Original Article

Inositol phosphatase INPP4A inhibits the apoptosis of in vitro neurons with characteristic of intractable epilepsy by reducing intracellular Ca\(^{2+}\) concentration

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Abstract: Epilepsy is a chronic neurological disorder characterized by recurrent seizures. Seizures can be controlled for most epilepsy patients after drug therapy, but at least 20% of patients develop intractable epilepsy (IE). The mechanism by which IE causes neuronal damage has not been completely understood. Inositol polyphosphate 4 phosphatase (INPP4A), a magnesium-dependent phosphatase, is shown to be associated with glutamate excitotoxicity. Herein, we show that INPP4A plays an essential role in seizure-induced neuronal apoptosis using an in vitro IE neuron model. In this model, INPP4A expression significantly decreased compared to normal neurons. Our results showed that overexpression of INPP4A significantly inhibited LDH activity and increased cell viability while knockdown of INPP4A markedly increased LDH activity and inhibited cell viability. Similarly, overexpression of INPP4A significantly enhanced G1 phase transition to S phase and inhibited apoptosis while knockdown of INPP4A significantly inhibited cell cycle progression and increased apoptosis in IE neurons. Furthermore, INPP4A-mediated inhibition of apoptosis might be associated with reduced intracellular Ca\(^{2+}\) concentration. Our findings thus support the results of the previous in vivo studies that INPP4A is linked to the pathogenesis and progression of intractable epilepsy, which suggest that INPP4A may be an important target against epilepsy, especially IE.

Keywords: Epilepsy, intractable epilepsy, INPP4A, cell cycle, apoptosis, Ca\(^{2+}\) concentration

Introduction

Epilepsy is a chronic neurological disorder characterized by recurrent seizures with diverse etiology that affects up to 1% of the world population [1]. Seizures can be controlled for most epilepsy patients after drug therapy, but at least 20% of patients develop intractable epilepsy (IE). Long-term repeated, uncontrollable seizures not only makes patients suffer physical and psychological damage, but also bring family and society a heavy burden [1].

The mechanism of seizures causes neuronal damage has not been completely understood. In both patients and animals with refractory temporal lobe epilepsy, it has been shown that neuronal damage is characteristic of apoptosis after seizures [2, 3]. Recurrent seizures can induce activation of excitatory glutamate receptor and calcium overload, which subsequently causes release of cytochrome C from the mitochondria into the cytoplasm. Cytochrome C interaction with other cytoplasmic components activates caspase 3, 6 and 7 and causes neuronal death or apoptosis [4]. However, the exact mechanism is little known about activation of excitatory glutamate receptor and calcium overload after seizures.

Inositol polyphosphate 4 phosphatase (INPP-4A), a magnesium-dependent phosphatase, plays an important role in the metabolism of inositol [5]. INPP4A is highly expressed in the central nervous system, which is shown to play a key role in glutamate excitotoxicity [5] and cell proliferation [6, 7]. Recently, Sasaki et al. showed that INPP4A knockout mice developed tonic and spastic-like activity. Administration of NMDA glutamate receptor antagonist MK801 improved the above symptoms, suggesting that INPP4A may play an important role in the devel-
opment of epilepsy [8]. Previous studies also showed that INPP4A mutant mice would die from epileptic seizures. The hippocampal CA1 region in this mutant mouse had significant neuronal loss that was one of the important features of hippocampal sclerosis (HS) [9]. Similarly, 69.2% of patients appeared HS in patients with refractory TLE surgery or autopsy [10, 11]. These findings suggest that INPP4A may play an important role in the pathogenesis and progression of refractory epilepsy.

Herein, we show that INPP4A plays an essential role in seizure-induced neuronal apoptosis using an in vitro seizure-cell model. In this model, overexpression of INPP4A significantly inhibits neuronal apoptosis through preventing calcium release. These findings further support the results of the previous in vivo studies that INPP4A is linked to the pathogenesis and progression of refractory epilepsy. Importantly, our study, for the first time, shows the mechanism of INPP4A-associated calcium release underlying seizure-induced neuronal apoptosis.

Materials and methods

Cultivation of rat hippocampal neuron

Newborn Wistar rat within 24 hours were purchased from the Animal Experimental Center of Southern Medical University, Guangdong Province. The rats were immersed in 75% alcohol solution for body disinfection and then the brain tissue was separated and placed in ice-cold D-Hanks buffer. According to the mouse brain anatomy, hippocampus was separated and digested with 2.5% Trypsin for 25 min at 37°C. Then the trypsin was removed and DMEM/F12K cell culture medium was added to end the digestion. After washing twice, cells were tipping gently for 20 times till no large tissue. According to the mouse brain anatomy, hippocampus was separated and digested with 2.5% Trypsin for 25 min at 37°C. Then the trypsin was removed and DMEM/F12K cell culture medium was added to end the digestion. After washing twice, cells were tipping gently for 20 times till no large tissue. The cells were collected after centrifugation at 1000 rpm for 5 min, and cell suspension was filtered with 200 mesh cell sieve. After that, cells were placed into 6-well plates coated by 0.1% polylysine. The medium was replaced with serum-free medium after incubating at 37°C for 12 h, then, half culture medium was replaced every 3 days.

Identification of rat hippocampal neuron

Hippocampal neuronal cells were harvested on day 5 and identified through immunofluorescence assay. Neuronal cells were gently rinsed with PBS 3 times and fixed with 4% paraformaldehide for 30 min. Cells were then blocked with normal goat serum at room temperature for 15 min. Cells were incubated with antibodies against microtubule-associated protein-2 (MAP-2) and 200-kDa neurofilament protein (NF-200) (Abcam, Cambridge, MA, USA) at 37°C for 150 min respectively, which were then incubated with FITC-conjugated secondary antibodies at room temperature for 1 h. Images were taken under the fluorescence microscope with HD camera (Leica, Wetzlar, Germany).

Establishment of IE cell model

Hippocampal neuronal cells cultured for 14 days were randomly divided into mock group and IE model group, with 6 well settled each group. Cells in mock group and IE model group were cultured by normal extracellular fluid and free-magnesium extracellular fluid respectively, and changed into serum-free maintenance medium after 3 days. The ingredients of normal extracellular fluid were as follows: NaCl 145 mmol/L, KCl 2.5 mmol/L, HEPES 10 mmol/L, CaCl₂ 2 mmol/L, glucose 10 mmol/L, glycine 0.002 mmol/L, MgCl₂ 1 mmol/L (Sigma-Aldrich, StLouis, MO, USA). Meanwhile, the free-magnesium extracellular fluid were consist of NaCl 145 mmol/L, KCl 2.5 mmol/L, HEPES 10 mmol/L, CaCl₂ 2 mmol/L, glucose 10 mmol/L, glycine 0.002 mmol/L.

Overexpression and siRNA knockdown of INPP4A

Homo INPP4A was over-expressed or silenced by transfection of pcDNA-INPP4A plasmid (VIPOTION, Guangzhou, China) or INPP4A siRNA (GenePharma, Shanghai, China), respectively, with pcDNA3.1 (VIPOTION, Guangzhou, China) vector and scrambled siRNA (GenePharma, Shanghai, China) transfected cells as control in vitro. IE cells grown in 6-well plate were transfected with 1 μg pcDNA-INPP4A plasmid or 50 nM INPP4A siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA). After 6 h, the transfection solution was replaced by complete culture medium.

Quantitative real-time RT-PCR

Total RNA was isolated from cells using Rneasy Mini Kit (Qiagen, Germantown, MD, USA). The
mRNA of INPP4A and GAPDH were amplified by quantitative real-time RT-PCR. It was carried out using SuperScriptIII Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen) with the following procedures: 50°C for 3 min, 95°C for 5 min, followed by 35 cycles of 95°C for 15 s, 60°C for 30 s. The relative mRNA amount of INPP4A was calculated by comparative Ct method after normalizing the quantity of β-actin. The primer sequences were used as follows: INPP4A: forward: 5'-AATGACAGCAAGAGAACAG-3', reverse: 5'-TGACACTCACAGCCACAAAC-3'; β-actin: forward: 5'-GGAGATTACTGCCCTGGCTCCTA-3', reverse: 5'-GACTCATCGTACTCCTGCTTGCTG-3'.

**Western blotting**

Total cellular proteins were extracted and the protein concentrations were determined by BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of protein were subjected to SDS-PAGE and then transferred to PVDF membrane (Millipore, Billerica, MA, USA). After blocked by 5% milk, the membranes were incubated with antibodies against INPP4A and GAPDH (Santa Cruz, Dallas, Texas, USA). Lastly, HRP-conjugated secondary antibodies were applied and the signals were detected using the ECL kit (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

**Lactate dehydrogenase (LDH) activity assay**

The LDH activity in cells grown in 96-well plates was measured by LDH-Cytotoxicity Assay Kit II (Abcam) with an ELISA reader (PerkinElmer, USA) at a wavelength of 490 nm. The percentage of LDH release was calculated as follows: %LDH release=experimental LDH release/maximum LDH release×100. Maximum LDH release was determined by complete solubilization of bovine hepatocytes with 0.1% TritonX-100 [2].

**CCK-8 assay for cellular viability**

Cell viability was measured using Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan). Cells (5×10³ cells/well) grown in 96-well cell plates for 24 h, 48 h or 72 h were washed with PBS three times. Then a 1:10 diluted CCK-8 solution was added and incubated at 37°C for 2 h. The results were measured by microplate reader at 450 nm and expressed as percentages of the mock group.

**Cell cycle detection assay**

Flow cytometry analysis was performed to determine the effect of INPP4A on cell cycle progression. Mock cells and IE cells plated into 6-well plates were transfected with pcDNA-INPP4A plasmid or INPP4A siRNA and incubated for 48 h. Then the cells were harvested and fixed in 70% ethanol at 4°C for at least 2 h. Before flow cytometry analysis, cells were stained with 1 ml of propidium iodide (PI) (15 mg/ml) containing RNase (2.5 mg/ml). DNA content was analyzed by a FACSscan (BD Biosciences, San Jose, CA, USA) and the proportion of cells in a particular phase of cell cycle was determined by Cell Quest software (BD Biosciences).

**Cell apoptosis detection assay**

Quantitative assessment of apoptosis was carried out using Flow cytometry analysis. Briefly, transfected cells grown in 6-well culture plates were collected and subjected to Annexin V and PI staining using an Annexin V-FITC Apoptosis Detection kit (BD Biosciences). Cells were then analyzed in a FACSscan and data were analyzed using Cell Quest software.

**Intracellular Ca²⁺ concentration assay**

Intracellular Ca²⁺ concentration was measured through flow cytometry analysis and immunofluorescent analysis using the fluorescent Ca²⁺ chelator Fluo-4 AM and Fluo-3 AM (Molecular Probes, Eugene, OR, USA) respectively. Fluo-4 AM and Fluo-3 AM can permeate into cells and change into Fluo-4 and Fluo-3, which combine with Ca²⁺ to form a fluorescent compound. The fluorescent intensity of Ca²⁺ formed compound was determined at the excitation wavelength of 340 nm and the emission wavelength of 510 nm in FACSc.
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Figure 1. INPP4A expression decreases in IE neurons and is linked to LDH activity and cell viability. A. Hippocampal neuron cells were identified by immunofluorescence, staining with MAP-2 and NF-200 antibody. B, C. The expression of INPP4A was detected in mock and IE neurons with pcDNA-INPP4A, INPP4A siRNA or negative control (NC). IE neurons were transfected with pcDNA-INPP4A (1 μg) or INPP4A siRNA (50 nM) for 48 h. The total RNA and protein were collected and INPP4A mRNA and protein expression were analyzed by qRT-PCR and western blot, respectively. D. The activity of LDH was detected at 24, 48 and 72 h in mock and IE cells with pcDNA-INPP4A, INPP4A siRNA or NC. Mock and IE cells were transfected with pcDNA-INPP4A, INPP4A siRNA or NC for 48 h. The activities of LDH in cellular supernatant at the indicated time points were detected by Elisa. E. The effect of INPP4A on cell viability in mock and IE cells with pcDNA-INPP4A, INPP4A siRNA or NC. Cells were transfected with pcDNA-INPP4A, INPP4A siRNA or NC. Cell viability at the indicated time points was detected by CCK8 assay. The experiments were performed in triplicate and each value represents mean ± SD. *P<0.05, **P<0.01, ***P<0.001 versus mock neurons with negative control (Mock + NC), #P<0.05, ##P<0.01, ###P<0.001 versus intractable epilepsy neurons with negative control (IE + NC).

cell viability at the indicated time points was detected by CCK8 assay. The experiments were performed in triplicate and each value represents mean ± SD. *P<0.05, **P<0.01, ***P<0.001 versus mock neurons with negative control (Mock + NC), #P<0.05, ##P<0.01, ###P<0.001 versus intractable epilepsy neurons with negative control (IE + NC).

scanning confocal microscope (Olympus America, Center Valley, PA, USA) at wavelength 488 nm for excitation and 516 nm for emission.
Statistical analyses

Statistical analyses were performed using GraphPad Prism 6.0 software. Results are expressed as mean ± SD. Data were analyzed by one-way ANOVA with multiple comparisons test (three or more data sets in a group) or two-way ANOVA with multiple comparisons test (grouped data). P<0.05 was considered significant.

Results

**INPP4A expression decreases in the IE neurons**

To study the expression of INPP4A in the neuron with treatment of free-magnesium solution, hippocampal neurons from neonatal rat were first isolated and cultured in vitro. Hippocampal neurons were identified by staining with MAP-2 and NF-200 antibody, which are both specific biomarkers of neurons. As shown in **Figure 1A**, we successfully obtained hippocampal neurons.

To establish in vitro IE cell model, hippocampal neurons were cultured for 3 h in free-magnesium medium to establish IE cell model. Next, the expression of INPP4A in the IE neurons was explored in the indicated condition (**Figure 1B and 1C**). Our results showed that the expression of INPP4A mRNA and protein significantly decreased in IE neurons compared with normal neurons (**Figure 1B and 1C**).
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Figure 3. INPP4A decreases Ca^{2+} concentration in IE neurons through flow cytometry analysis. A. The effect of INPP4A on Ca^{2+} concentration in IE neurons. Mock and IE neurons were transfected with pcDNA-INPP4A, INPP4A siRNA or NC for 48 h. Cells were then collected and Ca^{2+} concentration was detected by flow cytometry. B. The statistical analysis of the effect of INPP4A on Ca^{2+} concentration in IE neurons. The experiments were performed in triplicate and each value represents mean ± SD. *P<0.05, **P<0.01, ***P<0.001 versus Mock + NC; #P<0.05, ##P<0.01, ###P<0.001 versus IE + NC.

INPP4A inhibits LDH activity and increases cell viability

Next, to determine the biological function of INPP4A, we first studied effects of INPP4A on LDH activity and cell viability in the IE neurons using overexpression and knockout of INPP4A. The results showed that LDH activity in the IE neurons significantly increased compared to the normal neurons at 24 h, 48 h and 72 h (Figure 1D). Conversely, cell viability in the IE neurons was lower than that of normal neurons (Figure 1E). Furthermore, we found that overexpression of INPP4A significantly inhibited LDH activity and increased cell viability in the IE neurons at 24 h, 48 h and 72 h. On the contrary, knock out of INPP4A further increased LDH activity and inhibited cell viability (Figure 1D and 1E). These findings suggest that INPP4A may play an important role in the proliferation and apoptosis of IE neurons.

INPP4A accelerates cell cycle progression and inhibits apoptosis of IE neurons

We next assessed whether INPP4A was required for cell cycle progression in IE neurons through overexpression or knockdown of INPP4A and examined cell cycle distribution over time. As shown in Figure 2A and 2C, G1 phase population in IE neurons was significantly higher than that in mock neurons; conversely, S phase population in IE neurons was lower. Also, overexpression of INPP4A accelerated G1/S phase transition while knockdown of INPP4A enhanced accumulation of cells in the G1 phase of the cell cycle (Figure 2A and 2C).
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Figure 4. INPP4A decreases Ca\textsuperscript{2+} concentration in IE neurons. Mock and IE neurons were transfected with pcDNA-INPP4A, INPP4A siRNA or NC for 48 h in the presence of Ca\textsuperscript{2+} channel activator BayK8644 or Ca\textsuperscript{2+} channel inhibitor SKF96365. Cellular Ca\textsuperscript{2+} and nucleus were stained with fluo-3 AM and DAPI, respectively. The groups were indicated as follows: A. Ca\textsuperscript{2+} concentration in Mock neurons transfected with NC; B. Ca\textsuperscript{2+} concentration in IE neurons transfected with NC; C. Ca\textsuperscript{2+} concentration in IE neurons transfected with pcDNA-INPP4A; D. Ca\textsuperscript{2+} concentration in IE neurons transfected with INPP4A siRNA; E. Ca\textsuperscript{2+} concentration in IE neurons transfected with pcDNA-INPP4A in the presence of BayK8644; F. Ca\textsuperscript{2+} concentration in IE neurons transfected with INPP4A siRNA in the presence of SKF96365. The experiments were performed in triplicate.

The effects of INPP4A on apoptosis of IE neurons were investigated after they were treated with pcDNA-INPP4A, INPP4A siRNA or negative control (NC) for 48 h. The flow cytometry data (Figure 2B and 2D) showed that necrosis and apoptosis cells in IE neurons transfected with NC significantly increased compared to mock neurons transfected with NC while live cells in IE neurons with NC treatment significantly decreased. In addition, necrosis and apoptosis cells in IE neurons treated with pcDNA-INPP4A significantly decreased compared to the IE neurons treated with NC; meanwhile, live cells in IE neurons treated with pcDNA-INPP4A significantly increased. In line with this, treatment of INPP4A siRNA significantly increased early apoptosis cells in the IE neurons while significantly decreased live cells (Figure 2B and 2D). These findings suggest that INPP4A accelerates cell cycle progression and inhibits apoptosis of IE neurons.

**INPP4A decreases Ca\textsuperscript{2+} concentration in IE neurons**

Cell apoptosis is closely related with Ca\textsuperscript{2+} concentrations. Thus, the effects of INPP4A were tested on Ca\textsuperscript{2+} concentrations in the IE neurons. Our results demonstrated that Ca\textsuperscript{2+} concentrations significantly increased in the IE neurons compared to mock neurons (Figures 3A and 3B, 4A).
and 4B). Furthermore, the results that treatment of pcDNA-INPP4A significantly decreased Ca\(^{2+}\) concentrations in the IE neurons while treatment of INPP4A siRNA significantly increased Ca\(^{2+}\) concentrations (Figures 3A and 3B, 4C and 4D).

We next studied effects of BayK8644, a calcium channel agonist and SKF96365, a blocker of receptor-mediated calcium entry on Ca\(^{2+}\) concentrations in the IE neurons with pcDNA-INPP4A and INPP4A siRNA, respectively. Immunofluorescence analysis showed that BayK8644 restored decreased Ca\(^{2+}\) concentrations in the IE neurons with treatment of pcDNA-INPP4A (Figure 4E) while SKF96365 decreased increased Ca\(^{2+}\) concentrations in the IE neurons treated with INPP4A siRNA (Figure 4F). The above findings suggest that INPP4A accelerates cell cycle progression and inhibited apoptosis of IE neurons, at least partly by decreasing Ca\(^{2+}\) concentrations.

Discussion

The pathogenesis of intractable epilepsy has not been fully defined. Animal models are the main research methods to study physiological mechanisms of epilepsy development. Considering the problems of instability and difficult to control in animal models, the research of in vitro model is paid more and more attention. In 1995, Sombati successfully constructed IE model of cells with spontaneous recurrent discharge using primary hippocampal neurons cultured in magnesium-free medium [12]. This model is simple and repeatable and has been widely used in the study of the mechanism of intractable epilepsy and the development of drug [13-15]. Herein, we showed that INPP4A plays an important in proliferation and apoptosis in IE neurons with this model.

Animal and human studies show that downregulation or loss of INPP4A expression is linked to neurodegenerative disorders. For example, INPP4A mutant Weeble mice showed severe motor instability and significant deletions in the cerebellum and hippocampal CA1 neurons [16]. Consistent with this, INPP4A\(^{-/-}\) mice showed severe involuntary movement, including excessive physical movement such as throwing, chorea, neck and trunk contraction [6]. Recently, a human study showed that INPP4A expression was significantly lower in temporal lobe epilepsy (TLE) patients than in controls.

Moreover, INPP4A expression was downregulated in rat epilepsy induced by lithium-pilocarpine. Of note, this study just provides a phenomenon of INPP4A downregulation in patients and animals with epilepsy [17]. In the present study, we provided direct evidence showing that INPP4A accelerates cell cycle progression and inhibits apoptosis of IE neurons by overexpressing and silencing INPP4A expression. These findings thus suggest that INPP4A plays an essential protective role in neurons after seizures.

Neuronal apoptosis has been widely observed in animal epilepsy models, which is an important contributor to the neuronal loss in TLE patients [18]. Seizure-induced neuronal apoptosis is largely associated with excitotoxic glutamatergic neurotransmission gating excessive Ca\(^{2+}\) entry which damages DNA and protease activation leading to proteolysis of cell and organelle membranes, culminating in necrosis or apoptosis [19]. INPP4A has been shown a protective function in neurons to suppress excitotoxic cell death [6]. Thus, we speculated that INPP4A-mediated inhibition of apoptosis in IE neurons is associated with Ca\(^{2+}\) release. In agreement with this, our results showed that overexpression of INPP4A decreased Ca\(^{2+}\) release while its knockout increased Ca\(^{2+}\) release. Furthermore, BayK8644, a calcium channel agonist prevented INPP4A inhibition of Ca\(^{2+}\) release. However, the exact mechanism underlying INPP4A inhibition of apoptosis needs to be further studied.

Taken together, our study demonstrates that INPP4A plays a key role in protecting neurons from seizures-induced apoptosis. We also show that INPP4A inhibition of apoptosis is associated with inhibition of Ca\(^{2+}\) release in IE neurons. Combined with previous findings, INPP4A may be an important target against epilepsy, especially IE.

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Disclosure of conflict of interest

None.

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