Available online at www.sciencedirect.com
SCIENCE DIRECT
Neurochemistry International 48 (2006) 75–82
NEUROCHEMISTRY International
www.elsevier.com/locate/neuint

Depletion of reduced glutathione precedes inactivation of mitochondrial enzymes following limbic status epilepticus in the rat hippocampus

Hannah Sleven [a], Julie E. Gibbs [a], Simon Heales [b], Maria Thom [b], Hannah R. Cock [a,*]

[a] *Epilepsy Group, Centre for Clinical Neurosciences, St. George's, University of London, Cranmer Terrace, London SW17 0RE, UK*
[b] *Institute of Neurology, University College London, Queen Square, London WC1N 3BG, UK*

Received 23 August 2005; received in revised form 26 September 2005; accepted 3 October 2005
Available online 14 November 2005

Abstract

The time course and critical determinants of mitochondrial dysfunction and oxidative stress following limbic status epilepticus (SE) were investigated in hippocampal sub-regions of an electrical stimulation model in rats, at time points 4–44 h after status. Mitochondrial and cytosolic enzyme activities were measured spectrophotometrically, and reduced glutathione (GSH) concentrations by HPLC, and compared to results from sham controls. The earliest change in any sub-region was a fall in GSH, appearing as early as 4 h in CA3 (-13%, $p < 0.05$), and persisting at all time points. This was followed by a transient fall in complex I activity (CA3, 16 h, -13%, $p < 0.05$), and later changes in aconitase (CA1,-18% and CA3, -22% at 44 h, $p < 0.05$). The activity of the cytosolic enzyme glyceraldehyde-3-phosphate-dehydrogenase was unaffected at all time points. It is known that GSH levels are dependent both on redox status, and on the availability of the precursor cysteine, in turn dependent on the cysteine/glutamate antiporter, for which extracellular glutamate concentrations are rate limiting. Both mechanisms are likely to contribute indirectly to GSH depletion following seizures. That a relative deficiency in GSH precedes later changes in the activities of complex I and aconitase in vulnerable hippocampal sub-regions, occurring within a clinically relevant therapeutic time window, suggests that strategies to boost GSH levels and/or otherwise reduce oxidative stress following seizures, deserve further study, both in terms of preventing the biochemical consequences of SE and the neuronal dysfunction and clinical consequences.

Keywords: Glutathione; Mitochondrial dysfunction; Oxidative stress; Rat hippocampus; Status epilepticus

1. Introduction

Status epilepticus (SE) is a condition in which a single seizure, or a series of seizures, lasts for 30 min or more without restoration of consciousness, and is associated with a significant morbidity and mortality, including neuronal damage and dysfunction (Fountain, 2000; Shorvon, 1994). Evidence from both human and animal studies suggest that the rhythmic and synchronous neuronal activity characteristic of seizures is damaging to neurons on a local level, and that this damage is independent of more systemic factors in SE, such as hypoxia and acidosis (Meldrum, 2002). Different brain areas vary in their vulnerability to the neuronal damage associated with seizures.

Seizure related neuronal death is believed to involve excitotoxic mechanisms instigated by excessive glutamate activation of post-synaptic NMDA receptors, which leads to increased calcium uptake and impaired intracellular calcium handling, and culminates in the activation of cell death pathways leading to both necrosis and apoptosis (Cock et al., 2002; Meldrum, 1993). Mitochondrial dysfunction has been shown to be a critical component of excitotoxic cell death (Schinder et al., 1996) and oxidative stress is also believed to play an important role (Cock, 2002). However, the sequence of events in the early hours after SE is poorly defined, and thus the identification of critical determinants, essential for the development of rational neuroprotective strategies, remains uncertain. This is particularly important given the inter-dependence of mitochondrial respiratory chain function and redox status. We set out to further elucidate the sequence and mechanisms of neuronal damage following SE in an animal model, and to determine whether mitochondrial enzyme dysfunction might be cause or effect in terms of redox-homeostasis following SE.

* Corresponding author. Fax: +44 20 8181 6897.
E-mail address: hcock@sgul.ac.uk (H.R. Cock).
URL: http://www.sgul.ac.uk/depts/cn/epilepsygroup/index.htm

0197-0186/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2005.10.002

2. Experimental procedures

2.1. Animals

Male Sprague–Dawley rats (280–320 g; A.J. Tuck and Sons, UK) were individually housed in a regulated environment with a 12-h light:12-h dark cycle and free access to standard feed and water. All animal procedures followed local and Home Office regulations under the Animal (Scientific Procedures) Act, 1986, UK.

2.2. Limbic SE

Limbic SE was induced as previously described. Briefly, unilateral stimulating (perforant path) and recording (DG cell layer) electrodes were stereotaxically implanted under halothane anaesthesia with a subcutaneous earth. Recording electrode placement was adjusted to achieve the maximum amplitude population spike in response to low frequency stimulation, and electrodes secured into a six pin plastic pedestal (Plastics One, Sevenoaks, UK) before fixing with dental acrylic (Minerva Dental, Cardiff, UK) and skull screws. After a 5–8 day recovery period, limbic SE was induced in freely moving awake animals by electrical stimulation (2–6 mA, 20 Hz) of the perforant path. After an initial 2 h period of stimulation, self-sustaining SE was allowed to continue for a further 3 h before termination of SE with 20 mg/kg diazepam i.p. (Phoenix Pharma Ltd., Gloucester, UK). The animals were monitored continuously, with extracellular recordings from the DG cell layer displayed on an oscilloscope (Tektronix TDS 210). Spike frequency and the behavioural severity of seizures (classified on the Racine scale; Racine, 1972) were recorded for every 15 min period. All animals were given 5 ml of saline, s.c., at the end of SE to minimise dehydration. Where self-sustaining SE was not achieved, or it was achieved but could not be terminated at 5 h, these animals were excluded from the study.

2.3. Biochemical analysis

At 4, 16 or 44 h following the termination of SE, animals were sacrificed by decapitation under brief halothane anaesthesia. The brains were rapidly removed on ice and the hippocampus of each hemisphere isolated and dissected into DG/hilus (DG/H, combined), CA1 and CA3 sub-fields, as previously described (Davey et al., 1997), and snap frozen in liquid nitrogen. The sub-fields from each hemisphere were combined, homogenised and stored at −70 °C for later analysis. Samples from one SE and one sham animal at each time point were assayed in parallel, with the experimenter blinded to the origin of the sample. Spectrophotometric enzyme assays for aconitase (EC 4.2.1.3), α-ketoglutarate dehydrogenase (AKGDH, EC 1.2.4.2), complexes I (EC 1.6.5.3), II/III (succinate oxidase system, 1.3.5.1 and 1.10.2.2) and IV (EC 1.9.3.1), citrate synthase (CS, EC 2.3.3.1) and GAPDH (EC 1.2.1.13) were performed in triplicate on the samples within 5 days, and HPLC analysis of GSH undertaken as previously described (Cock et al., 1998; Heinz and Freimuller, 1982). Results were corrected for protein content (Lowry et al., 1951). CS, aconitase, AKGDH and GAPDH activities are expressed as nmol/min/mg protein; complexes I, II/III and IV activities are expressed as a ratio of CS activity to correct for mitochondrial content, and GSH is expressed as nmol/mg protein.

2.4. Histology

Acute neuronal injury (eosinophilia and pyknotic neurones) was assessed in haematoxylin and eosin stained sections throughout the hippocampus of perfusion-fixated brains as previously described, using a modified semi-quantitative scale. Using a Leica Orthoplan microscope and ×16 objective lens with an eyepiece graticule (grid area 0.25 mm^2) a blinded assessor counted the number of damaged neurons per ×16 field and recorded a grade for each sub-region: Grade 0, none; Grade 1, 1–2; Grade 2, 3–10; Grade 3, 11–20; Grade 4, 21–30; Grade 5, 31–40; Grade 6, >40. All sections including hippocampus from each animal (range 6–12 per animal) were evaluated, and the section scores used to calculate means and S.E.M. for each hippocampal region.

2.5. Materials

Stimulating and recording electrodes, pedestals and skull screws were obtained from Bilaney Consultants Ltd. (Sevenoaks, UK). Chemical reagents were at a minimum analytical grade and were purchased from the Sigma Chemical Company (Poole, UK) or VWR Merck (Leicestershire, UK).

2.6. Statistics

Results are presented as mean ± S.E.M. Comparisons were performed using a one-way multifactorial analysis of variance (ANOVA) on SPSS Version 10 for windows, followed by L.S.D. post hoc analysis for significant main effects. Variables entered were group (sham/status), brain region (CA1, CA3 and DG/H), case control set and protein; the outcome measure was the biochemical parameter.

3. Results

3.1. Behavioural and EEG features

A total of 39 rats were included in the biochemical study, with 19 sham-operated animals and 20 stimulated animals. All stimulated animals underwent the full 5 h of SE (as assessed by behavioural and electrographic criteria) and their SE was successfully stopped at the end by administration of diazepam. Within 15 min of commencing stimulation animals persistently show behavioural changes consistent with limbic SE, as previously described, including periods of behavioural arrest, stereotyped movements (continuous sniffing, paw licking, rearing and wet dog shakes), clonic movements of forelimbs, and sometimes progressing to rearing and/or falling. The combined operative and seizure related mortality was under 20%, and animals that did not survive either the procedure or SE with full recovery were not included in the analysis. The mean behavioural seizure severity did not significantly differ between experimental groups (Fig. 1). The mean spike

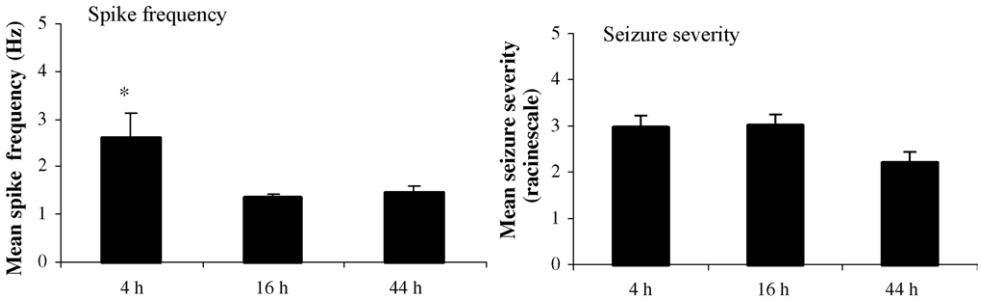

Fig. 1. Spike frequency and behavioural seizure severity, 4 h ($n = 5$), 16 h ($n = 10$) and 44 h ($n = 5$) after SE. Values are mean ± S.E.M., $^{*}p < 0.05$.