Epilepsy & Behavior 27 (2013) 378–384

Contents lists available at SciVerse ScienceDirect

Epilepsy & Behavior

journal homepage: www.elsevier.com/locate/yebeh

Changes in gene expression in the frontal cortex of rats with pilocarpine-induced *status epilepticus* after sleep deprivation

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ARTICLE INFO

Article history:
Received 3 February 2013
Revised 18 February 2013
Accepted 24 February 2013
Available online 28 March 2013

Keywords:
Epilepsy
Sleep deprivation
NADPH-oxidase
Nitric oxide
Superoxide dismutase
Catalase

ABSTRACT

Sleep and epilepsy present a bidirectional interaction. Sleep complaints are common in epilepsy, and sleep deprivation may provoke seizures. However, the mechanisms underlying this relationship are unknown. Thus, this study investigated the effects of paradoxical sleep deprivation (PSD24h) and total sleep deprivation (TSD6h) in the expression of genes related to reactive oxygen species and nitric oxide production in the frontal cortex of a rodent model of temporal lobe epilepsy (PILO). The data show that PILO rats had increased NOX-2 expression and decreased SOD expression, independent of sleep. Higher NOX-2 expression was observed only in PILO rats subjected to the control condition and TSD6h. Also, eNOS and DDAH1 were increased in the PILO group submitted to TSD6h. Moreover, CAT expression in the frontal cortex of PILO rats submitted to PSD24h was reduced compared to that of PILO rats that were not sleep-deprived. The molecular changes found in the frontal cortex of PILO rats following sleep deprivation suggest a mechanism via oxidative stress.

1. Introduction

Epilepsy is a chronic, life-threatening neurological disease that affects 1–3% of the population worldwide [1–3]. It is characterized by recurrent spontaneous seizures which can increase the content of reactive oxygen species (ROS) and superoxide generation in the brain, leading to oxidative stress [4]. Indeed, oxidative stress is regarded as a possible mechanism in the pathogenesis of epilepsy [5]. Studies have already verified that persistent seizures can increase mitochondrial oxidative stress, cause oxidative damage to susceptible targets (protein, lipids, and DNA), change redox potential, and decrease the level of ATP, leading to a collapse in brain energy production and supply. This decrease in energy supply, in turn, leads to subsequent cell damage [6–9].

Oxidative stress has also been associated with decreases in nitric oxide (NO) bioavailability and alterations in NO signaling pathways [10]. In the central nervous system (CNS), NO modulates cognitive functions, synaptic plasticity, sleep control, and body temperature [11–13] and plays a role in the pathophysiology of epilepsy through its influence on glutamatergic and GABAergic neurotransmission [11,12]. However, there is conflicting evidence in the literature about the anti- vs proconvulsive properties of NO [14]. Another important source of ROS is the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), which is a complex composed of a membrane-bound flavocytochrome b_{558} consisting of 2 subunits (p^{22phox} and gp^{91phox}) and cytosolic activating factors (p^{47phox} and p^{67phox}). In particular, the catalytic transmembrane isoform gp^{91phox}, also called NOX-2, has been shown to be a key ROS generator in experimental models of epilepsy [15]. Also, it is important to note that angiotensin II (AngII), one of the major actors in the renin–angiotensin system (RAS), is a potent activator of NOX-2 and is also involved in the pathophysiology of epilepsy [16]. However, the role of Ang 1–7, which is a peptide derived from AngII that opposes most of its effects, is unknown [17–21].

Epilepsy and seizures can also affect physiological patterns, such as the neuroendocrine system [22], memory and cognition [23], and sleep [24], in a complicated manner. Epileptic seizures and interictal discharges can cause sleep fragmentation and change sleep architecture [24–26], exacerbating some sleep disorders and leading to sleep deprivation [27]. Sleep deprivation, in turn, increases the likelihood of seizure recurrence [28,29]. Importantly, sleep loss is associated with changes in markers of oxidative stress, such as decreases in glutathione levels, changes in the activity of antioxidant enzymes [30,31], increased DNA damage [32], decreased ACE expression [33], and reduced plasma levels of AngII and Ang 1–7 [34,35], among others. However, the central molecular mechanisms involved in the detrimental association of sleep deprivation with epilepsy are still unclear. In this sense, the aim of the current study was to investigate the effects of two different protocols of sleep deprivation in the mRNA expression of NOX-2 and antioxidant enzymes as well as nitric oxide-related genes in the central nervous system of a rodent pilocarpine-induced *status epilepticus* (SE) model of epilepsy.

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http://dx.doi.org/10.1016/j.yebeh.2013.02.024

2. Materials and methods

2.1. Animals

Eight-week-old male adult Wistar–Hannover rats obtained from Centro de Desenvolvimento de Modelos Experimentais (CEDEME, Universidade Federal de São Paulo) served as subjects. The animals were maintained and treated in accordance with the guidelines established by the Ethical and Practical Principles for the Use of Laboratory Animals [36]. This study also complied with current Brazilian government guidelines (CONCEA). All procedures were approved by the Ethics Committee of the Universidade Federal de São Paulo (# 1363/10).

2.2. Experimental design

For induction of SE, we followed the protocol described by Matos et al. [37]. Briefly, a single dose of pilocarpine (350 mg/kg, i.p.) was administered to rats (PILO group, n = 31) 30 min after injection of methylscopolamine (0.1 mg/kg, s.c.). Only rats that presented convulsive and intermittent seizures after pilocarpine injection were included in the study. Seizure activity was monitored behaviorally and terminated after 3 h of convulsive SE with an i.p. injection of diazepam (10 mg/kg). The vehicle group (SAL group, n = 28) was injected with saline (0.9% NaCl, i.p.). This model is considered a reliable model of temporal lobe epilepsy (TLE) as animals treated with pilocarpine show similar alterations to hippocampal sclerosis, which is often present in patients with TLE [38]. Fifty days after the SE episode, the animals in the PILO group were assigned randomly to total sleep deprivation (PILO + TSD6h group, n = 8), paradoxical sleep deprivation (PILO + PSD24h, n = 12), or control conditions (PILO + CTRL, n = 11). Similarly, rats in the SAL group were assigned to the same conditions (SAL + TSD6h, n = 8; SAL + PSD24h, n = 9; SAL + CTRL, n = 11). Fig. 1 shows the experimental timeline. The total number of animals used was 59.

2.3. Sleep deprivation

Fifty days after SE induction, the PILO + TSD6h and SAL + TSD6h groups were subjected to total sleep deprivation through a gentle handling protocol. This procedure consists of touching the animals with the hand or a brush and shaking and tapping the cage and is widely accepted as a way to keep rodents awake for a period of hours while minimally disturbing potential ongoing activity [39]. Total sleep deprivation was carried out for 6 h (7 AM–1 PM). In parallel, the PILO + PSD24h and SAL + PSD24h groups were paradoxically sleep-deprived for 24 h using the modified multiple platform method [40]. Briefly, the PSD procedure consists of placing rats in a water tank (106 cm × 40 cm × 30 cm) containing 14 circular platforms (6.5 cm in diameter each). The rats can move around inside the tank by jumping from one platform to another. When the rats reach the paradoxical sleep phase, the resultant muscle atonia causes them to fall into the water and awaken. In a pilot study, 24 h was considered a safe period for PSD in rats with epilepsy (PILO groups), i.e., unlikely to result in multiple seizures and lead to drowning death of the rats in the water tank. The CTRL groups were maintained in their home cages in the same rooms as the PSD24h and TSD6h groups. We did not include large platform controls since they present disturbed sleep architecture that could interfere in this study [41].

2.4. Tissue collection and total RNA extraction

The animals were decapitated immediately after the sleep deprivation procedure or the equivalent time for CTRL rats. The frontal cortex was rapidly dissected from the brain, flash frozen in dry ice, and then stored at −80 °C until RNA extraction. Total RNA was extracted from the frontal cortex using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. After RNA extraction, RNA was evaluated by electrophoresis in agarose gel to assure integrity of the 18S and 28S ribosomal subunits.

2.5. Reverse transcription and quantitative real-time PCR (RTqPCR)

Total RNA (2 μg) was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed at 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min. Amplification (400 ng of cDNA) and detection was performed using an Assay-on-Demand and TaqMan 5 fluorogenic nuclease chemistry in an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems) according to the manufacturer's instructions. A two-stage cycle (hold stage of 50 °C for 2 min and 95 °C for 10 min followed by 95 °C for 15 s and 60 °C for 1 min) was repeated 40 times followed by a dissociation stage. Identifications of primers for rat gp^{91PHOX} (Cybb or NOX-2), rat nitric oxide synthase 2 (NOS2 or iNOS), rat nitric oxide synthase 3 (NOS3 or eNOS), rat dimethylarginine dimethylaminohydrolase 1 (DDAH1), rat superoxide dismutase 1 (SOD or CuZnSOD), rat catalase (CAT), and rat angiotensin converting enzyme 2 (ACE2) target genes were Rn00576710_m1, Rn00561646_m1, Rn02132634_s1, Rn00574200_m1, Rn00566938_m1, Rn00560930_m1, and Rn01416293_m1, respectively. Endogenous control genes chosen for normalization were β-actin (reference sequence: NM_031144) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (reference sequence: NM_017008), whose primer sequences (5′–3′) are AGCGTGGCTACAGCTTCACC/AAGTCTAGGGCAACATAGCACAGC and TGCCCCCATGTTTGTGATG/GCTGACAATCTTGAGGGAGTTGT, respectively. Nitric oxide synthase 2 was not detectable in the frontal cortex.

2.6. Statistical analysis

The expression fold changes of all genes were calculated by the $2^{-\Delta\Delta CT}$ method [42] using the arithmetic mean of the reference genes, GAPDH and β-actin, as the normalization factor since they did not differ between groups. The variables were first tested for normality and homogeneity distribution, and all of them fit the parametric criteria. Thus, to compare the effects of treatment (SAL and PILO), sleep (CTRL, TSD6h, and PSD24h) and treatment * sleep interaction (SAL + CTRL, SAL + TSD6h, SAL + PSD24h, PILO + CTRL, PILO + TSD6h, and PILO + PSD24h) in the relative gene expression values, a general linear model was used for a two-way analysis of variance (ANOVA). Tukey post hoc tests were applied when necessary to detect differences in sleep and treatment * sleep interaction effects. To establish the association between all gene expression variables, Pearson correlation analysis was used. The analyses were performed using the SPSS software (version 17, Chicago, IL, USA). Data are expressed as mean ± SEM. The level of significance was $p < 0.05$.

3. Results

Fig. 2 shows NOX-2 expression in the frontal cortex of rats from the SAL + CTRL, PILO + CTRL, SAL + TSD6h, PILO + TSD6h, SAL + PSD24h, and PILO + PSD24h groups. Analysis of variance revealed a significant treatment effect ($F_{1,53} = 7.38$, $p < 0.01$), demonstrating that PILO rats (1.28 ± 0.07) had increased NOX-2 expression in the frontal cortex independent of sleep condition when compared to CTRL rats (1.01 ± 0.07). Moreover, a significant treatment * sleep interaction effect was found ($F_{1,53} = 1.12$, $p < 0.05$), showing specifically that the PILO + CTRL group (1.39 ± 0.11) had greater cortical NOX-2 expression compared to the SAL + CTRL group (1.05 ± 0.07, $p < 0.05$), and the PILO + TSD6h group (1.42 ± 0.13) had greater NOX-2 expression compared to the SAL + TSD6h group (1.03 ± 0.10,