Seizure 18 (2009) 420–428

Contents lists available at ScienceDirect

Seizure

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

Preservation of mitochondrial integrity and energy metabolism during experimental status epilepticus leads to neuronal apoptotic cell death in the hippocampus of the rat

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ARTICLE INFO

Article history:
Received 28 September 2008
Received in revised form 19 February 2009
Accepted 20 March 2009

Keywords:
Apoptosis
Status epilepticus
Energy metabolism
Mitochondrial integrity
ATP
Hippocampus

ABSTRACT

Status epilepticus results in mitochondrial damage or dysfunction and preferential neuronal cell loss in the hippocampus. Since a critical determinant of the eventual cell death fate resides in intracellular ATP concentration, we investigated whether mitochondrial integrity and level of energy metabolism are related with apoptotic cell death in specific hippocampal neuronal populations. A kainic acid (KA)-induced experimental temporal lobe status epilepticus model was used. Qualitative and quantitative analysis of DNA fragmentation, TUNEL immunohistochemistry, double immunofluorescence staining for activated caspase-3, electron microscopy or measurement of ATP level in the bilateral hippocampus was carried out 1, 3 or 7 days after microinjection unilaterally of a low dose of KA (0.5 nmol) into the CA3 hippocampal subfield. Characteristic biochemical (DNA fragmentation), histochemical (TUNEL or activated caspase-3 staining) or ultrastructural (electron microscopy) features of apoptotic cell death were presented bilaterally in the hippocampus 7 days after the elicitation of sustained hippocampal seizure activity by microinjection of KA into the unilateral CA3 subfield. At the same time, CA3 or CA1 subfield on either side manifested a maintained ATP level; alongside relatively intact mitochondria, rough endoplasmic reticulum, Golgi apparatus or cytoplasmic membrane in hippocampal neurons that exhibited ultrastructural features of apoptotic cell death. Our results demonstrated that preserved mitochondrial ultrastructural integrity and maintained energy metabolism during experimental temporal lobe status epilepticus is associated specifically with apoptotic, not necrotic, cell death in hippocampal CA3 or CA1 neurons.

1. Introduction

Seizure is a major form of acute brain injury that could lead to a large number of changes and cascades of events at the cellular level, including gene expression, receptor composition, synaptic physiology and activation of late cell death pathways.[1,2] Clinical and epidemiological studies suggest that patients with chronic epilepsy undergo progressive brain atrophy that is accompanied by long-term behavioral changes and cognitive declines despite optimal antiepileptic drug therapy.[3,4] Prolonged and continuous epileptic seizure (status epilepticus) is a medical emergency associated with substantial morbidity and mortality.[5] Human and animal studies[4,6] showed that status epilepticus results in significant cerebral damage and increases the risk of subsequent epileptic episodes, alongside a characteristic pattern of preferential neuronal cell loss in the hippocampus. However, the nature of seizure-induced neuronal cell death is controversial. Necrosis is generally taken as the principal morphological phenotype of dying cells after seizures.[2] Despite the predominance of necrotic morphology in dying neurons after seizures, other studies suggest that apoptotic cell death plays an important role in seizure-induced brain damage.[1,7–9] Thus, research into the cell death fate after status epilepticus remains of critical importance.

From its role as the cellular powerhouse, the mitochondrion is emerging as a key participant in cell death because of its association with an ever-growing list of apoptosis-related proteins.[10,11] The

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doi:10.1016/j.seizure.2009.03.002

nature of cell death and its relationship with mitochondrial dysfunction and bioenergetic failure in the hippocampus during status epilepticus remains unknown. A critical determinant of the eventual cell death fate resides in intracellular ATP concentration, the production of which depends on the structural and functional integrity of the mitochondrion. Whereas ATP depletion is associated with necrosis, ATP is required for the development of apoptosis.[12–14] Based on an experimental model of temporal lobe status epilepticus, we demonstrated recently[15,16] that the prolonged seizure-like hippocampal electroencephalographic (hEEG) activity elicited by microinjection unilaterally of the glutamate agonist kainic acid (KA) into the CA3 subfield of hippocampus[17,18] is associated with dysfunction of Complex I respiratory chain enzyme and mitochondrial ultrastructural damage in the bilateral CA3 or CA1 field. It follows that the long-term cell death fate in the hippocampus after status epilepticus is dependent on whether mitochondrial ultrastructural integrity and energy metabolism are preserved. The present study was undertaken to address this issue. Based on complementary biochemical, histochemical and electron microscopic results, we demonstrated that apoptotic cell death induced by experimental temporal lobe status epilepticus is associated with preserved mitochondrial ultrastructural integrity and energy metabolism in hippocampal CA3 or CA1 neurons.

2. Materials and methods

All experimental procedures were in compliance with the guidelines for the care and use of experimental animals endorsed by our institutional animal care committee. All efforts were made to reduce the number of animals used and to minimize animal suffering during the experiment.

2.1. Animals

Experiments were carried out in 141 specific pathogen-free adult male Sprague–Dawley rats (237–316 g). Rats were obtained from the Experimental Animal Center of the National Science Council, Taiwan, Republic of China, and were housed in an animal room under temperature control (24–25 °C) and 12-h light–dark cycle. Standard laboratory rat chow and tap water were available *ad libitum*.

2.2. Experimental temporal lobe status epilepticus

We reported previously[15,16,19] that microinjection unilaterally of KA into the hippocampal CA3 subfield results in a progressive buildup of bilateral seizure-like hEEG activity. This model of experimental temporal lobe status epilepticus was again used in the present study. The head of the animal was fixed to a stereotaxic headholder (Kopf, Tujunga, CA, USA) after chloral hydrate (400 mg/kg) was given intraperitoneally to induce anesthesia, and the rest of the body was placed on a heating pad to maintain body temperature at 37 °C. KA (0.5 nmol; Tocris, Ellisville, MO, USA) dissolved in 0.1 M phosphate buffered saline (PBS, pH 7.4) was microinjected stereotaxically (3.3–3.6 mm posterior to bregma, 2.3–2.6 mm from the midline, and 3.4–3.8 mm below the cortical surface) into the CA3 subfield of hippocampus on the left side. The volume of microinjection was restricted to 50 nL and was delivered using a 27-gauge needle connected to a 0.5-μL Hamilton microsyringe (Reno, NV, USA). This consistently resulted in progressive and concomitant increase in both root mean square and mean power frequency values of hEEG signals recorded from the CA3 subfield on the right side.[15,19] As a routine, these experimental manifestations of temporal lobe status epilepticus were followed for 30–40 min. The wound was then closed in layers, and sodium penicillin (10,000 IU; YF Chemical Corporation, Taipei, Taiwan) was given intramuscularly to prevent postoperative infection. Animals were returned to the animal room for postoperative recovery in individual cages. Rats that received unilateral microinjection of 50 nL of PBS and did not exhibit seizure-like hEEG activities served as our vehicle controls. Animals that received choral hydrate anesthesia and surgical preparations without additional experimental manipulations served as sham-controls.

2.3. Collection of tissue samples from the hippocampal subfields

At pre-determined intervals (1, 3 or 7 days) after microinjection of KA or PBS into the hippocampus, rats were perfused intracardiacly with 50 mL of warm (37 °C) saline that contains heparin (100 U/mL). The brain was rapidly removed under visual inspection and placed on a piece of gauze moistened with ice-cold 0.9% saline for the removal of bilateral CA1 or CA3 subfield of hippocampus.[15] We routinely separated tissues collected from the hippocampal subfields on the left (injection side for KA) and the right side (recording side for hEEG). This allowed us to ascertain that the results from our biochemical, histochemical or ultrastructural analysis were consequential directly to experimental temporal lobe status epilepticus and not indirectly to KA excitatotoxicity.[17,18]

2.4. Qualitative and quantitative analysis of DNA fragmentation

Hippocampal tissues collected 1, 3 or 7 days after microinjection of KA or PBS into the left CA3 subfield were used for qualitative and quantitative analysis of DNA fragmentation.[16,19,20] After extraction of total DNA from the hippocampal tissues, nucleosomal DNA ladders were amplified by a PCR kit for DNA ladder assay (APO-DNA1, Maxim Biotech, San Francisco, CA, USA) to enhance the detection sensitivity, and were separated by electrophoresis on 1% agarose gel.[16,19,20] To quantify apoptosis-related DNA fragmentation, a cell death enzyme-linked immunosorbent assay (Roche Molecular Biochemicals, Mannheim, Germany) that detects apoptotic but not necrotic cell death[21] was used to assay the level of histone-associated DNA fragments in the cytoplasm.[22] In brief, proteins from the cytosolic fraction of the hippocampal samples were used as the antigen source, together with primary anti-histone antibody and secondary anti-DNA antibody coupled to peroxidase.[16,19,20] The amount of nucleosomes in the cytoplasm was quantitatively determined using 2,2′-azino-di-[3-ethylbenzthiazoline]sulfonate as the substrate. Absorbance was measured at 405 nm and referenced at 490 nm using a microtiter plate reader (Hitachi, Japan).

2.5. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining

As a histochemical marker for apoptotic cell death, animals were processed for TUNEL staining 1, 3 or 7 days after the induction of experimental temporal lobe status epilepticus. In brief, the hippocampus was removed and fixed in 30% sucrose in 10% formaldehyde-saline solution for ≥72 h. Six micrometer paraffin-embedded sections of the hippocampus were processed for TUNEL staining using an in situ apoptosis detection kit (ApopTag, Intergen Company, Purchase, NY, USA).[23] The total numbers of TUNEL-positive cells on each section were counted using an Olympus AX70 microscope and expressed as the TUNEL indices.[23,24]

2.6. Double immunofluorescence staining and laser confocal microscopy

Immunofluorescence staining was carried out in animals using procedures that were modified from those reported previously.[16,25] In brief, free-floating sections of the hippocampus were incubated with a rabbit polyclonal antiserum against activated caspase-3 (Cell Signaling, Danvers, MA, USA), together with a mouse monoclonal