Neuroscience 131 (2005) 887–897

CASPASE 6 EXPRESSION IN THE RAT HIPPOCAMPUS DURING EPILEPTOGENESIS AND EPILEPSY

S. NARKILAHTI[a] AND A. PITKÄNEN[a,b]*

[a]*A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, PO Box 1627, FIN-70 211 Kuopio, Finland*

[b]*Department of Neurology, Kuopio University Hospital, PO Box 1777, FIN-70 211 Kuopio, Finland*

Abstract—The molecular basis of neuronal circuit reorganization during epileptogenesis is poorly understood. Such data are, however, critical for the search of new targets for the prevention of epileptogenesis. Here, we extended our previous studies on caspases in epileptogenesis by investigating the expression and activity of caspase 6 at different phases of the epileptic process in rats. Epileptogenesis was triggered by kainate-induced status epilepticus (SE) under video-electroencephalography-monitoring. Caspase 6 activity was measured fluorometrically in the hippocampus 8 h, 24 h, 48 h, 1 week, and 4 weeks after SE. Caspase 6 expression was examined using Western blot and immunohistochemistry. Our data demonstrated that the SE-induced increase in the expression of cleaved caspase 6 and its intraneuronal localization were dependent on the time delay from SE induction. Double-labeling with a neuronal marker, NeuN, indicated that within the first 48 h, caspase 6 immunoreactivity was present both in the hippocampal pyramidal cells and hilar neurons, some of which were also terminal transferase dUTP-end labeling-positive. This was coincident with a transient 18-fold increase in caspase 6 enzymatic activity. At the 1-week and 4-week time points, elevated caspase 6 immunoreactivity was detected in the dendritic processes and neuropil. These findings indicate that caspase 6 expression remains elevated long after the occurrence of acute cell death during epileptogenesis and epilepsy. Further, caspase 6 protein is not exclusively located in the somata of neurons, but also in dendrites. These data suggest that caspase 6 has functions other than execution of programmed cell death in epileptogenic hippocampus.

Key words: apoptosis, dendrite, kainic acid, programmed cell death, seizure, status epilepticus.

Epilepsy is the second most common neurologic disorder, affecting 40–50 million individuals worldwide. About 60% of epilepsies are of focal onset and in most patients seizures begin in the temporal lobe (Engel, 1989). In a large majority of patients with temporal lobe epilepsy (TLE), the epileptogenic process is triggered by a brain-damaging insult such as traumatic brain injury, stroke, or status epilepticus (SE; Mathern et al., 1995). Brain damage is followed by a latency phase during which a large number of neurobiologic alterations take place. These include acute and delayed neuronal death, reactive gliosis, axonal and dendritic plasticity, neurogenesis, inflammation, revascularization, and molecular reorganization of cellular membranes and extracellular matrix (for review see Jutila et al., 2002). These alterations result in spontaneous recurrent seizures, that is, epilepsy.

Human TLE can be modeled in rats by triggering the epileptogenic process with SE (Ben-Ari, 1985). The acute neurodegeneration that is detected within hours to 3 days after SE is due to necrosis (Fujikawa, 1996; Fujikawa et al., 2000). It is still under dispute whether programmed cell death contributes to the delayed neurodegeneration that continues for several weeks after SE (Pitkänen et al., 2002; Gorter et al., 2003). Identification of molecules responsible for delayed neurodegeneration would offer novel targets for neuroprotective therapy aimed at disease modification during epileptogenesis. Over the past few years, caspases have been identified as a candidate group of proteolytic enzymes that contribute to neurodegeneration after epileptogenic insults, including SE, stroke, and traumatic brain injury (for reviews see Yakovlev and Faden, 2001; Liou et al., 2003). Most of these studies, however, investigated caspase activity within a short time window relative to the occurrence of epileptogenic insult, and therefore, it has been difficult to address their role in delayed neuronal damage and in other aspects of circuitry reorganization.

There are 14 members in the caspase family of proteins which according to the function are divided to initiator caspases (caspases 2, 8, 9, 10), executioner caspases (caspases 3, 6, 7) and caspases involved in cytokine processing (caspases 1, 4, 5, 11, 12, 13, 14; Wolf and Green, 1999). The executioner caspases are responsible for cleavage of cellular substrates leading ultimately to death of the cell. So far, most of the studies have focused on caspase 3 whose expression and activity is demonstrated to increase after epileptogenic insults (Faherty et al., 1999; Ferrer et al., 2000; Henshall et al., 2000; Narkilahti et al., 2003b). Nevertheless, inhibition of caspase 3 does not rescue all of the neurons or does not prevent epileptogenesis (Kondratyev and Gale, 2000; Henshall et al., 2000; Narkilahti et al., 2003a). Contradictory to caspase 7, which

*Correspondence to: A. Pitkänen, A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, PO Box 1627, FIN-70 211 Kuopio, Finland. Tel: +358-17-163296; fax: +358-17-163025.
E-mail address: asla.pitkanen@uku.fi (A. Pitkänen).

Abbreviations: Ac-VEID-AMC, *N*-acetyl-Val-Glu-Ile-Asp-7-amino-4-methylcoumarin; CST, Cell Signaling Technology; DAB, 3,3-diaminobenzedine; EDTA, ethylenediaminetetraacetic acid; EEG, electroencephalography; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, *N*-[2-hydroxyethyl]piperazine-*N′*-[ethanesulfonic acid]; ir, immunoreactivity; NeuN, neuronal nuclei protein; NGS, normal goat serum; SD, standard deviation; SE, status epilepticus; SEM, standard error of mean; TBS, Tris-buffered saline; TLE, temporal lobe epilepsy; TUNEL, terminal transferase dUTP-end labeling.

0306-4522/05$30.00+0.00 © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2004.12.013

is not activated by SE (Henshall et al., 2002) or does not induce neuronal or glial death (Zhang et al., 2000), caspase 6 expression and activation is reported after SE (Ferrer et al., 2000; Henshall et al., 2002; Troy et al., 2002). Moreover, even transient caspase 6 expression is capable of inducing neuronal death (Zhang et al., 2000).

Caspase 6 (Fernandes-Alnemri et al., 1995) is highly expressed in adult brain and localized both in the cytosol and nerve terminals (LeBlanc et al., 1999; Shimohama et al., 2001a,b). Caspases 3, 7, 8, 10 are able to activate caspase 6 *in vitro* (Cohen, 1997). Accordingly, caspase 6 activity is reduced by caspase 8 inhibitor z-IETD following SE *in vivo* (Henshall et al., 2002). In neuronal cultures, caspase 6 itself activates caspase 3 (Allsopp et al., 2000; Zhang et al., 2000). The best known substrate for caspase 6 is lamin A, a structural protein of the nuclear envelope (Orth et al., 1996). Caspase 6 also cleaves cytoskeletal and structural proteins (Van de Craen et al., 1999; Ruchaud et al., 2002), proteins that are related to cell adhesion (Gervais et al., 1998) as well as microtubule-associated protein tau and amyloid precursor protein that are associated with Alzheimer's disease (LeBlanc et al., 1999; Pellegrini et al., 1999; Guo et al., 2004; Horowitz et al., 2004). As the list of structurally heterogeneous caspase substrates is still growing, non-lethal functions have been suggested for caspases (for reviews see Fischer et al., 2003; McLaughlin, 2004). For example, caspase 6 among the other caspases may contribute to other functions relevant to epileptogenesis such as axon guidance, synaptic plasticity, and migration of newly born neurons.

Here, we hypothesized that caspase 6 possesses multiple functions in epileptogenic tissue, that is, it contributes to the reorganization and maturation of the epileptogenic network. The present study addressed three questions. First, what is the time course of caspase 6 activation during epileptogenesis? Second, what is the distribution of caspase 6 expression in epileptogenic hippocampus? Third, what is the cellular localization of caspase 6 at different phases of the epileptogenic process? Epileptogenesis was triggered with kainate-induced SE, and tissue was sampled for analysis at 8 h, 24 h, 48 h, 1 week, and 4 weeks after SE.

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague–Dawley rats (Harlan Sprague–Dawley, Horst, Netherlands) or male Han-Wistar rats (National Laboratory Animal Center, Kuopio, Finland) weighing 300–350 g were housed in individual cages at a temperature of 19–21 °C, humidity 50–60%, and lights on 07:00–19:00 h. Standard food pellets and water were freely available. All animal procedures were approved by the Animal Care and Use Committee of the University of Kuopio and conducted in accordance with the guidelines set by the European Community Council Directives 86/609/EEC. All efforts were made to minimize the number of animals used and their suffering.

Electrode implantation for electroencephalography (EEG) monitoring

Cortical electrodes were implanted in all animals 2 weeks before induction of SE. Briefly, the animals were anesthetized using an i.p. injection (6 ml/kg) of a mixture containing sodium pentobarbital (58 mg/kg), chloral hydrate (60 mg/kg), magnesium sulfate (127.2 mg/kg), propylene glycol (40%), and absolute ethanol (10%), and then, placed into a stereotaxic frame (lambda and bregma at the same horizontal level). Two stainless steel screw electrodes (E363/20; Plastics One Inc., Roanoke, VA, USA) were inserted into the skull above the frontal cortex [3 mm anterior to the bregma, ±2 mm lateral to midline according to the rat brain atlas of Paxinos and Watson (1986)]. Two electrodes inserted into the skull bilaterally over the cerebellum served as indifferent and ground electrodes. The electrodes were fixed with dental acrylic (Selectaplus; Dentsply DeTrey GmbH, Dreieich, Germany).

Induction of SE

Two weeks after electrode implantation, SE was induced in Sprague–Dawley rats with 10 mg/kg of kainic acid i.p. (K-0250; Sigma, Germany; dissolved in 0.9% NaCl) and in Wistar rats with 9 or 10 mg/kg of kainic acid i.p. (2020; Opika-1; Ocean Produce International, Shelburne, Canada). The controls received saline injections. Video-EEG monitoring during SE demonstrated that the severity of SE was comparable between both rat strains (see below). Only the animals with electrographic SE lasting for at least 4 h were included in the study.

Video-EEG monitoring of SE

Epileptiform activity during SE was monitored using a video-EEG system that included the Nervus EEG Recording system (Taugagreining, Iceland) connected to an ISO-1032 Amplifier (Braintronics, Netherlands), SVT-S3000P Hitachi Time Lapse 168 VCR (Japan), and Panasonic WV-CL350 Video Camera (Japan). The rats were monitored continuously until killed or at maximum 24 h.

Video-EEG monitoring of spontaneous seizures

Animals that were randomized to the chronic 4-week group were continuously (24 h/day) monitored for 2 weeks (weeks 3–4) to detect spontaneous seizures using the same video-EEG system as described above. At night, recordings were performed under infrared light. If an electrographic seizure was detected, its behavioral severity was scored according to a modified Racine's scale (Racine, 1972): score 0: electrographic seizure without any detectable motor manifestation; score 1: mouth and face clonus, head nodding; score 2: clonic jerks of one forelimb; score 3: bilateral forelimb clonus; score 4: forelimb clonus and rearing; score 5: forelimb clonus with rearing and falling.

Activity and cleavage of caspase 6 after SE

Sprague–Dawley rats injected with kainate (n=23) were decapitated at 8 h (n=4), 16 h (n=3), 24 h (n=3), 48 h (n=3), 1 week (n=4), or 4 weeks (n=6) after kainate injection. Two saline-treated controls were killed at each time point. The hippocampus was dissected from both hemispheres separately, frozen in dry ice, and stored at −70 °C until processed. Right hippocampi were used for measurement of caspase 6 activity and left hippocampi for Western blot analysis.

Enzyme assay of caspase 6. Hippocampal samples were homogenized in a lysis buffer, centrifuged, supernatant was collected, and the protein concentration was determined as described previously (Narkilahti et al., 2003b). Thirty micrograms of protein in 100 μl of caspase assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% [(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol) was mixed with 100 μl of 40 μM *N*-acetyl-Val-Glu-Ile-Asp-7-amino-4-methylcoumarin (Ac-VEID-AMC) substrate (218760; Calbiochem, La Jolla, CA, USA) and incubated for 1 h at 37 °C. Caspase 6 activity was determined by measuring fluorescent AMC cleavage from Ac-VEID-AMC. The