scale (Racine 1972) with only motor seizures being considered (Class I and II seizures were not scored). Briefly, motor seizure severity was characterized as follows: class III, animals displayed forelimb clonus with a lordotic posture; class IV, animals reared with concomitant forelimb clonus; and class V animals had a Class IV seizure and fell over. Only rats having at least one class III convulsive seizure during each consecutive 1-h period up to 3 h were included in the study,

although most rats had multiple seizures per 1-h period. Spontaneous seizures from a group of 12 Li-Pilo-treated and control rats were monitored by direct observation by an observer blind to treatment group from 24 h to 3 months following SE for random intervals totaling 8–10 h a week for seizure severity and duration used for data in Fig. 1. Seizure frequency was not quantified based on the observation protocol used. All Li-Pilo-treated rats used in this study for endpoints 7 days post-SE were observed to have a minimum of two spontaneous seizures during the observation period (24 h to 3 months) and defined as being chronically epileptic (Hellier *et al.* 1998). All rats used in this study were observed for 2 h before being killed to ensure a seizure free period.

mtDNA damage

Rats were killed at the indicated time points following SE and brain regions dissected and stored at -80°C . Genomic DNA was extracted from brain tissue using a commercially available Qiagen genomictip kit (Qiagen Inc., Valencia, CA, USA). mtDNA damage was quantified using the quantitative PCR (QPCR) by comparing the relative efficiency of the amplification of the large mtDNA gene fragment (13.4 kb) and normalized to 250 bp fragments from saline-treated controls which have a statistically negligible chance of sustaining oxidative stress-induced damage. The PCR conditions used in this study were based on previously reported sequences for mtDNA primers, with minor modifications (Ayala-Torres *et al.* 2000; Jarrett and Boulton, 2005). The primer sequences were as follows for the rat mitochondrial genome 5'-GGCAATTAAGAG-TGGGATGGAGCGAA-3' and 5'-AAAATCCCCGCAAACAAT-GACCACCC-3'. QPCR was performed on a DNA Engine thermal cycler with all reactions being a total volume of 100 μL containing 15 ng total genomic DNA, 1 unit of XL rTth polymerase, 3.3 XL PCR buffer II (containing potassium acetate, glycerol and dimethylsulfoxide) and final concentrations of 200 μM dNTP's, 1.2 mM $\text{Mg}(\text{OAc})_2$ and 0.1 μM primers.

The gene fragments were amplified using the following thermocycling profile: PCR was initiated with the addition of the 1 unit of XL rTth polymerase when samples had reached a temperature of 75°C followed by an initial penetration for 1 min at 94°C , cycles of denaturation at 94°C for 30 s and primer extension at 60°C for 13 min. Following the PCR cycles a final extension at 72°C for 10 min was performed. The mitochondrial gene products underwent 28 cycles of thermo-cycling. After the completion of the QPCR the gene products were resolved on a 1% agarose gel and digitally photographed on a UV transilluminator (UVi Tec, Cambridge, UK). The intensity of the PCR product bands was quantified with Scion Image analysis software (Scion Corp., Version Beta 4.0.2, Frederick, MD, USA).

Isolation of mitochondrial fractions

Hippocampi from each rat were pooled and homogenized with a Dounce tissue grinder (Wheaton, Milville, NJ, USA) in an isolation buffer (70 mM sucrose, 210 mM mannitol, 5 mM Tris-HCl, 1 mM EDTA; pH 7.4) and diluted 1 : 1 in 24% Percoll. Homogenates were centrifuged at 30 700 g at 4°C for 10 min. The sediment was subjected to Percoll gradient (19% on 40%) centrifugation at 30 700 g at 4°C for 10 min. The material located at the interface of the lowest two layers was slowly diluted 1 : 4 with mitochondrial isolation buffer containing 1 mg/mL bovine serum albumin and centrifuged at 6700 g at 4°C for 10

min to obtain final pellets consisting of respiring mitochondria. The final pellet was used immediately for H₂O₂ production studies.

Seizure-induced mitochondrial H₂O₂ production

H₂O₂ formation in mitochondrial fractions was measured using an Amplex Red reagent kit (Molecular Probes, Eugene, OR, USA) as previously described (Castello *et al.* 2007). Briefly, fluorometric detection of H₂O₂ production was achieved using the horseradish peroxidase-linked Amplex Ultra Red fluorometric assay. Isolated mitochondria (10 µg) were added to a 96-well plate containing 100 µL of reaction buffer containing 0.1 U/mL horseradish peroxidase, 50 µM Amplex UltraRed, and 2.5 mM malate plus 10 mM glutamate. A microplate reader equipped for excitation in the range of 530–560 nm and fluorescence emission detection at 590 nm was used to determine the production.

HPLC determination of GSH and GSSG

The measurement of GSH and GSSG was performed with an ESA (Chelmsford, MA, USA) 5600 CoulArray HPLC equipped with eight electrochemical detector cells as previously described in the literature (Lakritz *et al.* 1997; Liang and Patel 2006). The potentials of the electrochemical detector were set at 0/150/300/450/570/690/780/850 mV. Analyte separation was conducted on a 5 µM, 150 × 4.6 mm C-18 reversed-phase YMC ODS-A column (Waters Com., Milford, MA, USA). The mobile phase was composed of 100 mM sodium phosphate, 1% methanol; pH 3.0 and a flow rate of 0.6 mL/min was maintained. Samples were sonicated in ice cold 0.1 N perchloric acid and centrifuged at 16 000 *g* at 4°C for 10 min. Aliquots (20 µL) of the supernatant were subjected to HPLC. The GSSG peak was identified by the addition of glutathione reductase (GR) and H₂O₂ to the samples. Following a 30-min incubation with 0.15 unit/mL GR, the GSSG peak was decreased by 90%, whereas a 30-min incubation with 1 mM H₂O₂ resulted in a doubling of the GSSG peak (data not shown). The absolute ratios of tissue GSH/GSSG were consistent with previously reported values measured by enzymatic recycling methods (Sian *et al.* 1997).

Measurement of Reduced CoA (CoASH) and its GSH disulfide (CoASSG)

CoASH and CoASSG were determined by the HPLC method described previously by Rogers *et al.* (2000). Frozen hippocampus (50 mg) was placed in 200 µL 0.1 M sodium phosphate and 28.5 mM *N*-ethylmaleimide (NEM) buffer (pH 7.4) and sonicated immediately prior to thawing. The tissue was acidified with equal volumes of 4% HClO₄ and centrifuged at 12 000 *g* at 4°C for 20 min. Aliquots of supernatant were separated on a Zorbax SB-C₁₈ column using the following mobile phases: (A) 10% methanol and 10 mM tetrabutylammonium hydrogen sulfate (pH 5.0), (B) 85% methanol and 10 mM tetrabutylammonium hydrogen sulfate (pH 5.0). The gradient was 5 min at 100% A, 0% B, followed by a 30 min linear gradient to 40% A and 60% B. Peak height was quantified by UV detection at 254 nm. Briefly, the CoASSG standard was prepared by GSH (100 nmol) and CoASH (100 nmol) dissolved in 0.1 M sodium phosphate buffer, pH 7.4. Diamide (100 nmol) was added to a final volume of 1 mL followed by the addition of NEM (1000 nmol) and 10 µL H₃PO₄. The mixture was analyzed by HPLC. The retention time of the CoASSG peak preceded the CoASH-NEM peak by 2 min.

Results

Spontaneous behavioral seizures

A time-dependent increase in seizure severity and duration was observed in a representative subgroup of rats following Li-Pilo-induced SE, with the first spontaneous motor seizure noted by 3 weeks (Fig. 1). By 9 weeks following SE all observed rats were having spontaneous seizures characterized by bi-lateral clonus and loss of balance [P5 seizures based on a modified Racine scale (Racine 1972)]. Approximately 85% of Li-Pilo-treated rats were confirmed to have recurrent and spontaneous motor seizures and were included in the study. Saline-treated controls did not show any spontaneous seizure activity. It is likely that unobserved electrographic and/or behavioral seizures occurred prior to 3 weeks. Seizure frequency was not reported based on the need for 24/7 video/EEG monitoring to accurately quantify this parameter.

Mitochondrial oxidative stress during epileptogenesis

Oxidative stress was evaluated by measuring steady-state levels of H₂O₂ production in isolated hippocampal mitochondria to investigate the mechanism underlying oxidative damage associated with epileptogenesis. Li-Pilo-induced SE produced an initial increase in mitochondrial H₂O₂ production in the hippocampus that peaked by 96 h and returned to control levels at 7 days, during the so-called 'latent period,' and then significantly increased again coincident with spontaneous seizures at 21 days and 3 months during chronic epilepsy (Fig. 2a). KA-induced SE has been demonstrated to result in higher acute increases in H₂O₂ production than observed here (Jarrett *et al.* 2008). As diazepam was used to attenuate motor seizures in the current study 90 min following SE, it was hypothesized that the relative lack of increased H₂O₂ production at 24–48 h was because of less severe and/or frequent seizure activity. Hence, steady-state levels of mitochondrial H₂O₂ in the hippocampus of rats not treated with diazepam were measured. Figure 2(b) illustrates that levels of H₂O₂ production increased significantly from controls at 24–48 h when seizures were permitted to continue unabated during SE suggesting the seizure dependence of increased mitochondrial oxidative stress.

Redox status during epileptogenesis

The measurement of two subcellular redox couples was conducted as an indication of hippocampal tissue and mitochondrial redox status and biomarker of oxidative stress during epileptogenesis (Reed and Savage 1995). GSH catalyzes the removal of H₂O₂ in cells and is the most abundant intracellular non-enzymatic oxidant defense in the body (Meister and Anderson 1983). GSH is recycled from GSSG, its disulfide redox partner, by GR and NADPH oxidation. Li-Pilo treated rats showed a significant deficiency in hippocampal tissue GSH until 96 h post-SE, and remained decreased throughout the period of epileptogenesis although not reaching statistical significance (Fig. 3a). GSSG levels markedly increased following Li-Pilo treatment, particularly at 96 h and throughout the period of epileptogenesis and chronic epilepsy (Fig. 3b). GSH/GSSG decreased throughout epileptogenesis and chronic epilepsy (Fig. 3c), suggesting that redox-dependent processes may contribute to the progression of epileptogenesis in experimental TLE.

Although GSH/GSSG measurements are an important tool in the measurement of redox status and oxidative stress, non-specific GSH oxidation may occur during subcellular fractionation. This may be overcome by measurement of the thiol form of coenzyme A (CoASH) and its disulfide with GSH (CoASSG), which are primarily compartmentalized within mitochondria and exchange thiol/disulfide with GSH and GSSG (Wong *et al.* 2001; O'Donovan *et al.* 2002). Previous work in our laboratory comparing SE-induced alteration of GSH/GSSG from isolated hippocampal mitochondria with CoASH/CoASSG from whole hippocampal tissue suggests identical magnitude and time-course of the two redox couples (Liang and Patel 2006). Therefore, CoASH and CoASSG in tissue, which provides a reliable intramitochondrial redox status and overcomes artifactual changes in GSH and GSSG formation during subcellular fractionation were measured. It should be noted that tissue measurements can produce increased variability as seen at the 21 days and 3 months CoASSG time points (Fig. 4b), as well as an underestimation of changes. Following Li-Pilo treatment, CoASH levels and CoASH/CoASSG were significantly decreased as early as 24 h and persisted throughout epileptogenesis and chronic epilepsy (Fig. 4a and c) suggesting a role for mitochondrial specific oxidative stress and dysfunction in increasing seizure susceptibility and TLE onset.

mtDNA lesion frequency during epileptogenesis

Mitochondrial oxidative stress can produce mtDNA lesions and damage. Using a sensitive QPCR assay specific to a 13.4 kb mitochondrial fragment, it was determined whether mtDNA damage occurred in the hippocampus and piriform cortex during Li-Pilo-induced epileptogenesis. Figure 5(a,a1) illustrates that mtDNA lesion frequency increased acutely in the hippocampus following Li-Pilo-induced SE, decreased during the so-called 'latent period,' and increased in a time-dependent manner thereafter through the period of chronic epilepsy. This biphasic increase in oxidative mtDNA damage as a function of time coincided with the biphasic increase in mitochondrial H₂O₂ production in the hippocampus (Fig. 2). In order to determine the seizure dependence of oxidative damage to mtDNA, a subset of rats were not treated with diazepam to allow motor seizures to continue during SE. Figure 5(b) indicates that mtDNA lesion frequency in the hippocampus increased significantly at 24 h (data not shown) and 48 h when seizures continued unabated. Because of the widespread effects of pilocarpine in the rodent brain, mtDNA lesion frequency was also determined in the piriform cortex during epileptogenesis (Fig. 5c) where it followed the same biphasic pattern as observed in the hippocampus but was not as severe. In a group of rats whose motor seizures during SE continued without diazepam treatment (Fig. 5d), mtDNA lesion frequency increased significantly at 24 h (data not shown) and 48 h.

Discussion

Summary

This study demonstrated that tissue and mitochondrial redox status in the hippocampus is persistently altered throughout Li-Pilo-induced epileptogenesis and provided evidence supporting the role of mitochondrial oxidative stress and mtDNA damage in the onset of TLE. Specifically, Li-Pilo treated rats showed (i) a biphasic increase in steady-state levels of mitochondrial H₂O₂ as a function of time during epileptogenesis contingent on the severity

of SE, (ii) persistent alterations to GSH/GSSG and CoASH/CoASSG throughout epileptogenesis, and (iii) increased mtDNA lesion frequency that coincided with mitochondrial oxidative stress production in the brain. Together, these findings suggest that mitochondrial oxidative stress, mtDNA damage, and persistently altered redox status are active during epileptogenesis in the Li-Pilo animal model, and may contribute to the progression of TLE.

Behavioral seizures and mitochondrial oxidative stress during Li-Pilo-induced epileptogenesis

Motor seizure severity and duration progressively increased following Li-Pilo-induced SE with the first spontaneous seizure noted by 3 weeks. It is likely that electrographic and convulsive seizure events went undetected based on the observation protocol, particularly as non-convulsive electrographic seizures are known to precede the first convulsive motor seizure (Bertram and Cornett 1993, 1994). However, the results presented here confirm the presence of a 'latent period' and the typical increase in seizure frequency and duration consistent with the KA model and other experimental and human studies (French *et al.* 1993; Spencer and Spencer 1994; Hopkins and Shorvon 1995; Hellier *et al.* 1998; Jarrett *et al.* 2008; Williams *et al.* 2009) indicating the progressive nature of TLE (Engel *et al.* 1989; French *et al.* 1993). By observing and killing animals at varying time points throughout epileptogenesis, and not only acutely following SE treatment, the present study demonstrated oxidative mechanisms which may contribute to TLE onset and not merely be a consequence of chemoconvulsant treatment and/or SE.

Intramitochondrial $O_2^{\bullet-}$ flux can be measured in isolated mitochondria following spontaneous or enzymatic dismutation to H_2O_2 . The Amplex Red assay is a reliable measure of steady-state levels of H_2O_2 production, however, a number of factors may lead to an underestimation of its accurate concentration and levels of mitochondrial oxidative stress. First, the Amplex Red assay detects extramitochondrial levels of H_2O_2 which have been attenuated by diffusion through the mitochondrial membranes making it susceptible to degradation and scavenging by endogenous antioxidants such as GSH and peroxidases. Also, not all $O_2^{\bullet-}$ is converted to H_2O_2 and can alternatively react with other electron acceptors, form $ONOO^-$ in the presence of nitric oxide, and hydroxyl radical (OH^\bullet) through Fenton chemistry. Thus, there is a threshold of intramitochondrial $O_2^{\bullet-}$ formation that must be achieved before extramitochondrial measurement of H_2O_2 as a marker of mitochondrial oxidative stress can be made (Murphy 2009), and this may result in an underestimation of mitochondrial oxidative stress.

ROS function as second messengers in signal transduction but are also mediators of oxidative stress, damage, and inflammation. Seizure-induced overproduction of $O_2^{\bullet-}$ by the mitochondrial ETC (Liang *et al.* 2000) or extracellular sources such as the microglial NOX2 (Gao *et al.* 2003; Wu *et al.* 2003) can lead to the generation of more highly reactive species which may alter critical proteins involved in controlling neuronal excitability making them more susceptible to degradation by intracellular proteolytic systems and thereby increasing seizure susceptibility during epileptogenesis. While many of the acute oxidative consequences of seizures on cellular constituents have been demonstrated (Bruce and

Baudry 1995; Gluck *et al.* 2000; Liang *et al.* 2000; Patel *et al.* 2001; Tejada *et al.* 2007), the role of oxidative stress and mitochondrial dysfunction during epileptogenesis and as a contributor to TLE has been relatively unexplored. This study supports findings that mitochondrial oxidative stress and dysfunction are active during epileptogenesis and may contribute to the progression of chronic epilepsy (Patel 2004; Jarrett *et al.* 2008). In comparison with KA-induced epileptogenesis (Jarrett *et al.* 2008), Li-Pilo-induced epileptogenesis in this study resulted in a slower onset and lower magnitude of mitochondrial H₂O₂ production. The demonstration that removing diazepam treatment following SE markedly increased Li-Pilo-induced mitochondrial H₂O₂ production is sufficient to explain the slower onset of mitochondrial oxidative stress. The direct activation of glutamate receptors and resultant excitotoxicity produced by KA treatment may result in a higher magnitude of H₂O₂ production compared to Li-Pilo-induced cholinergic mechanisms. The overall pattern of increased mitochondrial oxidative stress, however, was similar between the two models. It is possible that levels of H₂O₂ production decreased at 7 days in both models because of an increase in neuronal death, however, the majority of neuronal death is thought to occur prior to that time point and measured redox couples in the present study were not similarly affected at 7 days.

Persistent alterations to redox status during epileptogenesis

An alternative to the measurement of oxidized cellular targets as markers of oxidative stress is to directly measure a subcellular redox couple as a sensitive index of redox status. GSH and CoASH have been demonstrated to decrease acutely following KA treatment (Skaper *et al.* 1999; Gilberti and Trombetta 2000; Ong *et al.* 2000; Liang and Patel 2006), but there is a lack of evidence demonstrating their levels during epileptogenesis and as a contributor to TLE. Hippocampal tissue GSH/GSSG following Li-Pilo-induced SE demonstrated in the present study decreased by 24 h and remained decreased throughout epileptogenesis. Similar experiments from the KA model demonstrated a significant decrease in hippocampal tissue GSH/GSSG only at 48 h in a 7-day study, while mitochondrial GSH/GSSG showed more dramatic and persistent decreases (Liang and Patel 2006). Also, CoASH and CoASH/CoASSG decreased in the KA model as early as 8 h and remained altered up until 7 days. Thus, redox status seems to be altered independent of animal models, although the effects of KA-induced epileptogenesis produced a profound and rather selective impairment of the mitochondrial redox status, while Li-Pilo-induced epileptogenesis resulted in persistent impairment of both mitochondrial and tissue redox status.

Measurement of intramitochondrial GSH and GSSG is complicated by *in situ* oxidation of GSH and disulfide exchange reactions occurring during the isolation procedure. This limitation is overcome by measurement of the thiol form of CoASH and its disulfide with GSH, CoASSG, which are primarily compartmentalized within mitochondria and exchange thiol/disulfide with GSH and GSSG (Wong *et al.* 2001; O'Donovan *et al.* 2002). The finding presented here that CoASH/CoASSG was persistently altered throughout epileptogenesis is consistent with the notion that mitochondria contribute disproportionately to SE-induced oxidative stress. Also, it was demonstrated that GSH/GSSG and CoASH/CoASSG were both decreased even when measurements of H₂O₂ production and mtDNA returned to control levels. Additionally, the changes in redox state during epileptogenesis observed here began

prior to the reported occurrence of neuronal death in the hippocampus (Liang *et al.* 2000) and may contribute to it. Neuronal loss that occurs beyond the initial acute period following Li-Pilo-induced epileptogenesis may result in decreased GSH or CoASH levels, however, it cannot account for increased GSSG and CoASSG levels. It is possible that surviving neurons and/or other cell types such as astrocytes could contribute to the observed redox changes. Astrocytes may be an important source of the H₂O₂ and redox imbalance observed during pilocarpine-induced epileptogenesis given that non-synaptic mitochondria used in our studies originate largely from glial cells (Rendon and Masmoudi 1985; Brown *et al.* 2006). These persistent alterations to mitochondrial redox status during epileptogenesis could cause significant mitochondrial dysfunction (Jain *et al.* 1991; Werner and Cohen 1993) affecting neuronal excitability through ETC dysfunction and decreased ATP production. Thus, the early onset and persistent decrease in mitochondrial redox status in the hippocampus suggests its ongoing contribution to epileptogenesis.

mtDNA damage during epileptogenesis

Epileptic seizures are present in several mtDNA disorders arising from mutations and stochastic mtDNA injury has been associated with acquired epilepsy (Wallace *et al.* 1992, 1994; Cock *et al.* 1999; Deschauer *et al.* 2003; Horvath *et al.* 2006). mtDNA lack protective histones, is located in close proximity to the inner mitochondrial membrane where ROS are generated, lacks introns, has a high transcription rate leading to an increased probability of mutations and/or deletions, and possesses less efficient repair mechanisms than nDNA under certain circumstances (Yakes and Van Houten 1997; Jarrett *et al.* 2008) making the mitochondrial genome particularly vulnerable to ROS-induced damage (Bohr *et al.*, 2002). Increased production of ROS causing mtDNA damage could lead to decreased activities of mitochondrial ETC complexes containing mtDNA-encoded subunits and selectively diminishing ATP production. This study demonstrates an initial rise in mtDNA damage, a return to control levels during the so-called 'latent period,' and re-emergence of mtDNA damage coincident with the onset of spontaneous seizures confirming previous observations that mitochondrial dysfunction occurs during chronic epilepsy (Kunz *et al.* 2000; Kudin *et al.* 2002; Kann *et al.* 2005; Jarrett *et al.* 2008). The increase in mtDNA lesion frequency largely coincided with mitochondrial H₂O₂ production, suggesting that mitochondrial oxidative stress is contributing to mtDNA damage. Although a similar pattern of mtDNA lesion frequency was observed in the hippocampus and piriform cortex, the damage was more severe in the former possibly because of relatively increased focal seizure activity. When motor seizures were allowed to continue during SE without diazepam treatment a significant increase in mtDNA lesion frequency occurred up to 48 h suggesting the seizure dependence of oxidative-induced mtDNA damage. It is possible that the apparent lack of mtDNA damage at 7 days is because of increased repair mechanisms that have been demonstrated following SE (Jarrett *et al.* 2008). It has not been determined whether and to what extent oxidative mtDNA damage may increase seizure susceptibility but it is conceivable that alterations in ATP production and increases in neuronal excitability through mechanisms such as altered Na⁺/K⁺ ATPase could be active during epileptogenesis. The seizure-induced accumulation of oxidative mtDNA lesions and resultant somatic mtDNA mutations during epileptogenesis could, over time, render the brain more susceptible to subsequent seizures in TLE.

Conclusions

The findings of the present study suggest that persistent impairment of mitochondrial and tissue redox status during the 'latent period' and acute and chronic phases of epileptogenesis may contribute to the progression of experimental TLE. Mitochondrial H₂O₂ production and mtDNA damage showed similar increases during epileptogenesis which both decreased during the 'latent period.' However, mitochondrial and tissue redox status remained impaired throughout epileptogenesis including the 'latent period' suggesting an oxidized environment which could alter critical macro-molecules involved in neuronal excitability and seizure susceptibility via redox signaling. The present study suggests that mitochondrial oxidative stress and dysfunction are active mechanisms during epileptogenesis that if attenuated may contribute to decreasing seizure susceptibility in TLE and provide an alternative therapy for patients refractory to current anti-epileptic drugs.

Abbreviations used

ETC	electron transport chain
GR	glutathione reductase
KA	kainic acid
Li-Pilo	lithium-pilocarpine
mtDNA	mitochondrial DNA
NEM	<i>N</i> -ethylmaleimide
QPCR	quantitative PCR
ROS	reactive oxygen species
SE	status epilepticus
TLE	temporal lobe epilepsy

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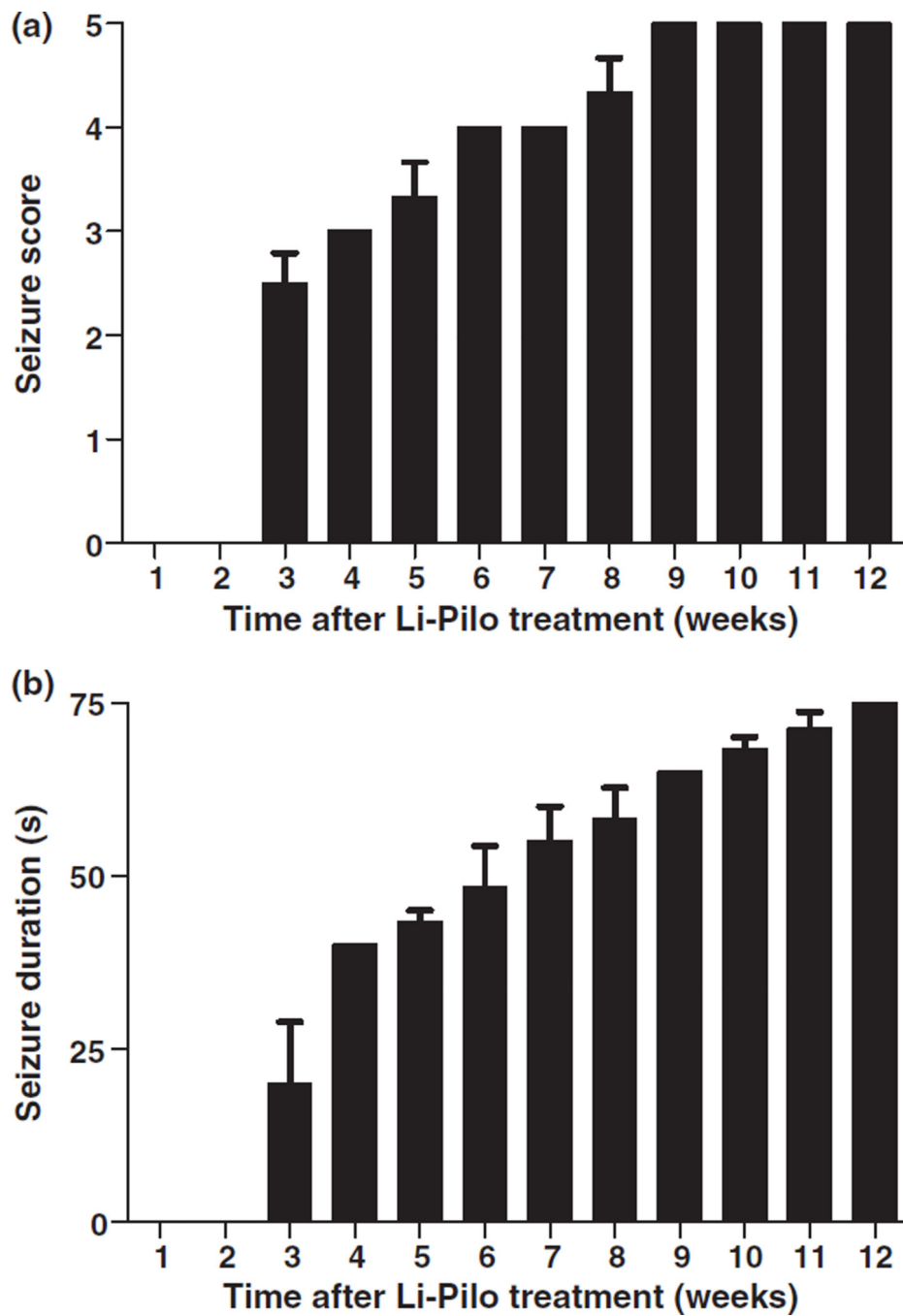


Fig. 1. Time-dependent increase in seizure activity following Li-Pilo-induced epileptogenesis. Rats were observed to have spontaneous motor seizures by 3 weeks post-SE which increased in (a) severity and (b) duration up to 12 weeks. Bars represent mean \pm SEM, $n = 12$.

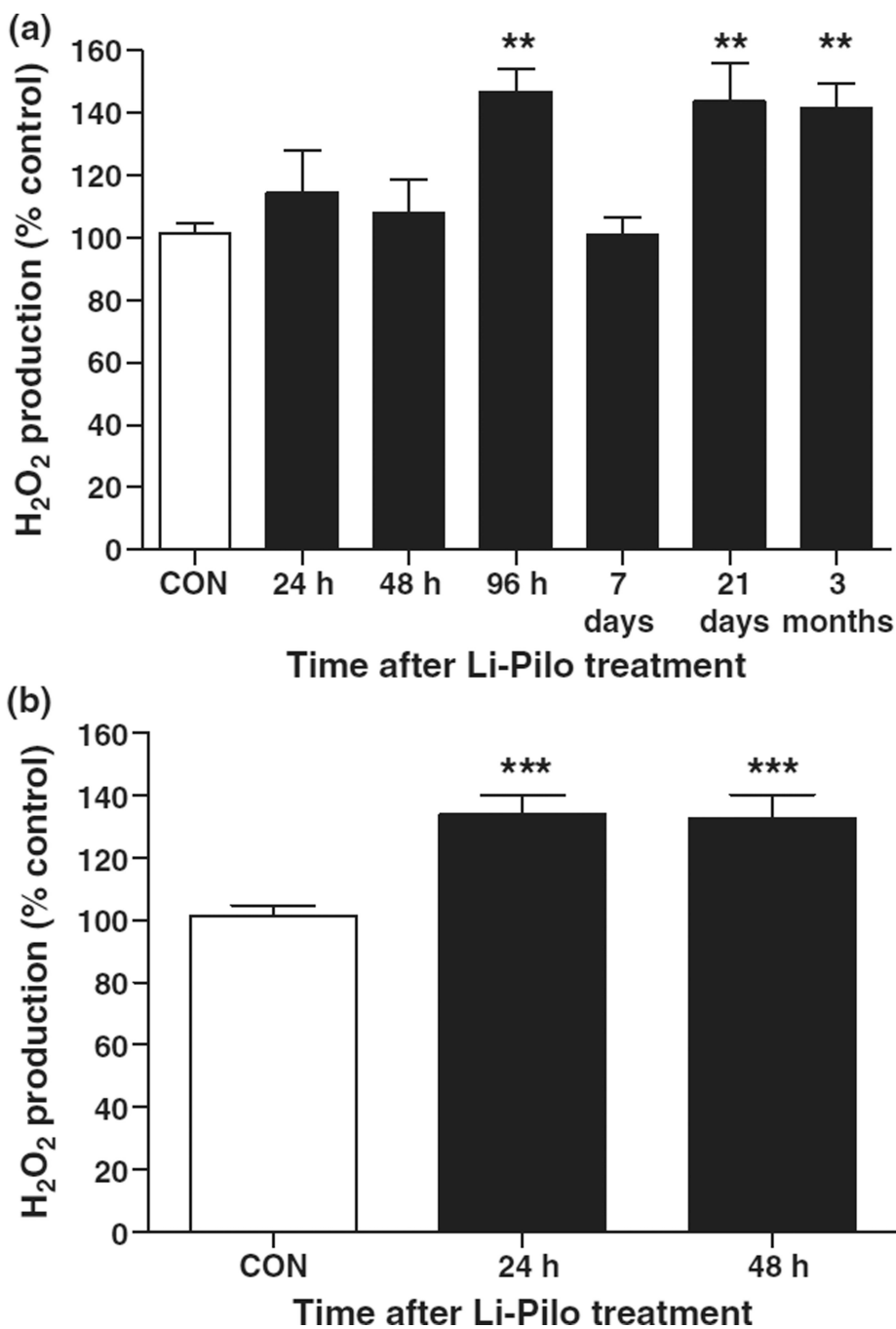


Fig. 2. Mitochondrial oxidative stress increased in a time-dependent manner following Li-Pilo-induced epileptogenesis. (a) Levels of steady-state mitochondrial H₂O₂ production from the hippocampus expressed as a percentage change compared to controls from 24 h to 3 months post-SE from rats receiving diazepam 90 min following the onset of SE. Bars represent mean ± SEM, $n = 5-6$ per time point. ** $p < 0.01$, one-way ANOVA. (b) Levels of steady-state mitochondrial H₂O₂ production from the hippocampus up to 48 h post-SE in a subset of rats not receiving diazepam to attenuate motor seizures during SE. Note that mitochondrial H₂O₂

production increased significantly when seizures were allowed to continue unabated during SE. Bars represent mean \pm SEM, $n = 5$ per time point, *** $p < 0.001$, one-way ANOVA.

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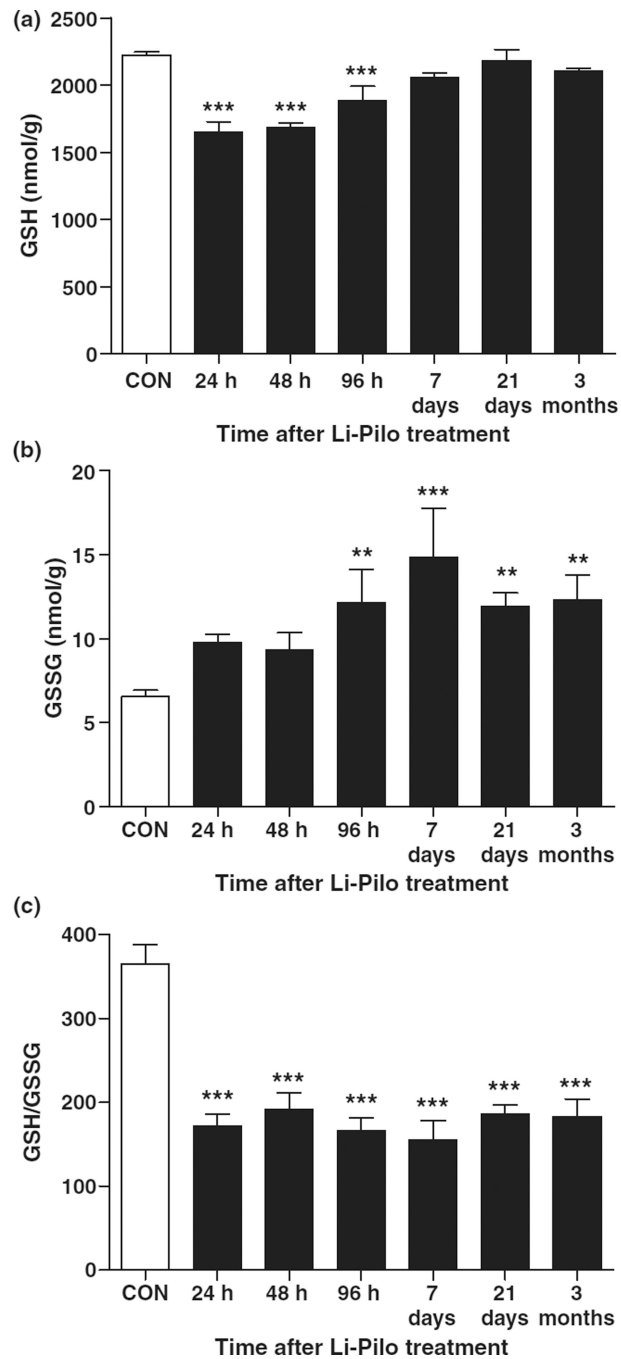


Fig. 3. Persistent alterations to redox status in the hippocampus throughout epileptogenesis. (a) GSH, (b) GSSG, and (c) GSH/GSSG from the hippocampus of rats at time points following Li-Pilo-induced SE versus saline-treated controls. Bars represent mean \pm SEM. $n = 5-6$ per time point, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA.

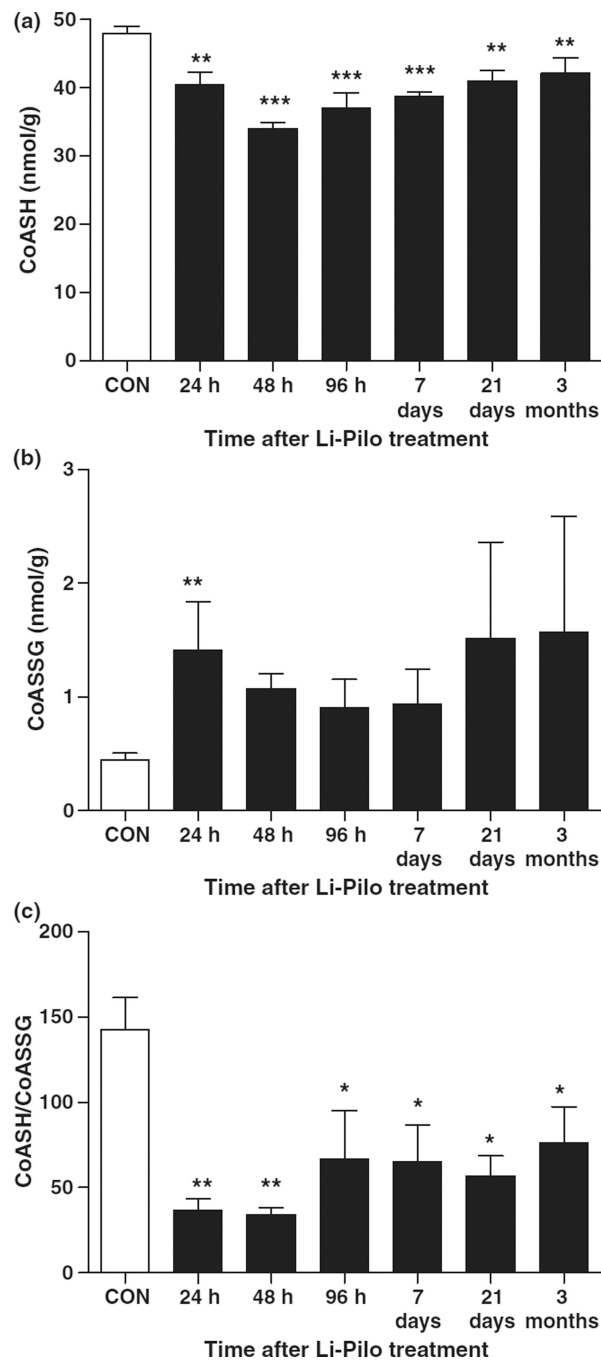


Fig. 4. Persistent alterations to mitochondrial specific redox status in the hippocampus throughout epileptogenesis. (a) CoASH, (b) CoASSG, and (c) CoASH/CoASSG in the hippocampus of rats at time points following Li-Pilo-induced SE versus saline-treated controls. Bars represent mean \pm SEM. $n = 5-6$ per time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA.

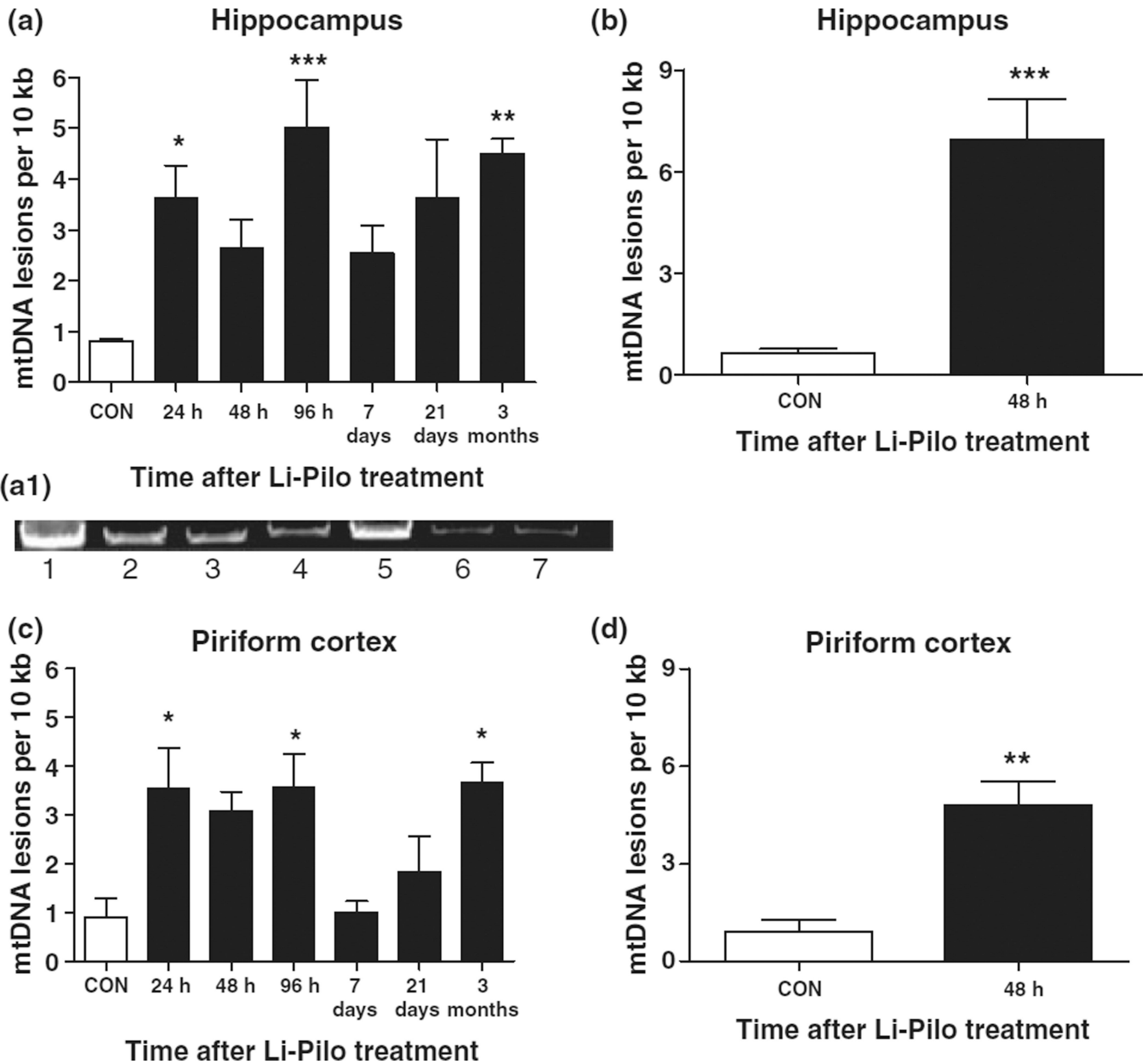


Fig. 5. mtDNA damage increased in a time-dependent manner during Li-Pilo-induced epileptogenesis. mtDNA lesion frequency determined using QPCR specific to a 13.4 kb mitochondrial fragment in the (a,a1) hippocampus and (c) piriform cortex during Li-Pilo-induced epileptogenesis in rats that received diazepam to attenuate SE. Panel (a1) is a representative agarose gel derived from QPCR analysis of hippocampal tissue after Li-Pilo treatment. Lane 1, CON; lane 2, 24 h; lane 3, 48 h; lane 4, 96 h; lane 5, 7 days; lane 6, 21 days; lane 7, 3 months. mtDNA lesion frequency at 48 h from rats that did not receive diazepam are shown in the (b) hippocampus and (d) piriform cortex. Note that lesion frequency increased significantly at 48 h when motor seizures were allowed to continue

during SE without diazepam treatment. Bars represent mean \pm SEM, $n = 5-10$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA.

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