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NEURONAL ZINC-α2-GLYCOPROTEIN IS DECREASED IN TEMPORAL LOBE EPILEPSY IN PATIENTS AND RATS

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Abstract—Zinc-α2-glycoprotein (ZAG) is a 42-kDa protein encoded by the *AZGP1* gene that is known as a lipid mobilizing factor and is highly homologous to major histocompatibility complex class I family molecules. Recently, transcriptomic research has shown that *AZGP1* expression is reduced in the brain tissue of epilepsy patients. However, the cellular distribution and biological role of ZAG in the brain and epilepsy are unclear. Patients with refractory temporal lobe epilepsy (TLE) and brain trauma were included in this study, and pentylenetetrazole (PTZ)-kindled rats were also used. The existence and level of ZAG in the brain were identified using immunohistochemistry, double-labeled immunofluorescence and western blot, and the expression level of *AZGP1* mRNA was determined with quantitative real-time polymerase chain reaction (qrt-PCR). To explore the potential biological role of ZAG in the brain, co-immunoprecipitation (Co-IP) of phosphorylated ERK (p-ERK), TGF-β1 and ZAG was also performed. ZAG was found in the cytoplasm of neurons in brain tissue from both patients and rats. The levels of *AZGP1* mRNA and ZAG were lower in refractory TLE patients and PTZ-kindled rats than in controls. In addition, the ZAG level decreased as PTZ kindling continued. Co-IP identified direct binding between p-ERK, TGF-β1 and ZAG. ZAG was found to be synthesized in neurons, and both the *AZGP1* mRNA and ZAG protein levels were decreased in epilepsy patients and rat models. The reduction in ZAG may participate in the pathogenesis and pathophysiology of epilepsy by interacting with p-ERK and TGF-β1, promoting inflammation, regulating the metabolism of ketone bodies, or affecting other epilepsy-related molecules.

Key words: zinc-α2-glycoprotein, *AZGP1*, neuron, temporal lobe epilepsy.

INTRODUCTION

Zinc-α2-glycoprotein (ZAG) is a 42-kDa, soluble, secretory protein expressed mainly in epithelial cells and encoded by the *AZGP1* gene located on chromosome 7q22.1 (Burgi and Schmid, 1961; Tada et al., 1991). The structure and amino acid sequence of ZAG are highly homologous to those of the major histocompatibility complex class I (MHC-I) family, which has an important function in immunity (Hassan et al., 2008). ZAG acts as a lipid mobilizing factor in adipocytes and plays a key role in lipid mobilization (Bao et al., 2005). AZGP1 mRNA was first detected by northern blot in the rat liver, but, at the time, it was not detected in the rat brain (Fu et al., 1994). ZAG expression is significantly higher in the cerebrospinal fluid (CSF) of patients with mild Alzheimer's disease (AD) and has been speculated to be a potential biomarker for the prediction and prognosis evaluation of mild and very mild AD, but the level of ZAG in CSF is not significantly correlated with its level in plasma (Hu et al., 2007). However, the origin of ZAG in CSF is still unclear. Maślińska and colleagues identified ZAG in astrocytes, Purkinje cells and around capillaries in the brain tissue of patients with Krabbe's disease but not in that of controls. In addition, they speculated that ZAG may enter the brain through the blood–brain barrier (BBB) (Maślińska et al., 2013). In contrast, a transcriptomic study found that the level of *AZGP1* messenger ribonucleic acid (mRNA) was lower in the brain tissue of epilepsy patients than in controls, suggesting that ZAG may be synthesized in the brain (Liu et al., 2016). Therefore, the origin and distribution of ZAG in the brain is conflicting.

ZAG has been proven to be involved in many epilepsy-related pathways and can regulate many epilepsy-related molecules, although not in studies on epilepsy. For example, in studies on tumors, ZAG was reported to block transforming growth factor-β (TGF-β)-mediated extracellular regulated protein kinase (ERK) phosphorylation (Kong et al., 2010) and suppress the activity of the mammalian target of rapamycin (mTOR)

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Abbreviations: AD, Alzheimer's disease; AED, anti-epileptic drug; BSA, bovine serum albumin; cDNA, complementary deoxyribonucleic acid; Co-IP, co-immunoprecipitation; CSF, cerebrospinal fluid; DAB, 3,3-diaminobenzidine; DAPI, 4,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; ILAE, international league against epilepsy; MHC-I, major histocompatibility complex class I complex; mTOR, mammalian target of rapamycin; OD, optic density; PBS, phosphate-buffered saline; p-ERK, phosphorylated extracellular regulated protein kinase; PP2A, protein phosphatase 2A; PPARγ, peroxisome proliferator-activated receptor-γ; PTZ, pentylenetetrazole; PVDF, polyvinylidene fluoride; qrt-PCR, real-time quantitative polymerase chain reaction; SD, standard deprivation; SDS, Sodium dodecylsulfate; TBST, tris-buffered saline with tween-20; TGF-β, transforming growth factor-β; TLE, temporal lobe epilepsy; TNFα, tumor necrosis factor α; ZAG, zinc-α2-glycoprotein.

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pathway by decreasing the levels of phosphorylated mTOR (Chang et al., 2014). Meanwhile, a transcriptome profiling study revealed TGF-β signaling involvement in epileptogenesis (Cacheaux et al., 2009). In addition, another study has demonstrated that up-regulation of TGF-β is present in neurons of amygdala-kindled rats (Plata-Salamán et al., 2000). The levels of phosphorylated ERK (p-ERK) and ERK in patients with refractory epilepsy are significantly higher than in controls (Xi et al., 2007). Several studies have also revealed that the mTOR signaling pathway is involved in both genetic and acquired epilepsy, and inhibition of the mTOR pathway may be a new therapeutic strategy for epilepsy and epileptogenesis (Citraro et al., 2016). Interestingly, according to studies on tumors, decreased expression of ZAG, which has also been found in epilepsy (Liu et al., 2016), leads to up-regulation of TGF-β, p-ERK and mTOR. Thus, ZAG may be a modulator involved in these signaling pathways in epilepsy.

ZAG may also influence epilepsy by promoting fatty acid β-oxidation and ketone body production, which have been identified in hepatocytes (Xiao et al., 2017; Rui, 2014), considering ketone bodies have been known to have anti-epileptic effects, especially in patients with refractory epilepsy (Pasca et al., 2016). Moreover, neurons are able to metabolize fatty acids and produce ketone bodies (Takahashi and Mashima, 2014). Thus, ZAG may also have an effect on epilepsy by regulating fatty acid metabolism in the brain. However, the expression and distribution of ZAG and its biological role have not been clearly illustrated in epilepsy.

In this study, we examined the *AZGP1* mRNA and ZAG protein expression level in patients with refractory temporal lobe epilepsy (TLE) and pentylenetetrazole (PTZ)-kindled rats compared to that in controls using quantitative real-time polymerase chain reaction (qrt-PCR), immunohistochemistry, and western blot. We also identified the cellular distribution of ZAG in the brain of refractory TLE patients and PTZ-kindled rats using immunofluorescence. To further explore the potential mechanism by which ZAG affects epilepsy, we also identified the interaction between p-ERK, TGF-β and ZAG in the brain using co-immunoprecipitation (Co-IP).

EXPERIMENTAL PROCEDURES

Ethical approval

The study was performed in accordance with the Guidelines of Chongqing Medical University established based on the Declaration of Helsinki (2013). This study protocol was approved by the Ethics Committee of The Second Affiliated Hospital of Chongqing Medical University (2017–009) after a review meeting and vote. In addition, all experimental processes were supervised by the ethics committee. Written consents were obtained from each patient and/or patient's legal guardians. The animal study was also approved by the Ethics Committee of The Second Affiliated Hospital of Chongqing Medical University (2017–009) and performed in accordance with the guidelines of Chongqing Medical University and Guide for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Patients

Fourteen refractory TLE patients who underwent surgery at The Second Affiliated Hospital of Chongqing Medical University were included. The refractory TLE patients were diagnosed following the definition of the International League Against Epilepsy (ILAE) (Kwan et al., 2010). Briefly, all patients were resistant to the maximum doses of at least three anti-epileptic drugs (AEDs) and were evaluated by detailed history, neurological examination, neuropsychological test and neuroimaging data. For presurgical evaluation and epileptogenic zone identification, a combined assessment of ictal semiology, brain magnetic resonance imaging, video-electroencephalography, sphenoidal electrode monitoring, intracerebral electroencephalography and intraoperative electrocorticography was used. Table 1 summarizes the clinical features of the intractable TLE patients.

Temporal neocortex samples were obtained from 20 brain trauma patients who underwent surgery as controls. The temporal neocortical tissues resected from the brain trauma patients were histologically normal but inactive due to brain trauma and had to be removed according to the judgement of the surgeons. All control patients had no history of epilepsy, did not experience seizures after the trauma, and did not have a history of any other neurological disease. The clinical features of the control patients are shown in Table 2.

All resected brain tissue was immediately frozen in liquid nitrogen and then stored at −80 °C.

Experimental animals and PTZ kindling

Adult, male, 200–300 g, specific-pathogen-free Sprague–Dawley rats (Experimental Animal Center of Chongqing Medical University) were maintained in the laboratory with the temperature maintained at 27 °C with ad libitum access to food and water. All rats were housed for 1 week before the experiment and were divided randomly into the control (n = 20) and PTZ-treated group (n = 100).

Rats in the PTZ-treated group received a daily injection of PTZ (Sigma–Aldrich, St. Louis, USA) (35 mg/kg, intraperitoneally) for 28 days. Each day after injection, all rats were observed for 30 min in plastic cages to assess the seizure severity according to a modified Racine's scale (Racine et al., 1977). Rats that exhibited stage 4 or 5 seizures on three consecutive days were considered to be fully kindled, and these fully kindled rats who received the PTZ injection for more than 21 days were defined as the PTZ-kindled group. Control rats received an equal amount of saline injected intraperitoneally daily.

Rats were sacrificed at 1 day, 1 week, 2 weeks, 3 weeks or 4 weeks after PTZ injection initiation.

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