Neurobiology of Disease 102 (2017) 1–10

Contents lists available at ScienceDirect

Neurobiology of Disease

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

A calpain inhibitor ameliorates seizure burden in an experimental model of temporal lobe epilepsy

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ARTICLE INFO

Article history:
Received 15 November 2016
Revised 27 January 2017
Accepted 20 February 2017
Available online 22 February 2017

Keywords:
Epileptogenesis
Calpain
Proteolysis
Epilepsy
Spontaneous seizures

ABSTRACT

In this study, we used the pilocarpine model of epilepsy to evaluate the involvement of calpain dysregulation on epileptogenesis. Detection of spectrin breakdown products (SBDPs, a hallmark of calpain activation) after induction of pilocarpine-induced *status epilepticus* (*SE*) and before appearance of spontaneous seizure suggested the existence of sustained calpain activation during epileptogenesis. Acute treatment with a cell permeable inhibitor of calpain, MDL-28170, resulted in a partial but significant reduction on seizure burden. The reduction on seizure burden was associated with a limited reduction on the generation of SBDPs but was correlated with a reduction in astrocytosis, microglia activation and cell sprouting. Together, these observations provide evidence for the role of calpain in epileptogenesis. In addition, provide proof-of-principle for the use of calpain inhibitors as a novel strategy to prevent epileptic seizures and its associated pathologies.

1. Introduction

Epilepsy is a chronic disease characterized by the occurrence of spontaneous recurrent seizures (SRS) arising from abnormal neuronal hyperexcitability and synchronization (Kumar and Buckmaster, 2006; El-Hassar et al., 2007; Fritschy, 2008; Williams et al., 2009; O'Dell et al., 2012). In humans, almost half of individuals experiencing de novo *status epilepticus* (*SE*) develop epilepsy after a seizure-free interval (Annegers et al., 1987; French et al., 1993; Tsai et al., 2009). A short seizure-free interval (latent period) also precedes the appearance of spontaneous seizures in chemoconvulsant (pilocarpine or kainate) models of temporal lobe epilepsy (TLE) (Sharma et al., 2007; Loscher and Brandt, 2010; O'Dell et al., 2012). In addition to recurrent seizures, rodents enduring chemoconvulsant-induced epilepsy demonstrate brain lesions (neuronal loss, astrogliosis, mossy fiber sprouting and hippocampal sclerosis) highly isomorphic to the human condition (Sharma et al., 2007; Curia et al., 2008; Pitkanen and Lukasiuk, 2009).

The *cal*cium-dependent proteases with pa*pain*-like activity (*calpains*) belong to a family of non-lysosomal cysteine proteases activated by calcium (Campbell and Davies, 2012; Ono and Sorimachi, 2012). Two major calpain isoforms are ubiquitously expressed in the brain: calpain-1 and calpain-2 (Liu et al., 2008; Saatman et al., 2010; Baudry and Bi, 2016). Following a brain injury, a sustained increase in intracellular calcium results in calpain activation (Liu et al., 2008; Saatman et al., 2010). Calpain activation can be readily detected following brain injury induced by chemoconvulsants (pilocarpine or kainate) typically used to triggered *SE* (Bi et al., 1996; Araujo et al., 2008; Wang et al., 2008). As such, there is evidence that sustained calpain activation contributes to both chronic and acute neurodegeneration in a wide range of pathologic conditions including *SE* (Vanderklish and Bahr, 2000; Bevers and Neumar, 2008; Vosler et al., 2008; Saatman et al., 2010). Pharmacological inhibition of calpain after chemoconvulsant-induced *SE* provides neuroprotection (Araujo et al., 2008; Wang et al., 2008), suggesting that calpains have an active contribution to the acute neurodegenerative process. Moreover, analysis of tissue obtained from patients with epilepsy showed increased calpain expression (Feng et al., 2011; Das et al., 2012). Despite this knowledge, the contribution of calpain overactivation to the epileptogenic process remains poorly studied.

Due to the existence of a close correlation between calpain activation and a broad range of proteins that can be cleaved by this protease, calpain inhibition is an attractive therapeutic target (Vosler et al., 2008; Saatman et al., 2010). As an additional feature, calpain inhibition is expected to have few side effects since the basal levels of calpain activation prevailing in the normal brain is relatively low (Saatman et al., 2010). Here, we report the effects of a calpain inhibitor on several aspects linked to epileptogenesis including seizure burden and cellular pathologies associated to seizure occurrence. Our findings suggest that

Abbreviations: TLE, temporal lobe epilepsy; SE, status epilepticus; CA1, Cornus Ammonis 1; DG, dentate gyrus; FJB, Fluoro-Jade B; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adaptor molecule 1; EEG, electroencephalogram; SBDPs, α-spectrin breakdown products; ZNT3, Zinc Transporter 3; IL-1, interleukin 1; PBS, phosphate buffered saline.

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Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2017.02.003
0969-9961/

pharmacological inhibition of calpain represents a novel therapeutic approach to reduce seizure burden.

2. Methods

2.1. Pilocarpine-induced status epilepticus

Male Sprague Dawley rats (Charles River, Wilmington, MA) were housed in a controlled environment with food and water ad libitum. Animal experiments were performed in accordance with Institutional Animal Care and Use Committee regulations and protocols approved by the University of Colorado Anschutz Medical Campus. *Status epilepticus* was induced at 8–9 weeks of age according to a previously reported protocol (Brooks-Kayal et al., 1998; Shumate et al., 1998). To reduce the peripheral effects of pilocarpine, an intraperitoneal (i.p.) injection of scopolamine methyl nitrate (1 mg/kg, Sigma, St. Louis, MO) was applied 30 min before administration of pilocarpine hydrochloride (385 mg/kg i.p, Sigma, St. Louis, MO). If rats did not exhibit convulsive seizures 1 h after pilocarpine injection, a second or third dose of pilocarpine (192.5 mg/kg) was administered in order to achieve seizure equivalence between animals. To slow down seizure progression and decrease mortality, diazepam (6 mg/kg, i.p.; Hospira, Lake Forest, IL) was administered 1 h after *SE* onset and additional doses (3 mg/kg, i.p.) were administered every 2 h if seizures persisted. Control rats were handled similarly but received a subconvulsive dose of pilocarpine (38.5 mg/kg, i.p.) and 1/10 of the dose of diazepam (0.6 mg/kg, i.p.). As criteria for inclusion, all rats used had confirmed stage 5 behavioral seizures.

MDL-28170 is cell permeable peptide that inhibits both calpain-1 and calpain-2 (Markgraf et al., 1998; Thompson et al., 2010). To evaluate if MDL-28170 (50 mg/Kg, i.p., Bachem, Torrance, CA) prevented cellular alterations linked to epileptogenesis, two treatment paradigms were used: a *low*-dose treatment consisting of *two acute* injections applied at 1 and 5 h after *SE* onset with a final dose the following morning; and, a *high*-dose treatment that consisted of *four acute* doses at 1, 3, 5 and 9 h after *SE* onset with a final dose the following morning. A third group, *SE* plus vehicle (Veh), was a composed of rats that received vehicle injections (DMSO) with the same frequency of the *low*- and *high*-dose. The vehicle group was predicted to undergo all pathophysiological events promoting SRS and was used as reference to evaluate possible disease-modifying effects of MDL-28170. To determine if MDL-28170 altered seizure burden, in addition to the acute doses, daily doses of the drug were administered at 1, 2 and 3 days post-*SE*. Animals were randomly assigned to each of these groups. The concentration and administration frequency were chosen based in studies describing MDL-28170 delivery to the brain (Li et al., 1998; Markgraf et al., 1998; Araujo et al., 2008). MDL-28170 has a plasma half-life of 1 to 2 h and is capable of penetrating the blood-brain barrier and cell membranes. In naïve rats, a single intravenous bolus administration of MDL-28170 (30 mg/kg) resulted in protease inhibition within the brain in 30 min and declined over a period of 4 h with a half-life of approximately 2 h and no apparent toxicity (Markgraf et al., 1998).

2.2. Tissue lysates

Following the rapid isolation of hippocampus from the rest of the brain, hippocampal slices (600 μm) were prepared using a McIlwan tissue chopper. Each individual slice was then microdissected to isolate the Cornus Ammonis 1 (CA1); the region of hippocampus where more prominent levels of calpain activation and neuronal death can be detected after SE (Araujo et al., 2008). For microdissection, the CA3 region was separated from the CA1 and DG; then, the CA1 region was separated from DG through the hippocampal sulcus (Silva et al., 2001). All CA1 pieces collected from the same rat were pooled together, frozen on dry ice and stored at −80 °C. Whole tissue lysates were prepared by brief tissue sonication in RIPA buffer containing a mixture of protease and phosphatase inhibitors. To remove cell debris, lysates were cleared by centrifugation at 17,000 ×*g* for 20 min. Protein concentration was determined using the Bio-Rad RC/DC reagent kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Western blot

Protein samples were separated in SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were blocked with 5% non-fat dry milk in Tris-buffered saline (pH 7.4) plus 0.05% Tween 20. Blots were then incubated overnight at 4 °C with primary antibodies diluted in 1% non-fat dry milk. A polyclonal rabbit antibody, AB38, which recognizes calpain-cleaved spectrin fragments of ~150 kDa was produced and characterized previously (Roberts-Lewis et al., 1994) and was a generous gift from Dr. David R. Lynch (University of Pennsylvania, PA). A rabbit monoclonal antibody that detects full-length and cleaved α-spectrin was obtained from Epitomics (Cat. No. 2507-1, Burlingame, CA). To estimate potential variability in protein content and loading, blots were re-probed with an anti-actin antibody (Sigma, St. Louis, MO). Following incubation with the primary antibody, blots were washed and then incubated at room temperature for 1 h with the appropriate secondary antibodies. Anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase were from GE Health Care (Piscataway, NJ) or Jackson Immunoresearch laboratories (West Grove, PA), respectively. Immunoreactive bands were visualized using Super Signal West Dura chemiluminescent substrate (Pierce, Rockford, IL, USA) and film. After scanning the films, immunoreactive bands of the appropriate size were quantified using ImageJ (NIH, Bethesda, MD, USA). Immunoreactivity for the bands of interest was normalized to actin immunoreactivity and compared to control values.

2.4. Histological analysis

Rats were deeply anesthetized and transcardially perfused, first with ice-cold PBS and then with ice-cold 4% PFA in 0.1 M phosphate buffer pH 7.4. Brains were removed from the skull and post-fixed overnight in 4% PFA solution. Fixed brains were cryoprotected in 30% sucrose solution and embedded in OCT compound (Tissue-Tek, Sakura Finetek, Torrance, CA). Whole brains were serially sectioned to obtain 15 μm coronal sections. For staining, three mounted sections were selected from a 1-in-15 series starting at approximately the same level of hippocampus (2.8 mm posterior to Bregma). For consistency and to minimize variability in the staining procedure, control and *SE* brains were processed and stained in parallel. Following staining, cell counts were conducted blinded to the administered treatment. The number of cells counted in three sections was averaged and the average number of cells is the reported value for each animal. Controls where the primary antibodies were omitted were run to confirm that the staining was dependent on the primary antibody. Images were obtained using a Nikon Eclipse TE2000-U fluorescence microscope.

To detect degenerating neurons, sections were stained with a simple, reliable, and sensitive technique using the anionic fluorochrome Fluoro-Jade B (FJB, Cat. No. 1FJB, Histo-Chem Inc., Jefferson, AR). Mounted sections were dried at room temperature and rehydrated with 100% ethanol for 10 min, 70% ethanol for 2 min and finally rinsed in distilled water for 2 min. Sections were immersed in 0.06% potassium permanganate for 10 min, rinsed with distilled water for 2 min and finally immersed in 0.0004% FJB staining solution for 10 min. Following staining, sections were rinsed with distilled water, dried and immersed in CitriSolv (Fisher, Pittsburgh, PA). After staining, tissue sections were mounted on slides using Permount (Fisher Scientific, Pittsburg, PA, USA).

To estimate astrogliosis, brain sections were stained with an antibody to detect GFAP (a marker for astrocytes). Tissue sections were blocked with PBS containing 10% normal goat serum, 0.1% BSA, 0.01% glycine and 0.3% Triton X-100. Sections were then incubated overnight with a mouse monoclonal anti-GFAP (Cat. No. G3893, Sigma, St. Louis,