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Temporal and Spatial Increase of Reactive Nitrogen Species in the Kainate model of Temporal Lobe Epilepsy

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Abstract

Steady-state levels of reactive oxygen species (ROS) and oxidative damage to cellular macromolecules are increased in the rodent hippocampus during epileptogenesis. However, the role of reactive nitrogen species (RNS) in epileptogenesis remains to be explored. The goal of this study was to determine the spatial and temporal occurrence of RNS i.e. nitric oxide levels in a rat model of temporal lobe epilepsy (TLE). Rats were injected with a single high dose of kainate and monitored by video for behavioral seizures for 6 weeks to determine the onset and severity of chronic seizures. RNS and tissue/mitochondrial redox status (glutathione redox couple and coenzyme A:glutathione redox couple) were measured in the hippocampus at 8 hr, 24 hr, 48 hr, 1 wk, 3 wk and 6 wk following kainate to assess the level of reactive species in subcellular compartments. We observed a biphasic increase in RNS levels with a return to control values at the 48 hr time point. However, both tissue and mitochondrial redox status showed permanent and significant decreases during the entire time course of epilepsy development. 3 nitrotyrosine (3NT) protein adducts were found to gradually increase throughout epileptogenesis, conceivably as a result of the local environment under oxidative and nitrosative stress. Colocalization of 3NT immunostaining with neuron- or astrocyte-specific markers revealed neuron-specific localization of 3NT in hippocampal principal neurons. Persistent and concurrent glutathione oxidation and nitrosative stress occurs during epileptogenesis suggesting a favorable environment for posttranslational modifications.

Keywords

temporal lobe epilepsy; mitochondria; nitrogen species; reactive oxygen species; posttranslational modification; oxidative stress

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Introduction

Temporal lobe epilepsy (TLE), the most common form of acquired epilepsy, is initiated by an injury such as head trauma, hypoxia, complex febrile seizures or status epilepticus (SE) (Delgado-Escueta et al., 1999). These precipitating injuries initiate molecular, biochemical, and structural alterations which result in the development of spontaneous recurrent seizures i.e. epilepsy. The process whereby injury culminates in network excitability or epileptogenesis is thought to involve several processes such as neuronal loss, gliosis, gene regulation, axonal sprouting, inflammation and neurogenesis. However, the role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in epileptogenesis is poorly understood (Waldbaum and Patel, 2010).

Abundant and overlapping endogenous antioxidants exist to overcome normal cellular production of reactive species; yet excessive production of ROS and RNS can overwhelm antioxidant defenses, shift the redox state of the local cellular environment and cause oxidation of vulnerable cellular targets. Studies from our laboratory and others have shown that oxidative stress arising from mitochondria and the plasma membrane/extracellular space via Nox2 contributes to seizure associated neuronal damage (Liang et al., 2000; Patel et al., 2005). Using surrogate markers of target oxidation in two separate TLE models (kainate and lithium-pilocarpine), we also showed that indices of ROS increase throughout epileptogenesis (Liang et al., 2000; Liang and Patel, 2006; Patel et al., 2008).

Much less is known about the link between nitrosative stress, or increased RNS, and epileptogenesis. One reason for this is the difficulty associated with accurately measuring various RNS species in biological systems. Current techniques each have their own limitations and it is critical to understand the specificity, limit of detection, and range at which RNS can be measured before experimentation. However, studies have shown that SE can result in a rapid increase of brain nitric oxide (NO) (Alderton et al., 2001; Sharma et al., 2008). The pathological effects of excess NO are proposed to be through the generation of the highly reactive nitrating species peroxynitrite (ONOO^-) in the presence of superoxide ($\text{O}_2^{\bullet -}$) (Szabo et al., 2007). Adverse consequences of RNS include induction of apoptotic pathways, potentiation of excitotoxicity through inhibition of glutamate reuptake, modulation of phosphorylation pathways and aberrant posttranslational modifications (PTMs) (Aguilar et al., 2012).

Irreversible ROS- or RNS-mediated PTMs are observed in disorders of the nervous system and are hypothesized to cause protein inactivation or degradation (Dalle-Donne et al., 2003; Ischiropoulos and Gow, 2005). For example, we and others have shown that protein carbonylation, an oxidative modification to most basic amino acids, occurs in human neurological diseases such as Parkinson's and Alzheimer's disease as well as rodent models of epilepsy (Gluck et al., 2000; Keeney et al., 2006; Sultana et al., 2006). We have recently shown mitochondrial specific increased carbonylation in the rat hippocampus at acute and chronic time points of epileptogenesis which coincided with periods of high seizures and inhibition of CI activity (Ryan et al., 2012). However, much less is known regarding the generation and potential role for protein nitration in epileptogenesis, another irreversible modification to tyrosine amino acids (noted as 3NT) that is commonly classified as a

biomarker for disease (Ischiropoulos, 1998; Souza et al., 2008). Protein nitration occurs in the presence of ONOO⁻ but its incidence has also been linked with alterations in redox status (Chinta and Andersen, 2006). Studies have shown that protein nitration is increased following seizures and in epilepsy models (Chavko et al., 2003; Liang et al., 2012).

The role of RNS in epileptogenesis and development of chronic epilepsy has been largely unexplored. Simultaneous assessment of RNS, GSH and GSSG levels can determine if conditions that favor posttranslational modifications occur during epileptogenesis which may serve a deleterious or protective role via oxidative damage and redox signaling, respectively. The goal of this study was to determine the spatial and temporal occurrence of NO levels and protein nitration in a rat model of temporal lobe epilepsy (TLE). Additionally, we sought to determine the temporal relationship between RNS and ROS (assessed by redox status).

Experimental Procedures

All materials were obtained from Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ) unless otherwise noted.

Induction of epileptogenesis

Animal housing and all experimentation was conducted in compliance with the University of Colorado Anschutz Medical Campus Animal Care and Use Committee under protocol number B-64011(10)1E. Male Sprague Dawley rats (~300-350g) were subcutaneously administered saline (vehicle) or 11mg/kg kainate (Nanocs KA-0002; New York, NY) dissolved in sterile buffered saline (pH 7.4). Animals were observed for status epilepticus (SE) and given moistened chow with subcutaneous saline injections 24 hr later to decrease mortality which was 10–15%. The rats were sacrificed after 1 minute of carbon dioxide inhalation followed by immediate decapitation at 8 hr, 24 hr, 48 hr, 1 wk, 3 wks and 6 wks after injection to encompass the acute, latent and chronic periods of epileptogenesis. Hippocampi were recovered from these animals, immediately frozen and stored at –80°C or crude mitochondria was isolated from 50 mg hippocampal tissue according to Liang and colleagues (Liang and Patel, 2006) and immediately prepared for biochemical analysis.

Monitoring of behavioral seizures

Behavioral (convulsive) seizures during SE were evaluated by direct observation for 6 hr after kainate (KA) injection 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 loss of balance. All animals having at least 3 Class III convulsive seizures each hour for at least 3 hr are included in the study. Direct observation confirmed that animals did not present behavioral seizures 8 hr, 24 hr, 48 hr and 1 wk post kainate treatment although most animals did have seizures by week 3. Rats were monitored by 24/7 video in the University of Colorado *In Vivo* Neurophysiology Core for quantification of seizure number, duration, and severity through the 6 wk time point to

confirm epilepsy. Rats observed to have 2 spontaneous and behavioral seizures a week after kainate administration were considered epileptic.

Measurement of NO levels

Tissue NO levels were measured by the production of nitrite in a Sievers Nitric Oxide Analyzer (NOA) 280i (GE Analytical Instruments; Boulder, CO). Nitrite levels were measured from 50 mg of hippocampal tissue homogenized in 300 μ l ice-cold PBS. Samples were filtered to remove high molecular weight proteins and 50 μ l of sample was injected into a Sievers NOA according to manufacturer's instructions and previously published methods (Huang et al., 2005). Chemiluminescence that resulted from the reaction of ozone with NO was measured via a photomultiplier. Data collection and analysis was conducted according to manufacturer's protocols by creating a nitrite standard curve with sodium nitrite. The level of tissue nitrite was calculated as percent to control.

HPLC determination of GSH and GSSG

Reduced and oxidized forms of GSH were measured by HPLC as described previously by Liang and Patel (Liang and Patel, 2006). Average GSH levels quantified for tissue was 2168.99 ± 19.6 nmol/g tissue. Tissue GSSG from control samples averaged 12.6 ± 2.15 nmol/g tissue in control samples. Control values were normalized to one hundred and data is represented as percent control (% control).

HPLC determination of Coenzyme A (CoASH) and CoASSG

The mitochondrial redox status was measured by HPLC equipped with UV detection as previously described Liang and Patel (Liang and Patel, 2006). Average control CoASH level quantified for tissue was 36.1 ± 0.92 nmol/g tissue while CoASSG was 0.22 ± 0.02 nmol/g tissue. Control values were normalized to one hundred and data is represented as percent control (% control).

HPLC determination of 3NT

Hippocampal tissue or mitochondrial pellets were sonicated in ice cold 0.1 N PCA and centrifuged at 16000 g at 4°C for 10 min. Aliquots (20 μ l) of the supernatant were injected into an ESA 5600 CoulArray HPLC (Chelmsford, MA) equipped with eight electrochemical detector cells using methods described previously in the literature (Beal et al., 1990). Average control tyrosine levels in tissue were 68.82 ± 9.70 nmol/g tissue and 3NT levels were 0.40 ± 0.15 nmol/g tissue. Data was expressed as a ratio of 3NT to tyrosine (3NT/Tyrosine); for controls, the average ratio value was 0.005 ± 0.001 .

Immunofluorescence staining

Brain blocks containing the hippocampus (~4 mm thick) were fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4. The hippocampal blocks were frozen and cut into 30 μ m sections on a cryostat and immunostained with primary antibodies to neurons (anti-NeuN polyclonal, rabbit, ABN78, Millipore, dilute 1:500), glia (anti-GFAP antibody, rabbit, G 9269, Sigma, dilute 1:80) or to 3NT (anti-3 NT monoclonal, mouse, 05-233, Millipore, dilute 1:100). Secondary antibodies used included Rhodamine Red labeled goat anti-mouse

and FITC labeled goat anti-rabbit (Jackson Immuno Research Inc., dilute 1:100). In parallel, control or kainate treated sections, anti-3 nitrotyrosine primary antibody was replaced by blocking buffer to verify its specificity. Prior to imaging, sections were covered with fluorescent mounting media (VectorShied, VectorLabs) and images were captured utilizing an Olympus FV-1000 confocal microscope (Olympus, Tokyo, Japan) at 488 and 544 nm with objectives of 20× (0.70 NA, Plan Apochromat) or 60× (1.40 NA, Plan Apochromat). Images were exported to a TIFF format and colocalization analysis was performed using Fluoview FV1000 software, version 5.0 (Olympus, Tokyo, Japan). Three hippocampal sections from each rat (n=3; 9 total sections), separated by 100 µm in total, were analyzed for co-localization of NeuN/3 nitrotyrosine or GFAP/3 nitrotyrosine.

Statistical analyses

For all biochemical analyses, one-way ANOVA was used with GraphPad Prism 5 software. P values less than 0.05 were considered significant.

Results

Spontaneous behavioral seizures are progressive during kainate induced epileptogenesis

Development of spontaneous seizures in the rat kainate model of TLE was confirmed by 24/7 videotaping. Spontaneous behavioral seizures were observed as early as 12 days post kainate injection and the average seizure onset was 17.6 days (median 16.5 days; data not shown), characterized by bi-lateral clonus and/or loss of balance [Class IV and V seizures based on a modified Racine scale (Racine, 1972)]. Seizure frequency (seizures/week) increased over time and all rats which had full status epilepticus were confirmed to be epileptic by 6 wks of treatment. At the final time point, animals demonstrated an average of ~10.3 seizures per week (median 9 seizures per week) (Figure 1).

Increased RNS during epileptogenesis

NO is a gaseous chemical messenger in the brain and is involved in a variety of physiological processes. Elevated brain NO has been reported to have detrimental effects in various disease states (Hess et al., 2005; Jaffrey and Snyder, 2001). When levels of NO were measured in hippocampal tissues following kainate-induced epileptogenesis, early and dramatic increases were noted at 8 and 24 hrs as compared to control. NO levels remained higher than controls at 48 hrs but values were not statistically significant ($p > 0.05$). Interestingly, NO levels remained significantly elevated at 1 wk, 3 wk, and 6 wk post kainate treatment (Figure 2).

Impaired tissue redox status during epileptogenesis

To determine the relationship between RNS and ROS species formation during epileptogenesis, we measured tissue and mitochondrial redox status. Glutathione (GSH) is the most abundant intracellular non-enzymatic oxidant defense in the body (Meister and Anderson, 1983) and its oxidation to GSSG, a disulfide redox partner can be measured in tissue by HPLC analysis. The GSH/GSSG ratio is commonly used as a biomarker of oxidative stress in numerous biological systems (Liang and Patel, 2006; Reed and Savage, 1995) and decreases have been correlated with structural damage to cellular and

mitochondrial membranes, oxidant-induced posttranslational modifications to proteins, DNA damage, and enzyme inactivation which can all potentially affect neuronal excitability (Waldbaum and Patel, 2010). Whole hippocampal tissue GSH decreased during the acute, latent, and chronic stages of experimental TLE (Figure 3A). Hippocampal tissue GSSG levels were increased substantially at 48 hrs post kainate treatment ($p < 0.01$). Acute GSSG was increased at 8 and 24 hr time points. During the latent period, GSSG was elevated from 1-6 weeks after kainate treatment (Figure 3B). Finally, the GSH/GSSG ratio was significantly decreased in kainate treatment groups from 24 hr through 6 wks (Figure 3C). Previously, we found a similar pattern of GSH/GSSG depletion in rats treated with lithium-pilocarpine to induce epileptogenesis (Waldbaum et al., 2010) and these results indicate that redox status is permanently decreased during epileptogenesis.

Impaired mitochondrial redox status during epileptogenesis

CoASH and its disulfide with GSH (CoASSG) are primarily compartmentalized within the mitochondria (Wong et al., 2001). Therefore their measurement in intact hippocampal tissue was used to assess the mitochondrial redox status with minimal disruption of the tissue, as opposed to measuring GSH and GSSG in isolated crude mitochondria. Previous work in our laboratory has shown reliable measurement of CoASH and CoASSG redox couples in the lithium-pilocarpine model of epileptogenesis as well as acute responses to kainate-induced status epilepticus (Liang and Patel, 2006; Waldbaum et al., 2010). The data presented in this study of kainate-induced epileptogenesis demonstrates a permanent change in mitochondrial redox status and strengthens observations from our previous work that mitochondria are more susceptible to oxidative stress (Figure 4).

CoASH levels were decreased significantly (Figure 4A) while CoASSG levels were increased at all time points (Figure 4B). The ratio of CoASH to CoASSG was calculated and the results show a significant shift in the mitochondrial redox status towards an oxidizing environment (Figure 4C). The results indicate that mitochondrial redox status is severely impaired during kainate-induced epileptogenesis which favors the likelihood for reactive species damage to mitochondrial macromolecules and overall dysfunction.

Quantification of protein nitration

3NT is an indicator for protein nitration, a posttranslational modification specific to the tyrosine amino acid which can yield protein dysfunction or turnover. A primary source and major contributor to tyrosine nitration in physiological and pathological events *in vivo* is through ONOO⁻ production, a reaction by-product of NO and O₂^{•-} (Sawa et al., 2000). When tissue levels of 3NT were examined in saline and kainate treated rats by HPLC, a gradual increase was observed over the course of epileptogenesis (Figure 5A). Compared to control, the 3NT/tyrosine ratio was increased in kainate treated rats at all time points. As an experimental control, the ratio was examined in rats which were treated with kainate that did not have full status epilepticus. At 24 hr post kainate, rats with a Racine score of less than Class III had similar hippocampal 3NT levels to saline treated rats (means $\pm$ S.E.M of 3NT/tyrosine $\times 1000$ in controls = 4.76 ± 0.51 (n=4) and in KA injected non-status rats = 4.57 ± 0.48 (n=4); $p = 0.8$ with t-test).

In order to assess mitochondrial specific 3NT levels, hippocampal mitochondria was isolated from control and kainate treated rats at the selected time points of 48 hr, 1 wk, and 6 wks to capture each phase of epileptogenesis. Compared to controls, the ratio of mitochondrial 3NT/tyrosine was increased at each time point after kainate treatment, although the increase was only statistically significant at 6 wks (Figure 5B). Although mitochondrial specific NO levels could not be quantified in these studies, the presence of 3NT suggests that NO levels are elevated in this subcellular compartment. These data suggest that elevated hippocampal NO, in the presence of increased cellular and mitochondrial ROS, may be contributing to accumulation of protein nitrosative damage during the process of epileptogenesis.

Localization of protein nitration

Hippocampal 3NT was also examined by immunofluorescence staining to corroborate the HPLC method and to determine the cell type contributing to its formation. Limited 3NT positively stained cells were observed in the CA3, CA1 and hilar regions of the hippocampus after saline treatment. However, a significant increase in 3NT positive cells was observed in the CA3 24 hr after kainate treatment (Figure 5C). Increased 3NT positive cells were also observed in the CA1 and hilus after kainate treatment (image not shown).

To investigate which cell type was forming 3NT protein adducts, the double staining of neurons or glia (astrocytes) with 3NT was also performed. The merged images indicate 3NT positive cells were highly co-localized with neurons (Figure 6A), not glial cells (Figure 6D) in the CA3 region of the hippocampus. Co-localization analysis results show the degree of NeuN and 3NT staining overlap in the CA3 region of the hippocampus was $83.7 \pm 2.4\%$. In contrast, the degree of GFAP and 3NT staining overlap in the CA3 region of the hippocampus was only $6.10 \pm 1.90\%$. Evidence of 3NT co-localization with NeuN was also detected in the dentate gyrus and CA1 24 hr after kainate treatment (Figure 6B and 6C). An image of the colocalization staining of neurons and 3NT in the whole hippocampus of rats 24 hr after kainate treatment is shown in Supplemental 1.

Discussion

The present study uncovers a number of important findings related to the progression of kainate induced epileptogenesis. First, a biphasic increase in NO and 3NT occurs in the hippocampus rapidly (8 hr) following chemoconvulsant-induced SE, returning close to control values 48 hr post-SE and remaining elevated 6 weeks coinciding with chronic epilepsy. Secondly, the hippocampal tissue and mitochondrial redox status remained persistently oxidized throughout the epileptogenic period (24 hr - 6 wks). Finally, increased 3NT following SE originated from hippocampal pyramidal neurons, not astrocytes. Together, these findings demonstrate for the first time (to our knowledge) the spatial and temporal occurrence of RNS during the epileptogenic process and raise the possibility that RNS-induced posttranslational modifications may contribute to the development of chronic epilepsy following injury.

Previous reports from our laboratory confirm ROS production and macromolecule oxidation in two chemoconvulsant models of epileptogenesis, the kainate and lithium-pilocarpine models (Jarrett et al., 2008; Liang and Patel, 2006; Waldbaum et al., 2010). These reports

focus on the occurrence of mitochondrial ROS in the process of epilepsy development following brain injury. However, the role of RNS in acquired epilepsy has been relatively unexplored. RNS, like many ROS species, have physiologic and pathologic roles in the brain depending on cellular localization (i.e. cell type vs. subcellular compartment vs. the local environment) as well as the magnitude and type of species generated. NO is a well-known modulator of cerebral blood flow and plays a role in neurotransmitter release, synaptic plasticity, regulation of gene expression and signaling events because it can transverse cellular membranes as a relatively weak oxidant (Valko et al., 2006). Brain NO is increased in several animal seizure models including arginine, hyperbaric oxygen, pentylenetetrazol and bicuculline exposures (Chavko et al., 2003; Mollace et al., 1991; Osonoe et al., 1994; Wang et al., 1994). In addition, kainate and pilocarpine treated rats are reported to have increased brain NO levels shortly after treatment (Chuang et al., 2007; Maggio et al., 1995). In a study by Mülsch and colleagues, brain NO was shown to increase 6-fold within 60 min of kainate treatment (Mulsch et al., 1994). Interestingly, pre and/or post-treatment with various nitric oxide synthase (NOS) inhibitors in these animal models has shown that NO can act as a pro- or anti-convulsant, which complicates the role of NO in seizures and epileptogenesis. Our data shows that NO and 3NT levels increased early following SE (8 hr) before returning to control values at the 48 hr time point and remained elevated thereafter. The time course of RNS differs from the time course of ROS i.e. mitochondrial hydrogen peroxide (Jarrett et al., 2008) which also shows a biphasic response but increases ~24 hr following SE and return to control values at 1 wk and not 48 hr. This indicates that increased NO and related RNS can be detected much earlier than ROS perhaps due to lower tissue oxygen levels which show a similar reciprocal time course as NO (Patel et al., 2008).

Since NO is a weak oxidant on its own, it is plausible that it is produced in or diffuses to a cellular environment already under substantial oxidative stress to exert RNS-mediated damage (3NT adducts). During the course of this study, cellular and mitochondrial redox status (ratio of GSH to GSSG and CoASH to CoASSG) was shown to be significantly and permanently disrupted following kainate administration. This novel finding provides a hypothetical scenario (ex. critical oxidizing cellular environment) for which many ROS/RNS-induced protein PTMs can occur and promote deleterious subcellular events that progress epileptogenesis. PTMs associated with changes to redox status are frequently observed to occur on low pKa cysteine (Cys) residues or thiols in proteins. For example, glutathionylation is the addition of GS- to Cys while nitrosylation of protein thiols is the addition of NO to a Cys. These reversible reactions occur under basal conditions or following moderate oxidative/nitrosative stress and play a key modulatory role on protein function that has been studied extensively *in vitro*. On one hand, thiol modifications may serve as protection from further oxidation reactions such as irreversible oxidation to sulfinic/sulfonic acid and aberrant redox signaling (Giustarini et al., 2004). Alternatively, if the modified Cys residue is functionally important, glutathionylation/nitrosylation can inhibit protein function. One example of the dual effects of these PTMs is with mitochondrial complex I (CI), an enzyme complex of the electron transport chain which is critical for ATP production and has been implicated in several neurological disorders (Orth and Schapira, 2001; Petruzzella et al., 2012). CI has been shown to contain reactive thiols in the hydrophilic subunits that become glutathionylated upon exposure to bolus GSSG or

nitrosylated in the presence of NO (Dahm et al., 2006; Hurd et al., 2008). These modifications of CI have been suggested to protect the enzyme from further oxidation due to their reversible nature but inhibit the enzyme *in vitro*. These redox-dependent modifications are of particular interest in PD and other disorders of the nervous system associated with depletion of GSH, which has been linked with inhibition of CI (Chinta and Andersen, 2011; Jha et al., 2000). With growing evidence suggesting that CI function may have a role in epileptogenesis (Kunz et al., 2000; Ryan et al., 2012) and the observations of altered ROS/RNS as well as GSH in this study, it is conceivable to suspect that CI and other proteins have reversible PTMs in various temporal and spatial profiles that contribute to disease progression. Attempts were made but technical limitations prevented our group from identifying CI redox modifications *in vivo*.

Irreversible protein PTMs are also suspected during epileptogenesis. Irreversible modifications are usually associated with permanent loss of function and may lead to the elimination of the damaged proteins by the proteasome system or to their accumulation as insoluble aggregates (Dalle-Donne et al., 2006). We recently demonstrated that protein ROS-derived carbonyl derivatives were generated in hippocampal mitochondria of kainate treated rats and described a correlation between carbonylation of a specific CI subunit, loss of CI activity, and seizures (Ryan et al., 2012). In this study, we assessed protein nitration (3NT), the irreversible addition of a nitro group to tyrosine amino acid side chains in the presence of ONOO⁻. Protein nitration is a well-established biomarker of ONOO⁻ mediated protein damage and has been reported to accumulate in brain tissue isolated from PD patients and in rodent models of PD, traumatic brain injury and cerebral ischemia (Coeroli et al., 1998; Giasson et al., 2000; Hall et al., 2004; Pennathur et al., 1999). Excess NO production in the presence of oxidizing cellular conditions (altered redox status) during epileptogenesis increases the likelihood of this PTM to occur. For example, Aquilano and colleagues suggest a link between nitrosative damage, neuronal cell viability and GSH homeostasis in the brain by reporting that GSH depletion in neural cells and in the brain of mice causes upregulation of 3NT adducts (Aquilano et al., 2011a; Aquilano et al., 2011b). Furthermore, in a dopaminergic cell culture model of PD, acute glutathione depletion was hypothesized to cause protein nitration on CI subunits that was correlated to a loss in activity suggesting a link between protein nitration and GSH levels (Bharath and Andersen, 2005). A recent review highlighted potential mechanisms and consequences of 3NT adducts on CI and the intricate role of GSH for disorders of the nervous system (Chinta and Andersen, 2011).

One aspect of nitrosative stress that is critical to understanding mechanism is the location of NO production and 3NT adducts in the hippocampus of kainate treated rats. Two ways for NO to be increased following status epilepticus are through 1) over activation of neuronal N-methyl-D-aspartate-type glutamate receptors (NMDARs) which causes elevated intracellular calcium, activation of neuronal nitric oxide synthase (nNOS) and production of NO (Chuang et al., 2007; Nakamura et al., 2012) and 2) activation of glial cells of the hippocampus. Microglia and astrocytes can be induced by many factors including ROS, cytokines, or ischemia to upregulate inducible nitric oxide synthase (iNOS) for excess NO generation (Brown and Neher, 2010; Moncada and Bolanos, 2006). Since our results show that 3NT is localized in neuronal cells of the hippocampus, we hypothesize that there is an early upregulation of nNOS following status or seizures and that gliosis and inflammation may

likely contribute to the accumulation at chronic stages of disease progression. Further studies are required to uncover the exact contributions of hippocampal cell types, NO production and cellular proteins which could be modified during epileptogenesis. Additionally, the measurement of seizure-induced NO production in these studies was performed in animals without implantation of brain electrodes to record real-time electroencephalography (EEG) activity. Thus, one limitation of this work is that subclinical seizures and NO production during epileptogenesis cannot be correlated in this study. It is possible that subclinical seizures increase NO production, especially during the noted latent period where behavioral or convulsive seizures were not observed. Future work will be to include the use of EEG in conjunction with video to elucidate the exact time of the seizure – NO production relationship in hippocampal neurons.

In this study, we report an increased ratio of 3NT to tyrosine in whole hippocampal tissue and mitochondrial fractions indicative of protein nitration. Furthermore, 3NT accumulates in hippocampal CA3, CA1 and hilar neurons following kainate-induced SE. This finding highlights that protein damage related to 3NT is only occurring in the neuronal cell population of the hippocampus and could therefore contribute to the dysfunction and/or loss of pyramidal cells commonly observed in chemoconvulsant models of epileptogenesis. The demonstration of a profound and persistent oxidation of GSH to GSSG and depletion of glutathione during epileptogenesis likely favors reversible (nitrosylation and/or glutathionylation) and irreversible (nitration) PTMs of targets such as complex I and suggest that this may play a key role in epileptogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Reactive oxygen and nitrogen species elevated in the kainate model of epilepsy
- Hippocampal nitric oxide levels are increased during epileptogenesis
- Cellular/mitochondrial redox status is permanently impaired during epileptogenesis
- Protein nitration is progressively increased and specific to neuronal cells

Figure 1. Behavioral (convulsive) seizure frequency increases throughout epileptogenesis in rats treated with a single high dose of kainate

Rats were treated with 11mg/kg kainate and monitored by 24/7 video; seizures with a behavioral correlate [Class III-V seizures, according to Racine's scale] were counted and averaged over a 6 wk period, n=5 kainate. Separate groups of rats were treated with saline as controls to each study, n=7.

Figure 2. Nitric oxide is increased during epileptogenesis

Hippocampal tissue nitric oxide was measured with a Sievers nitric oxide analyzer in 200 μ g protein from homogenized tissue according to manufacturer's protocols. Data are represented as changes in nitric oxide compared to saline control, n = 3 rats per group. Statistics: * p 0.05; **p 0.01; ***p 0.001; one-way ANOVA with Dunnett's Multiple Comparison test.

Figure 3. Cellular redox status is impaired during epileptogenesis

Levels of tissue (A) GSH, (B) GSSG, and the (C) ratio of GSH/GSSG were measured by HPLC with electrochemical detection in rat hippocampus, n = 5 rats per group. Animals were treated with 11mg/kg kainate or saline control and monitored for indicated times. Statistics: * p 0.05; **p 0.01; ***p 0.001; one-way ANOVA with Dunnett's Multiple Comparison test.

Figure 4. Mitochondrial redox status is significantly and persistently impaired during epileptogenesis

Tissue (A) coenzyme A (CoASH) and its disulfide couple with GSH, (B) CoASSG were measured in rat hippocampus by HPLC equipped with a UV detector, $n = 5$ rats per group. Animals were treated with 11mg/kg kainate or saline control and monitored for indicated times. The ratio between the reduced and oxidized molecules (C) CoASH/CoASSG was calculated to assess overall redox status within mitochondria during epileptogenesis. Statistics: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; one-way ANOVA with Dunnett's Multiple Comparison test.

Figure 5. Hippocampal 3NT is significantly elevated during epileptogenesis

Levels of (A) cellular (tissue) 3NT and (B) mitochondrial (mito) 3NT were measured by HPLC with electrochemical detection in rat hippocampus, n = 5 rats per group for cellular 3NT and n=3–4 rats per group for mitochondria samples. Animals were treated with 11mg/kg kainate or saline control and monitored for indicated times. Data are represented as the ratio between levels of 3NT and tyrosine (3NT/Tyrosine). Statistics: * p < 0.05; **p < 0.01; ***p < 0.001; one-way ANOVA with Dunnett's Multiple Comparison test. Representative immunofluorescence staining (C) for 3NT in the hippocampal CA3 region of the rats at 24 hr after receiving either saline or KA treatment. Bottom pictures are the enlarged image from the top row. n=3 rats per group; 3 sections (separated by 100μm) per rat.

Figure 6. 3NT colocalizes with neurons of the hippocampus

Representative NeuN (green) and 3NT (red) immunofluorescence images in the hippocampal CA3 (A), dentate gyrus (B) and CA1 (C) region of rats 24 hr after receiving 11mg/kg kainate (images from saline treated rats are not shown). The insets on the upper or lower right corner of each picture are the enlarged image from the white rectangle to show colocalization (yellow) of NeuN and 3NT. Representative GFAP (astrocytes=green) and 3NT (red) immunofluorescence images in the hippocampal CA3 (D) region of rats 24 hr after receiving 11mg/kg kainate (images from saline treated rats are not shown). The insets on the upper right corner of each picture are the enlarged image from the white rectangle to show absence of colocalization (yellow) between GFAP and 3NT. n=3 rats per group; 3 sections (separated by 100μm) per rat.