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PI3Kγ deficiency enhances seizures severity and associated outcomes in a mouse model of convulsions induced by intrahippocampal injection of pilocarpine

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ABSTRACT

Phosphatidylinositol 3-kinase (PI3K) is an enzyme involved in different pathophysiological processes, including neurological disorders. However, its role in seizures and postictal outcomes is still not fully understood. We investigated the role of PI3Kγ on seizures, production of neurotrophic and inflammatory mediators, expression of a marker for microglia, neuronal death and hippocampal neurogenesis in mice (WT and PI3Kγ$^{-/-}$) subjected to intrahippocampal microinjection of pilocarpine. PI3Kγ$^{-/-}$ mice presented a more severe *status epilepticus* (SE) than WT mice. In hippocampal synaptosomes, genetic or pharmacological blockade of PI3Kγ enhanced the release of glutamate and the cytosolic calcium concentration induced by KCl. There was an enhanced neuronal death and a decrease in the doublecortin positive cells in the dentate gyrus of PI3Kγ$^{-/-}$ animals after the induction of SE. Levels of BDNF were significantly increased in the hippocampus of WT and PI3Kγ$^{-/-}$ mice, although in the prefrontal cortex, only PI3Kγ$^{-/-}$ animals showed significant increase in the levels of this neurotrophic factor. Pilocarpine increased hippocampal microglial immunolabeling in both groups, albeit in the prelimbic, medial and motor regions of the prefrontal cortex this increase was observed only in PI3Kγ$^{-/-}$ mice. Regarding the levels of inflammatory mediators, pilocarpine injection increased interleukin (IL) 6 in the hippocampus of WT and PI3Kγ$^{-/-}$ animals and in the prefrontal cortex of PI3Kγ$^{-/-}$ animals 24 h after the stimulus. Levels of TNFα were enhanced in the hippocampus and prefrontal cortex of only PI3Kγ$^{-/-}$ mice at this time point. On the other hand, PI3Kγ deletion impaired the increase in IL-10 in the hippocampus induced by pilocarpine. In conclusion, the lack of PI3Kγ revealed a deleterious effect in an animal model of convulsions induced by pilocarpine, suggesting that this enzyme may play a protective role in seizures and pathological outcomes associated with this condition.

Introduction

Status epilepticus (SE) is characterized by prolonged seizures, which represents a life-threatening condition. This severe condition is associated not only with short-term consequences, but different studies have also demonstrated that a significant percentage of the patients developed epilepsy after SE (Aicardi and Chevrie, 1970; Lowenstein, 1999; Verity et al., 1993).

A plethora of events may occur after the occurrence of SE, such as neuroinflammation, neuronal cell death, altered production of neurotrophic factors and neurogenesis (Scharfman and Gray, 2007; Simonato

Abbreviations: BDNF, brain-derived neurotrophic factor; DCX, doublecortin; DG, dentate gyrus; EAAT3, excitatory amino acid transporter 3; FJC, Fluoro-Jade® C; GLT1, glutamate transporters type 1; IFN-γ, interferon γ; IL, interleukin; NGF, nerve growth factor; PI3K, phosphatidylinositol 3-kinase; SE, *status epilepticus*; TNFα, tumor necrosis factor α.

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et al., 2006; Vezzani, 2005). In epileptic patients, these pathological outcomes are associated with epileptogenesis and long-term behavioral alterations (Pitkanen and Sutula, 2002).

The pathophysiology of the seizures, as well as the postictal complications, is not fully understood. In addition, the epileptogenic process is not controlled by antiepileptic drugs which are refractory in thirty percent of the patients (Sander, 2003). Therefore, it would be important to investigate molecular pathways that might act as regulators of these complex events raising the possibility of identifying new and more effective pharmacological targets.

Phosphatidylinositol 3-kinase (PI3K) is a key regulator of a plethora of actions in physiological and pathological conditions. The PI3K family is divided into three classes according to their structure and lipid substrate specificity, namely class I, class II and class III, from which the class I PI3K is the most studied. Class IA consists of heterodimers of a catalytic p110 subunit (p110α, p110β or p110δ) bound to a particular regulatory subunit (p85α, p55α, p50α, p85β or p55γ) (Marone et al., 2008). Different stimuli can activate PI3K, which, in turn, phosphorylates phosphatidylinositol 4,5-bisphosphate to generate phosphatidylinositol-3,4,5-trisphosphate. This latter molecule binds to the pleckstrin homology domain of one of the Akt (also known as protein kinase B) isoforms and facilitates the phosphorylation of Akt1, Akt2 or Akt3 at $Thr^{308/309/305}$ and $Ser^{273/474/472}$ residues, respectively, by the phosphatidylinositol-dependent kinases 1 and 2 (Laine et al., 2002).

Previous studies have shown an increased Akt phosphorylation in the hippocampus of patients with temporal lobe epilepsy (Shinoda et al., 2004) and in the cortex of rats injected with kainic acid into the basolateral nucleus of the amygdala (Henshall et al., 2002). However, the role of PI3K in seizures, as well as in postictal events, is still not fully understood. It is suggested that the mechanisms of action of anticonvulsive drugs might be dependent on PI3K activation. Lee et al. (2005) demonstrated that carbamazepine increased the activation of excitatory amino acid transporter 3 (EAAT3) in transfected oocytes and in C6 glioma cells that constitutively express this transporter. LY294002, a non-selective PI3K inhibitor, inhibits the activation of the transporter and the glutamate uptake in this model (Lee et al., 2005). Moreover, anticonvulsant drugs activate or modulate Akt expression. For instance, valproic acid activates Akt (Creson et al., 2009), and phenytoin treatment increases Akt expression in rats (Mariotti et al., 2010). However, the relevance of these findings for the anticonvulsant effect of these drugs has not been established yet.

PI3K are also involved in the production of inflammatory mediators by central nervous system cells (de Oliveira et al., 2008, 2012; Zhao et al., 2014). Therefore, considering the key role of inflammation in seizures and postictal events, it is possible to argue that PI3K could be involved in the development of these pathological conditions.

In contrast to the other isoforms, which are ubiquitously expressed, PI3Kγ expression is more restricted to the brain, immune, hematopoietic and cardiovascular systems (Ruckle et al., 2006). This might indicate that this enzyme presents a key role in brain physiology and pathology. For example, PI3Kγ has been implicated in the long-term depression and behavioral flexibility (Kim et al., 2011). PI3Kγ deficiency has been shown to reduce the blood–brain barrier leakage and tissue damage, and to improve neurological outcome in an animal model of stroke (Jin et al., 2011).

Thus, in the present study, we aimed to evaluate the role of PI3Kγ in seizures and associated outcomes induced by a convulsive stimulus.

Material and methods

Animals and surgical procedures

All experiments were performed in accordance with the Brazilian Institutional Ethics Committee (CEUA), protocol number 068/11. Every effort was made to avoid unnecessary pain or stress to the animals. Adult male C57Bl/6 and $PI3K\gamma^{-/-}$ mice (25–30 g), aged ten to twelve weeks, were obtained from Animal Care Facilities of the Institute of Biological Sciences, Federal University of Minas Gerais (ICB-UFMG), Belo Horizonte, Brazil. Animals were anesthetized with ketamine:xylazine (80 mg/kg:8 mg/kg, i.p. Syntec®, Cotia, Brazil), placed in a stereotaxic apparatus (Insight®, Ribeirão Preto, SP, Brazil), and implanted with guide cannula in both hippocampi. Stereotaxic coordinates for injection were: AP: −1.9 mm, ML: ±1.5 mm, DV: −2.3 mm, bregma as reference, according to Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates, Second Edition, 2001 (Paxinos and Franklin, 2001). Animals were allowed to recover for 5 days before the intrahippocampal administrations of pilocarpine through the cannulae.

Microinjection procedures

We used a 10 μL syringe (Hamilton, Sigma, Reno, NV, USA) connected to a pump (Insight®, Ribeirão Preto, SP, Brazil) adjusted to a flow of 0.2 μL/minute (min). After the end of microinjection, the syringe remained connected to the cannula for approximately 2 min to avoid reflux of the drug. All animals were gently restrained during the injection procedures. The experimental group received pilocarpine (Sigma-Aldrich, St. Louis, MO, USA) through the cannulae at different doses [5, 10, 20 and 40 μg/0.2 μL/site (corresponding to concentrations of 25, 50, 100 and 200 μg/μL, respectively)] while the control group received saline. Ninety minutes after *status epilepticus* (SE) onset, animals received a single i.p. injection of diazepam (10 mg/kg; Hipolabor, Belo Horizonte, Brazil). Working dose was defined in this dose response curve experiment, and then all following experiments were performed using the dose of 20 μg/0.2 μL/site of pilocarpine.

Evaluation of Akt protein activation by western blotting

Hippocampal tissues of the animals were carefully dissected 30 min or 24 h after the SE, homogenized in a lysis buffer (1% Triton X-100; 100 mM Tris/HCl, pH 8.0; 10% glycerol; 5 mM EDTA; 200 mM NaCl; 1 mM DTT; 1 mM PMSF; 25 mM NaF; 2.5 μg/ml leupeptin; 5 μg/ml aprotinin; and 1 mM sodium orthovanadate) and stored in −80 °C until the beginning of the analysis. Protein concentration was determined by using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). 60 μg of protein samples were separated on 10% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. After blocking in 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature, membranes were incubated overnight at 4 °C with primary antibodies against anti-$pAkt^{Ser473}$ (1:1000; Cell Signaling Technology, Danvers, MA, USA) and anti-β-actin (1:5000; Sigma-Aldrich, St. Louis, MO, USA). Following three washes with TBST, membranes were incubated with the appropriate peroxidase-conjugated secondary antibodies (1:3,000). Finally, membranes were incubated with enhanced chemiluminescence ECL-Plus (GE Healthcare, Piscataway, NJ, USA). The optical densities of detected bands were quantified using the ImageJ software. The results were normalized to the quantity of β-actin in each sample lane.

Seizure scoring

Immediately after saline or pilocarpine injection, we recorded the seizures of all animals with a camera (Sony DCR-SR68, Brazil) up to 90 min after SE onset. Behavioral assessment was made from the videotape analysis by an investigator blind to the genotype and treatments. We used a set of acrylic cages that allowed the simultaneous observation of ten animals at the same time. The seizures computed were of those who achieved a score of at least 3 according to Racine's scale (Racine, 1972). This scale categorizes five stages of severity, and it is based on the behavioral repertoire of the animals during a seizure, including “mouth and facial movements” (intensity score 1); “head nodding” (score 2); “forelimb clonus” (score 3); seizures characterized by rearing (score 4); and seizures characterized by rearing and falling