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ORIGINAL ARTICLE

Spatio-temporal Expression Study of Phosphorylated 70-kDa Ribosomal S6 Kinase (p70S6k) in Mesial Temporal Lobe Epilepsy[△]

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Objective To determine the spatio-temporal expression of p70S6k activation in hippocampus in mesial temporal lobe epilepsy.

Methods Temporal lobe epilepsy model was established by stereotactically unilateral and intrahippocampal injection of kainate acid (KA) in adult male C57BL/6 mice. Latent and chronic epileptogenesis were represented by mice 5 days after KA injection ($n=5$) and mice 5 weeks after KA injection ($n=8$), respectively. Control mice ($n=5$) were injected with saline. Immunohistochemical assays were performed on brain sections of the mice.

Results Hippocampus both ipsilateral and contralateral to the KA injection displayed significantly up-regulated pS6 immunoreactivity in dispersed granule cells in 5-day and 5-week model mice.

Conclusion The activation of p70S6k is mainly located in the dentate gyrus in KA-induced mouse model of temporal lobe epilepsy, indicating that the activation may be related with the disperse degree and hypertrophy of granule cells.

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EPILEPSY affects almost 1% people around the world, and mesial temporal lobe epilepsy (mTLE) is the most common form in adult.¹ Majority of these patients have seizures that can not be well controlled with classical antiepilepsy drugs,² except by the surgical resection of sclerous hippocampus.³ Thus, it is very important to investigate the process of epileptogenesis, and develop an effective strategy for treatment. In recent years, the focus of related researches has been shifted from the ion channel to intracellular molecules that may contribute to mTLE.⁴

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates cell growth, proliferation, survival, differentiation, and homeostasis.⁵⁻⁷ In nervous system, mTOR can regulate the neuron development, neurite outgrowth, and synaptic plasticity.^{8,9} Recent studies using the model of mTLE suggest that overactivation of mTOR signaling pathway may contribute to epileptogenesis.^{10,11} In the model mice, inhibition of mTOR suppresses dentate granule cell axon sprouting.¹⁰ Rapamycin administered prior to kainite can decrease kainite-induced neuron death, neurogenesis, mossy fiber sprouting, development of spontaneous epilepsy, and epilepsy-related hypertrophy.¹² The mTOR pathway is certainly involved in temporal lobe epileptogenesis. Obtaining insight into the downstream pathway of mTOR, and its spatio-temporal pattern in the hippocampus will increase our understanding of the mechanism and the process of mTLE. The present study addressed this topic by evaluating the expression and distribution of phosphorylated 70-kDa ribosomal S6 kinase (p70S6k), a downstream molecule of mTOR, with immunohistochemical assays.

MATERIALS AND METHODS

Experimental animals and kainite acid (KA)-induced mouse model of mTLE

Adult male C57BL/6 mice ($n=18$, Beijing Vitalrive Co., Ltd) of 8-week and weighing 20-24 g were treated in accordance with the institutional guidelines of the Beijing Administration Office of Laboratory Animals and acclimated for 2 weeks before experiments. The use of mice in the present study was approved by Ethics Committee of Chinese Academy of Medical Sciences and Peking Union Medical College.

The mice were first anesthetized by intraperitoneal injection of 10% chloral hydrate solution (5 mL/kg), and stereotaxically unilateral injection of 200 ng KA (Sigma, Shanghai, China) in 50 nL saline into the right hippocampus (anteroposterior = -2.0 mm, mediolateral = -1.8

mm, dorsoventral = -2.3 mm). The scalps were sutured up. All efforts were made to minimize animal suffering and reduce the mortality of model mice. We selected two time points after the KA injection (5 days, $n=5$; 5 weeks, $n=8$) to represent latent and chronic phases of epileptogenesis, respectively. At least 5 KA-injected mice were sacrificed at each time point for immunohistochemical evaluation. Control mice ($n=5$) were injected with saline alone prior to sacrifice.

Immunohistochemical assays

Mouse hippocampus tissues were incubated for at least 48 hours in 4% paraformaldehyde (Sigma) and embedded in paraffin. Paraffin-embedded tissues were sectioned into 2- μ m slices for immunohistochemical study. Sections with stereotypical hippocampus structure were used for immunohistochemistry. p70S6k antibody (1 : 200 in mouse section, Cell Signaling Technology, Danvers, MA, USA, #2708) was used. After incubation with the primary antibody at 4°C overnight, the tissues were treated with polymer helper (Golden Bridge, Beijing, China) for 20 minutes, and then treated with poly peroxidase-anti-goat IgG (Golden Bridge) for 20 minutes. Subsequently, the tissues were incubated with diaminobenzidine Horseradish Peroxidase Color Development Kit (Golden Bridge) for 2 minutes. Counterstaining was conducted with hematoxylin (Golden Bridge). After each incubation step, the sections were washed with Tris-buffered saline with Tween for 3 times, 5 minutes for each time.

RESULTS

Two mice died in the chronic phase after KA injection. The mortality of model mice was 11%.

Control mouse hippocampus ($n=5$) displayed p70S6k immunoreactivity (IR) in neuron of dentate gyrus (DG) and Cornu Ammonis (CA) (Fig. 1A-C). In the 5-day model mice ($n=5$), increased p70S6k IR was observed in the ipsilateral DG compared with control mice (Fig. 1D). But p70S6k IR was absent in CA, and neuron death is indicated by nuclear pyknosis (Fig. 1E,F)

In the 5-week model mice ($n=6$), intensified p70S6k IR was observed in both ipsilateral DG and contralateral DG (Fig. 2A, B). In addition, p70S6k IR in ipsilateral DG was higher in 5-week model mice than in 5-day model mice (Fig. 2A). Granule cells in the contralateral hippocampus displayed dispersion to a certain extent (Fig. 2C). In 5-week model mice, the granule cells in the ipsilateral hippocampus were found larger than those in the contralateral hippocampus, and the shape turned to spindle-like (Fig. 2C).

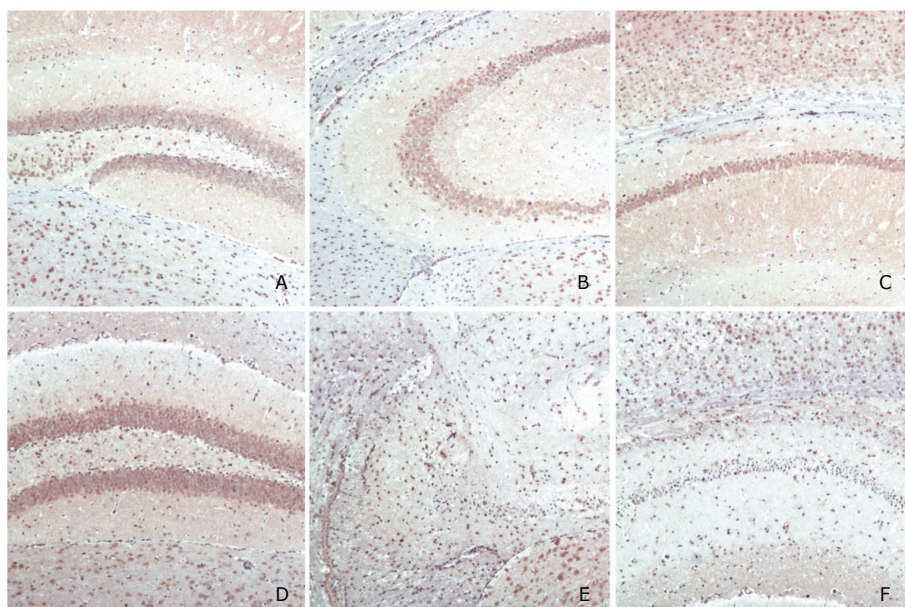


Figure 1. Distribution of p70S6k immunoreactivity (IR) in the hippocampus of saline-injected control mice ($n=5$) and 5-day kainite acid (KA)-induced mesial temporal lobe epilepsy (mTLE) model mice ($n=5$). hematoxylin $\times 40$
In control mice, the density of pS6 IR varies in dentate gyrus (DG) (A), Cornu Ammonis (CA) 3 (B), and CA2+CA1 (C). Hippocampus of 5-day mTLE model mice shows increased p70S6k IR in the DG (D), but absent pS6 IR in CA (E, F). Neuron death is indicated by nuclear pyknosis (E, F).

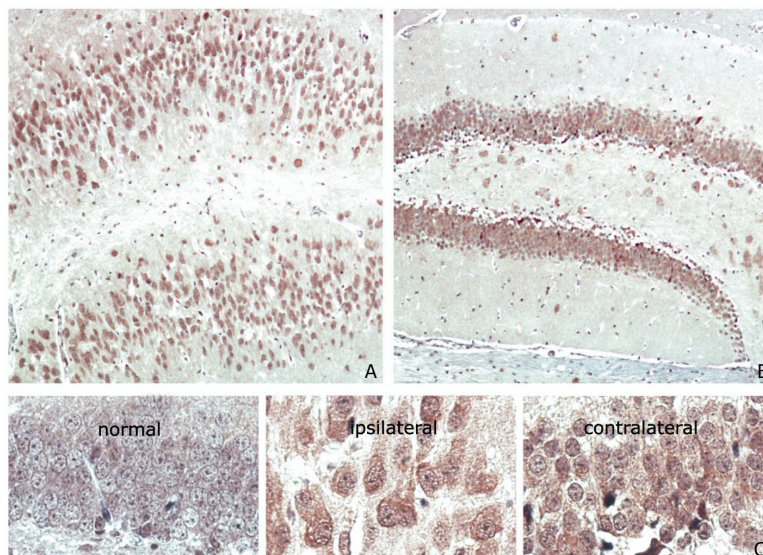


Figure 2. Distribution of p70S6k IR in the hippocampus of 5-week mTLE model mice ($n=6$). hematoxylin $\times 400$
The hippocampus shows largely increased p70S6k IR in the ipsilateral DG to KA injection (A). The contralateral hippocampus also displays increased p70S6k IR (B). In the ipsilateral hippocampus, granule cells are larger than in the contralateral hippocampus, meanwhile the shape turns to spindle-like (C).

DISCUSSION

Increasing evidence suggested that mTOR signaling pathway may be involved in the pathogenesis of temporal lobe epilepsy.¹³ Hippocampus sclerosis, such as neuron loss, granule cells dispersion, astroglia hyperplasia, is the hallmark pathological feature of mTLE.¹⁴ In this study, we

investigated the spatio-temporal distribution of p70S6k in the mouse model of KA-induced mTLE. The activation of p70S6k was found intensified in the dispersed granule cells of DG in the chronic phase model mice. And the dispersed granule cells with intensified p70S6k IR in the ipsilateral hippocampus of 5-week model mice are larger than the granule cells in the control mice. Progressive p70S6k IR in granule cells of DG in model mice was demonstrated by the

immunohistochemical assays in the control, latent phase mice (5 days), and chronic phase mice (5 weeks). Ribosomal protein S6 (S6), a downstream molecule of p70S6k, can regulate cell growth.¹⁵ It has been reported that rapamycin suppresses DG hypertrophy,¹⁶ which is a hallmark of mTLE.^{17,18} According to the results, p70S6k activation may be associated with cell size. In the model mice, the granule cells hypertrophy maybe caused by excessive phosphorylation of S6 controlled by p70S6k IR. Further investigations are needed to confirm whether the observed up-regulation of p70S6k IR in the mTLE mouse model also occurs in mTLE patients with hippocampal sclerosis (HS), whether the observed change in cell size and morphology in the model mice could be found in mTLE patients with HS as well, and whether the over-activation of p70S6k in granule cells contributes to other aspects, such as the excitatory neural circuitry.

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