Int J Clin Exp Pathol 2019;12(2):599-605
www.ijcep.com /ISSN:1936-2625/IJCEP0064014

Original Article

Role of endoplasmic reticulum stress-associated gene TRIB3 in rats following kainic acid-induced seizures

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Received August 19, 2017; Accepted September 29, 2017; Epub February 1, 2019; Published February 15, 2019

Abstract: TRIB3 (tribblespseudokinase 3) is a pseudokinase that affects several cellular functions, and its expression is increased during endoplasmic reticulum stress (ER stress). How recurrent seizures affect the regulation of TRIB3 in the hippocampus during epilepsy remains unclear. In this study, we investigated the role of TRIB3 in the kainic acid (KA)-induced seizures and related brain injury. In a rat model of kainic acid-induced seizures, neuronal excitotoxic injury and apoptosis, and increases in the expression of TRIB3 and ER stress markers glucose-regulated protein 78 (GRP78) and C/EBP homologous binding protein (CHOP) were observed in the hippocampus by 24 h to 30 d after KA administration. Furthermore, phosphorylation of AKT, which is inhibited by TRIB3, was decreased in the hippocampus after KA-evoked seizure. These results indicate that ER stress, TRIB3 and AKT signaling are involved in the acute and prolonged hippocampal injury following KA induced seizure, suggesting that the ER stress-associated gene TRIB3 plays an important role in neuronal apoptosis after seizure.

Keywords: TRIB3, ER stress, neuronal injury, epilepsy, AKT

Introduction

Epilepsy is one of the most common chronic neurological disorders in children. It is characterized by recurrent seizures and consequent neuronal cell death [1], which may contribute to the impairment of cognitive function [2]. Neuronal cell death has been detected in the course of acute brain injury, as well as the neurodegeneration and long-term adaptive processes associated with epilepsy [3]. However, the precise mechanisms underlying seizure-induced neuronal cell death have not been fully elucidated.

Endoplasmic reticulum stress (ER stress) can induce neuronal cell death in association with many neurological diseases [4-7]. ER stress-induced cell death involves the activation of C/EBP homologous protein (CHOP) [8, 9]. Although the mechanisms by which CHOP targets apoptosis are not completely understood, it has been shown that TRIB3 (tribblespseudokinase 3), a novel ER stress-inducible gene, is induced via an ATF4-CHOP pathway and is involved in cell death [10].

TRIB3 is an intracellular pseudokinase that modulates the activity of several signal transduction cascades [11], and is highly activated by a variety of stresses, including neurotrophic factor deprivation, hypoxia, and ER stress [12-15]. TRIB3 expression is upregulated in the brains of patients with Parkinson's disease [16], and in rats with recurrent febrile seizures [17], ordemyelization [18]. By interfering with AKT, TRIB3 contributes to insulin resistance and inhibition of tumorigenesis [19, 20]. However, the role and the proapoptotic activity of ER stress-associated gene TRIB3 in the process of epilepsy remains unclear. To address the detailed pathogenesis of neuronal cell death occurring after kainic acid-induced seizures [21, 22], we examined the expression level of TRIB3 and explored the effect of TRIB3 due to ER stress on neuronal cell death under acute and prolonged hippocampal injury following KA induced seizures.

Materials and methods

Animals

Animal care and experimental protocols complied with the Animal Management Rules of the Ministry of Health, China and the Animal Care Committee of Peking University First Hospital, Beijing, China. All efforts were made to minimize the number of animals used and their suffering. Sprague-Dawley male rats, 21 days of age, were randomly divided into a control group and seizure group. The rats in the seizure group were administered kainic acid (KA) (10 mg/kg, i.p. Sigma, USA) [21, 22]. Then animal behavior changes were observed over a period of 3 h. Rats exhibited standing and jumping manifestations, such as wet dog shakes similar to limbic seizures, followed by tonic and generalized seizures observed at 30 min-3 h after KA application. Onset of seizures was determined by the presence of stage according to the scale developed by Racine [23]. Rats in the control group were injected i.p. with the same volume of 0.9% saline. Rats were euthanized at different time points from 6 h to 30 d.

Electron microscopy

Rats were anesthetized with 10% choral hydrate and perfused through the heart with 0.9% saline followed by 3% (PFA) and 1% glutaraldehyde in PBS, and then the brains were removed. Hippocampal tissues were removed and cut into approximate 1 mm^3 cubes after immersion in 3% glutaraldehyde in PBS. Hippocampi were washed three times in sucrose and post-fixed in 1% osmium tetroxide for 2 h, and then dehydrated in graded ethanol solutions and embedded overnight in Epon 812 at 37°C. Semi-thin sections (2 μm) were stained with toluidine blue. These sections were used for selection of the CA1 of the hippocampus. Ultrathin sections (60-90 nm) were stained with uranylacetate and lead citrate, and closely examined under a transmission electron microscope (JEM-100CX, JEOL, Japan).

TUNEL assay

Rats were deep anesthetized and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde (PFA) for 30 min, and then brains were fixed in 4% PFA for 20 h, successively dehydrated overnight in 20 and 30% sucrose in phosphate-buffered saline (PBS), frozen in liquid nitrogen and then stored at -70°C for further analysis. Coronal sections of 10 μm thickness were used for evaluation of apoptosis. The number of apoptotic neurons was determined using terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) with an in situ cell death detection kit (Roche Applied Science, Germany) according to the protocol provided by the manufacturer. Nuclear staining with DAPI, and apoptotic cells labeled with TUNEL (green), were examined with a confocal laser scanning microscope (Olympus Fluoview FV1000, Japan). The number of TUNEL-positive cells in hippocampal CA3 region was counted six counting frames that were randomly selected on images at ×400 magnification.

Western blotting

Hippocampal tissues were lysed using ice-cold radio immunoprecipitation assay buffer (RIPA supplemented with a protease inhibitor mixture). Equal amounts of protein were subjected to SDS-PAGE and blotted onto nitrocellulose filters (Pall). Subsequently, filters were first incubated for 1 h in 5% skimmed milk and then overnight at 4°C with the following primary antibodies: rabbit anti-GRP78 (1:1000; Sigma, USA); mouse anti-CHOP (1:250; Santa Cruz Biotechnology); rabbit anti-TRIB3 (1:500; LifeSpanBioSciences, USA), rabbit anti-phosphorylated (p)-AKT (1:500) and rabbit anti-AKT (1:500; both from Cell Signaling), rabbit anti-caspase-3 (1:1000; cell signaling); rabbit anti-Bcl2 (1:1000; Cell Signaling); rabbit anti-Bax (1:1000; Abcam) and mouse anti-β-actin (1:1000; Zhong Shan Golden Bridge Biotechnology, China). On the following day, after washing three times (TBST, 10 min per time), filters were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000; Zhong Shan Golden Bridge Biotechnology, China) for 1 h, and washed with TBST three times (10 min per time), followed by detection using enhanced chemiluminescence. Quantification was performed using ImageJ software. The relative amount of GRP78, CHOP, TRIB3 and p-AKT in the control group was arbitrarily assigned as 1 for comparison.

Quantitation and statistics

Quantitative results were expressed as mean ± SEM. Statistical comparisons were performed using one-way ANOVA, followed by a post hoc

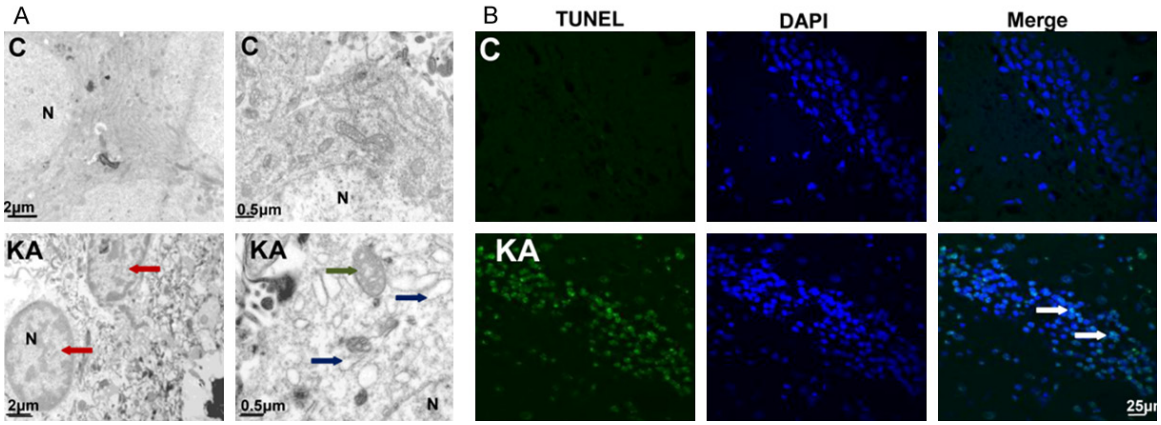

Figure 1. Excitotoxic neuronal injury and apoptosis after KA injection. A. Ultrastructure of hippocampal CA1 neurons stained with uranylacetate and lead citrate under transmission electron microscopy. Control: normal ultra-structure of hippocampal CA1 neurons. KA-treated: disrupted or shrunken nuclei (red arrow), swollen mitochondria (blue arrow), with dissolved and ruptured ridges, the appearance of vacuoles, aciculate rough endoplasmicreticulum (RER) (green arrow). B. TUNEL staining showing apoptotic neurons in the rat hippocampus: number of apoptotic neurons was significantly increased in the KA group compared with that of control group. C, control group. KA, KA group. N, nucleus.

analysis (Bonferroni's test). A $P<0.05$ was considered significant.

Results

Excitotoxic neuronal injury in hippocampi of kainic acid-induced seizure rats

For determination of neuronal damage in the hippocampus after KA-induced seizure, different ultrastructural changes in the hippocampus were characterized using a transmission electron microscope. In the KA-treated group, swollen mitochondria with dissolved and ruptured ridges, the appearance of vacuoles, and swollen rough endoplasmic reticulum (RER) were observed in electron micrographs (**Figure 1A**). TUNEL assay showed neuronal apoptosis in the hippocampus had increased in the KA group compared with the control group (**Figure 1B**). These results suggest that recurrent seizures induce excitotoxic neuronal injury and apoptosis in the hippocampus of rats following KA-induced seizures.

Induction of ER-stress and TRIB3 expression, and reduction of phospho-AKT in the hippocampus of KA-induced seizure rats

It has been previously demonstrated that endoplasmic reticulum stress (ER stress) mediates neuronal apoptosis in many neurological diseases [7]. To further elucidate the potential mechanisms by which seizures induce hippocampal injury following KA administration, we examined the induction of (ER stress) on neuronal apoptosis in KA-induced seizure rats. The ER chaperone GRP78 was elevated in the rat hippocampus, beginning at 24 h after KA administration, and continuously increasing until 30 d (**Figure 2A**). CHOP, a stress-inducible gene, is involved in ER stress-mediated apoptosis [9], and was increased in the hippocampus, peaking at 24 h and then decreasing to control levels at 3 d after KA injection (**Figure 2B**). These results suggest that ER stress is involved in KA-induced neuronal apoptosis and that the apoptosis could be mediated by the activation of CHOP.

In addition, TRIB3, a novel target of CHOP was upregulated obviously in the hippocampus from 24 h and continuously increased up to 30 d after KA administration (**Figure 2C**). To further investigate the mechanisms underlying neuronal damage, we examined the activation levels of AKT, a possible target of TRIB3. Phosphorylated AKT decreased significantly from 24 h and maintained lower levels through 30 d in the KA group as compared with the control group (**Figure 2C**). These results suggest that neuronal apoptosis induced by ER stress is regulated by the induction of CHOP and TRIB3, ER stress associated gene TRIB3 may enhance neuronal apoptosis by interacting with AKT in the hippocampus following kainic acid-induced seizures.

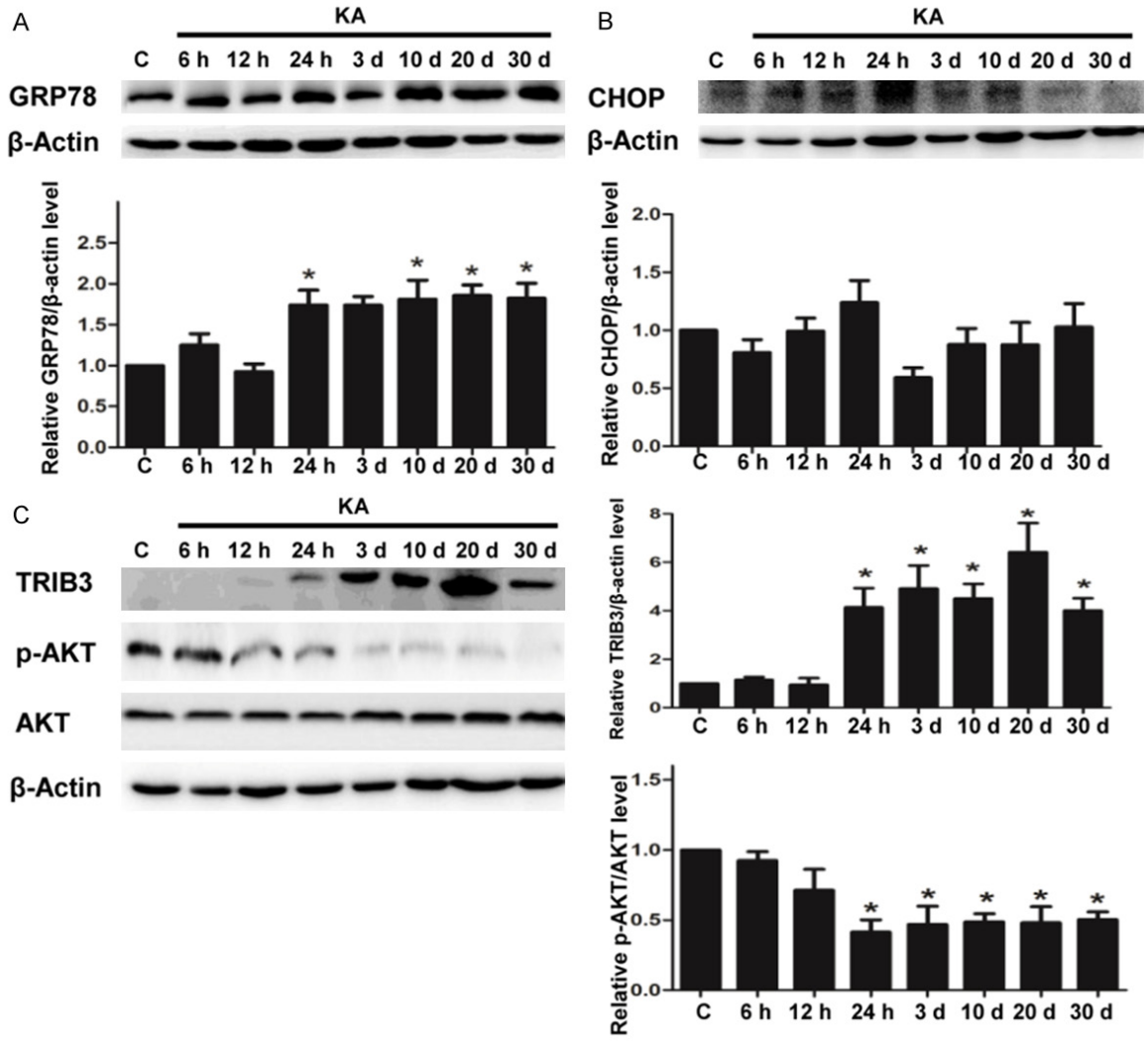

Figure 2. Induction of ER-stress and TRIB3 expression, and reduction of phospho-AKT in hippocampal neurons following kainic acid administration. GRP78 (A) and CHOP (B) in the hippocampus at different times after KA, as detected by western blotting; *P<0.05 for control versus from 24 h to 30 d after KA (n=6). (C) TRIB3 and phospho-AKT in the hippocampus at different times after KA, as detected by Western blotting; *P<0.05 for control versus from 24 h to 30 d after KA (n=6). C: control group. KA: KA group.

Induction of the pro-apoptotic protein Bax and the activation of caspase-3 in the hippocampus following kainic acid-induced seizures

To gain insights into the mechanisms underlying the pro-apoptotic effect of seizure, we detected the expression of Bcl-2 and Bax which have been recognized as downstream components of AKT and mediators contributing to cell apoptosis [24]. In the present study, no changes were observed in the expression of Bcl-2 in the KA group, compared with the control group. Bax protein level clearly increased by 24 h and continued to rise up to 30 d after KA administration (**Figure 3B**). Caspase-3 has been reported involves in neuronal death after seizure [25, 26]. Using western blot analysis, we observed increased activation of caspase-3 at 24 h, followed by a decrease to normal levels in hippocampus of kainic acid-induced seizure rats (**Figure 3A**). These findings suggest that the mechanisms underlying neuronal apoptosis during seizure is associated with the induction of Bax and the activation of caspase-3.

Discussion

In the present study, the major findings are that the ER stress pathway is involved in seizure-related neuronal apoptosis, as judged by ele-

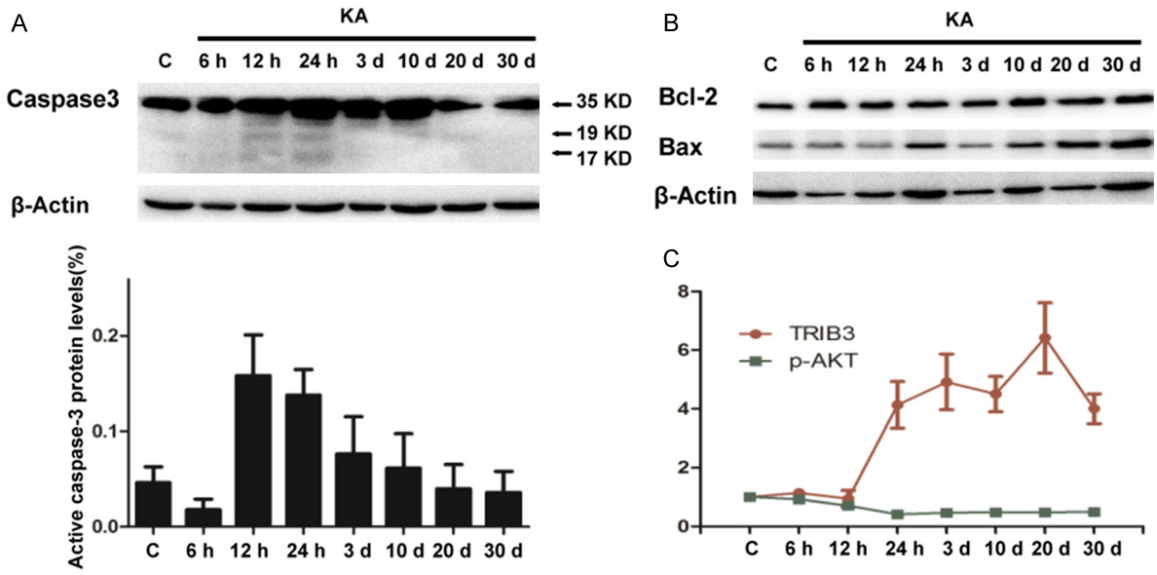

Figure 3. Induction of pro-apoptotic protein Bax and activation of caspase-3 in hippocampal neurons following kainic acid administration. A. Activated caspase-3 in the hippocampus at different times after KA, as detected by Western blotting. B. Bax and Bcl-2 in the hippocampus at different times after KA administration, as detected by western blotting. C. TRIB3 and phospho-AKT in the hippocampus at different times after KA administration. C: control group. KA: KA group.

vated expression of GRP78 and CHOP, that-TRIB3 is elevated due to ER stress in kainic acid-induced seizures, and that downregulation of phospho-AKT, and upregulation of proapoptotic proteins Bax and cleaved caspase-3 occurs. Thus, neuronal apoptosis induced by ER stress may be regulated by TRIB3-mediated inhibition of AKT phosphorylation.

Epilepsy refers to a group of neurological disorders of varying etiology, is characterized by recurrent seizures and results in brain dysfunction. Hippocampal injury is very important in the pathogenesis of epilepsy, and results in severe incapacitation and cognitive dysfunction [3]. We demonstrate hippocampal injury, following kainic acid-evoked seizures, consistent with excitotoxic neuronal injury involving apoptosis (**Figure 1**). ER stress has been widely studied for its role in mediating neuronal excitotoxic injury [6, 7], and has been shown to be an important factor in neurological diseases [27]. However, the mechanisms by which the ER stress triggers neuronal injury remain to be fully elucidated. The ER chaperone GRP78 and CHOP are associated with all three ER stress pathways [28], Therefore, we examined the their expression in the hippocampus following kainic acid-induced seizures. Western blot analysis revealed GRP78 and CHOP to be upregulated from 24 h to 30 d following kainic acid-induced seizures (**Figure 2A**, **2B**), the results suggesting that induction of ER stress may be related to seizure-induced hippocampal injury.

CHOP is known to be involved in ER stress-induced apoptosis [28]. Several studies show that CHOP alters the transcription of TRIB3 and mediates apoptosis [15]. TRIB3 induction antagonizes cardiac glucose metabolism and cardiacmyocyte survival [15], and is essential for ER stress-induced apoptosis in PC12 pheochromocytoma cells [29]. TRIB3 is induced by ATF4-CHOP and mediates cell death in Parkinson's disease models [16]. In addition, TRB3 overexpression due to endoplasmic reticulum stress inhibits activation of AKT kinase, known to enhance survival, in tongue squamous cells [20]. Thus, ourdata, showing an increase in TRIB3 during the acute brain injury phase, as well as the long-term adaptive phase following KA-induced seizure, suggests an involvement of TRIB3 in hippocampal injury following kainate-induced seizures.

ER stress results in the induction of TRIB3, a pseudokinase that affects a number of cellular functions by interacting with a host of molecules, such as AKT, FoxO and MLK3 [13, 30-32]. To further investigate the mechanisms underly-

ing neuronal damage after excitotoxicity, we examined possible signal transduction pathways involved. AKT plays a critical role in cellular growth, survival and metabolism [19, 24]. Loss of TRIB3 results in an increase in the phosphorylation of AKT that contributes to tumorigenesis [33]. Our previous study shows that recurrent febrile seizures may cause injury to hippocampal cells by interfering with AKT activation through ER stress-mediated upregulation of TRIB3 [17]. The present study provides in vivo confirmation that the levels of AKT phosphorylation decrease concomitantly with increased TRIB3 (**Figure 3C**). Taken together, these results indicate that TRIB3 may promote hippocampal apoptosis possibly by interfering with AKT phosphorylation.

In summary, this study demonstrates that the ER stress-associated gene TRIB3 is involved in hippocampal injury following kainic acid-induced seizure. TRIB3 induction, due to ER stress, may mediate neuronal apoptosis by interfering with AKT phosphorylation during epilepsy. Our study suggests that TRIB3 may be a potential and promising therapeutic target for excitotoxic neuronal injury caused by seizures.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (81200998), Beijing Natural Science Foundation (7092105 and 7112131), Key Clinical Project from the Ministry of Public Health (2010-12), and National Key Technology R&D Program (2012BAI03B02).

Disclosure of conflict of interest

None.

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