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Contribution of protease-activated receptor 1 in status epilepticus-induced epileptogenesis

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ABSTRACT

Clinical observations and studies on different animal models of acquired epilepsy consistently demonstrate that blood–brain barrier (BBB) leakage can be an important risk factor for developing recurrent seizures. However, the involved signaling pathways remain largely unclear. Given the important role of thrombin and its major receptor in the brain, protease-activated receptor 1 (PAR1), in the pathophysiology of neurological injury, we hypothesized that PAR1 may contribute to status epilepticus (SE)-induced epileptogenesis and that its inhibition shortly after SE will have neuroprotective and antiepileptogenic effects. Adult rats subjected to lithium–pilocarpine SE were administrated with SCH79797 (a PAR1 selective antagonist) after SE termination. Thrombin and PAR1 levels and neuronal cell survival were evaluated 48 h following SE. The effect of PAR1 inhibition on animal survival, interictal spikes (IIS) and electrographic seizures during the first two weeks after SE and behavioral seizures during the chronic period was evaluated. SE resulted in a high mortality rate and incidence of IIS and seizures in the surviving animals. There was a marked increase in thrombin, decrease in PAR1 immunoreactivity and hippocampal cell loss in the SE-treated rats. Inhibition of PAR1 following SE resulted in a decrease in mortality and morbidity, increase in neuronal cell survival in the hippocampus and suppression of IIS, electrographic and behavioral seizures following SE.

These data suggest that the PAR1 signaling pathway contributes to epileptogenesis following SE. Because breakdown of the BBB occurs frequently in brain injuries, PAR1 inhibition may have beneficial effects in a variety of acquired injuries leading to epilepsy.

Introduction

Impairment of blood–brain barrier (BBB) integrity is commonly observed in conjunction with traumatic brain injury, stroke, tumors and infections; conditions which can lead to seizures and the development of epilepsy (Albayrak et al., 1997; Chodobski et al., 2011; Latour et al., 2004; On et al., 2013; Stolp and Dziegielewska, 2009; Vezzani and Friedman, 2011). Short- and long-lasting increases of BBB permeability during seizures and status epilepticus (SE) have been demonstrated in different animal models of chronic epilepsy (Friedman, 2011) and in patients with epilepsy (Mihály and Bozóky, 1984; Oby and Janigro, 2006). Moreover, BBB opening or intracerebral injection of blood components may directly evoke seizures and lead to the generation of an epileptic focus (Lee et al., 1997; van Vliet et al., 2007). However, the specific pathways activated as a consequence of BBB disruption participating in the development of chronic epilepsy remain unclear.

There is now substantial evidence that besides its key role in coagulation, serum-derived protein thrombin participates in many mechanisms important for normal brain functioning and during pathological conditions involving abnormal neuronal synchronization, neurodegeneration and inflammation (Luo et al., 2007; Turgeon et al., 2000). Among the possible blood components involved in seizure generation during BBB opening (albumin, iron, thrombin) only thrombin has been shown to produce early-onset seizures (Lee et al., 1997; Tomkins et al., 2007; Willmore et al., 1978). Depending on the concentration, the effect of thrombin in the CNS might be protective or deleterious (Xi et al., 2003). At low concentrations, thrombin rescues neurons from death after brain insults (Jiang et al., 2002). In contrast, the alteration of BBB integrity during pathological conditions may lead to dramatic increases of thrombin levels in the CNS (Woitzik et al., 2011). Thrombin, through the activation of specific protease-activated receptors (PAR) expressed by neurons and glial cells, is implicated in the exacerbation of brain damage, seizures and induction of

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inflammation and neurogenesis, all processes that frequently occur during epileptogenesis (Rohatgi et al., 2004a, 2004b). Finally, deficiency of PAR1, a major thrombin receptor in the brain, protects against neuronal damage and neurologic deficits in different models of experimental brain insults thus indicating a significant role of PAR1 signaling in the development of neurodegenerative disorders (Chen et al., 2012; Junge et al., 2003; Manaenko et al., 2013; Olson et al., 2004; Wang et al., 2012).

Here we tested the hypothesis that PAR1 contributes to SE-induced neuronal damage and epileptogenesis. Using the lithium–pilocarpine model of SE, which was shown to be associated with BBB dysfunction (Ndode-Ekane et al., 2010), we have found a marked increase in the thrombin level and decrease in PAR1 immunoreactivity at 48 h after SE. Repetitive injection of the PAR1 antagonist after SE results in a decrease in animal mortality rate, improvement of functional recovery, decrease of SE-induced cell loss and suppression of epileptiform activity and seizures following SE. Our data indicates that PAR1-mediated signaling is involved in epileptogenesis induced by SE and that PAR1 could be a potential novel molecular target for antiepileptic drug therapy.

Material and methods

Animals

All experimental procedures were performed in accordance with the guidelines set by the National Institute of Health for the humane treatment of laboratory animals and approved by the Animal Care Committees of Bogomoletz Institute of Physiology and the University of Vermont College of Medicine. Eighty adult (P50–70) male Wistar rats were used throughout all aspects of histological (n = 12), behavioral (n = 40) and electrophysiological (n = 28) studies.

Lithium–pilocarpine status epilepticus model

In order to decrease animal mortality, SE initiation was performed by repeated administration of low doses of pilocarpine as described previously (Glien et al., 2001). Rats were injected intraperitoneally (ip) with lithium chloride (127 mg/kg, 1 ml/kg) 19–20 h before administration of pilocarpine. Pilocarpine was injected (10 mg/kg, ip) at 30 min intervals until SE was induced. The total dose of pilocarpine administration ranged from 10–50 mg/kg. SE was considered to start when the rat developed stage V seizures according to Racine's scale (Racine, 1972). Only 2 of 76 rats did not develop stage V seizures. These animals were excluded from further analysis. The SE onset time ranged from 26–150 min (average onset time was 78.8 ± 4.5 min). Seizures were terminated with sevoflurane at 90–120 min after SE onset for histological and behavioral studies (SE was terminated before 120 min if the rat developed wild running and severe myoclonic jerks with falling, typically a sign of impending death if the SE continues). For the electrophysiological studies of animals which underwent intrahippocampal electrode implantation, seizures were terminated at 1 h after SE onset (Salami et al., 2014). This shorter duration of SE for the electrophysiological studies was based on previous studies (Chauvière et al., 2012; Salami et al., 2014) showing a duration of SE of 60 min or less is sufficient to study the dynamics of the interictal spikes and spontaneous seizures in pilocarpine-induced epileptogenesis. After SE, rats were injected with 5% dextrose in lactate Ringer's solution three times a day for about 3 days and fed with milk (chocolate flavor) and moistened rodent chow three times a day until they were able to eat independently. The food and water intake (determined from the weight of the uneaten chow and leftover water in each cage) and rats' weight were examined daily by a technician for 11 days after SE.

Drug administration

The PAR1 specific blocker, SCH79797, or appropriate volume of vehicle was ip injected 20–30 min after SE termination and thereafter injections were repeated once a day for 10 consecutive days. SCH79797 was injected at a concentration of 25 μg/kg. This concentration was shown to be optimal for the reduction of myocardial necrosis observed during experimental ischemia and for the decrease of brain water content and amelioration of neurological deficits following surgical brain injury (Manaenko et al., 2013; Strande et al., 2007).

Preparation of sections for light and confocal microscopy

Eight rats with SE (SE + vehicle group: n = 4, and SE + SCH group: n = 4) and age-matched control rats (n = 4) that received all injections, except for pilocarpine which was substituted by saline, were used in the histological studies. Animals with SE received two injections (at 20–30 min after SE termination and 24 h latter) of SCH79797 or vehicle before sacrifice at 48 h following SE. For deep anesthesia rats were given ip 70 mg/kg ketamine (Pfizer, Finland) and 2.5 mg/kg xylazine (Bayer, Germany). Anesthetized rats were transcardially perfused with 20 ml phosphate-buffered saline (PBS; 0.1 M, pH 7.4) with heparin (15 units/ml) followed by 50 ml of 4% paraformaldehyde (PFA, Sigma, USA) solution in PBS. Hippocampi were removed, post-fixed for 24 h at 4 °C in 4% PFA and thoroughly washed in 0.1 M PBS. Transversal sections 30–50 μm thick from each rat were cut with a step 250 μm starting at 1200–1500 μm from the dorsal end of hippocampus using vibratome (Leica Biosystems, USA). The sections were kept in 0.1 M PBS with 0.02% sodium azide 4–8 weeks at 4 °C prior to immunohistochemistry or Nissl staining with thionin.

Histological analysis

The sections were washed in 0.1 M phosphate buffer, cryoprotected in a mixture of 20% DMSO, 2% glycerol in PBS for 60 min and permeabilized by freezing/thawing three times at −20 °C. Sections were then blocked with 0.5% normal goat serum and 1% albumin bovine serum in 0.1 M PBS (blocking solution) for 1 h at room temperature, and incubated overnight at 4 °C in primary antibodies: anti-thrombin HC polyclonal goat antibody (1:100; sc-23335, Santa Cruz Biotechnology, USA); anti-thrombin R (PAR) polyclonal rabbit antibody (1:100; sc-5605, Santa Cruz Biotechnology, USA); anti-NeuN monoclonal antibody, clone A60 (1:1000; MAB377, Millipore, USA); anti-Glial Fibrillary Acidic Protein (GFAP) monoclonal antibody, clone GA5 (1:1000; MAB360, Millipore, USA) in blocking solution. Sections were thoroughly rinsed in 0.1 M PBS and incubated for 90 min at room temperature in 0.1 M PBS containing 1% albumin bovine serum and appropriate secondary antibodies (all from Invitrogen, USA): Alexa 568-conjugated donkey anti-goat antibody (1:800; A-11057); Alexa 647-conjugated donkey anti-rabbit antibody (1:800; A-31573); Alexa 488-conjugated donkey anti-mouse antibody (1:800; A-21202). Cell nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole, 100 nM). Sections were finally rinsed three times in 0.1 M PBS and then mounted onto glass microscope slides in Dako fluorescent mounting medium (Dako, Denmark) for confocal microscopy. The immunofluorescent images were captured using a confocal microscope FluoView™ FV1000 (Olympus America Inc., Center Valley, PA) and histological measures were performed using ImageJ software (National Institutes of Health, USA). Immunohistochemical analysis was performed in hippocampal CA1 pyramidal region and adjacent zones (no more than 200 μm from the pyramidal CA1 area layer.) The area of analysis is shown on Fig. 1A. Images were taken at a resolution of 1024 × 1024 and have similar brightness and contrast (oil objective — 60×). Data are presented as immunopositive area of fluorescence (*i.e.*, Thrombin+, PAR+) per mm^2 (a.u.).