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Original Article

MiR-7 involves the activation of Nrf2 pathway by targeting Keap1 in epileptic seizure

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Abstract: MicroRNAs (miRNAs) are increasingly recognized as diagnostic biomarkers of epileptogenesis as well as targets to prevent or disrupt epilepsy. Keap1, a cytoplasmic inhibitory protein called Kelch-like ECH-associated protein 1, is also reported associated with epilepsy. However, the possible involvement of miRNAs in Keap1-mediated molecular basis for protection from epileptic seizure-induced brain damage is less understood. In the present study, Wistar rats were rapidly kindled in the amygdala. We found a dramatic upregulation of miR-7 and downregulation of Keap1 in the hippocampus of kindled rats, compared with control and sham group. Moreover, luciferase reporter gene assays identified that miR-7 was able to target 3'-untranslated region (3'-UTR) of Keap1 mRNA. To investigate the role of miR-7 in the protection of epilepsy mediated by Keap1/Nrf2 pathway, we used a transfection approach to overexpress miR-7, and then detected a consequential decrease in Keap1 mRNA and protein which subsequently results in an increased Nrf2 expression and cytoprotective enzymes (HO-1 and NQO1) expression. These results indicate that miR-7 may involve the protection of protection through targeting Keap1 and the subsequent activation of Nrf2 pathway.

Keywords: MiR-7, Nrf2, Keap1, epileptic seizure

Introduction

Epilepsy, one of the most common neurological disorders, is a chronic disorder of abnormal electrical activity in the brain, characterized by recurrent unprovoked seizures [1]. The mechanisms underlying epileptogenic pathogenesis have been proved to be complex, including oxidative stress, glutamate excitotoxicity, calcium overload and so on [2]. Given that pathological processes involved in epileptogenesis are complex and interrelated, to find a target, which can interrupt multi-mechanisms underlying seizure, is desirable.

MicroRNA (miRNA), an abundant group of endogenous non-coding single strand RNAs with approximately 19~25 nucleotides, regulates the expression of genes at post-transcriptional level by translational repression or degradation of target mRNA. They are critical for normal neuronal development and may be involved in many neurological diseases whose mechanisms remain to be explored. For example, Agostini et al. found that pro-apoptotic miR-34a regulates neurite outgrowth, spinal morphology and function [3], revealing the importance of miR-34a in neuronal differentiation and synaptogenesis. Recently, accumulating studies have investigated the role of miRNAs in neurological diseases such as epilepsy. For example, miR-34a was reported to be up-regulated during seizure-induced neuronal death [4]. However, they suggested that miR-34a is most likely not important for seizure-induced neuronal death in the mouse model. Aronica et al. investigated upregulation of miR-146a activation in a rat model of TLE as well as in human TLE [5]. Alsharafi et al. revealed the dynamic expression of MicroRNAs (183, 135a, 125b, 128, 30c and 27a) in the rat pilocarpine model and temporal lobe epilepsy patients [6]. MiR-134 blockade also reported to prevent status epilepticus like-activity and is neuroprotective in cultured hippocampal neurons [7]. MiR-7 was previously shown to play a protective role in cellular models of PD by directly targeting and downregulating the expression of α-synuclein [8] and RelA

[9, 10]. However, the expression and role of miR-7 in the epileptic seizure remains unclear.

In the present study, we show that miR-7 targets Keap1 mRNA and reduces its expression, resulting in the activation of the Nrf2 pathway, indicating a protective effect of miR-7 in the epileptic seizure.

Materials and methods

Animals

Adult male Wistar rats weighing 250-300 g (n = 50) were obtained from the Hebei Medial University. Rats were housed in a room with constant temperature (25 ± 1°C) and humidity (40-60%), and were kept on a 12 h light/dark cycle, with lights on at 8:00 AM and with free access to food and water. Animal experiments were performed according to the regulations of laboratory animal management promulgated by the Ministry of Science and Technology of the People's Republic of China No. 134, which coincides with internationally recognized NIH guidance. The rats were randomly assigned to three groups. Control group (n = 16) did not receive any treatment. Electrodes were implanted in the remaining 34 rats. Two rats could not be included in the experiments because of death during anesthesia or misplacement of the electrode. The remaining 32 rats were randomly distributed to the following groups: sham group (n = 16) did not receive any electrical current administration; kindling group (n = 16) were subjected to experimental kindling procedure as follow. Twenty-four hours after the last stimulation, all rats were sacrificed. Eight rats in each group were prepared for spectrophotometry, Western blot and real-time fluorescence quantitative PCR analysis. Animal care and sacrifice were conducted according to methods approved by the Animal Care and Use Committee, Xiangya Medical College, Central South University. All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Electrode implantation

For implantation, animals were anesthetized with chloral hydrate (300 mg/kg, i.p.), the sham and kindling groups were stereotactically implanted with twisted stainless steel wire bipolar stimulating electrodes in the left basolateral amygdale complex (Bregma: anterior: -2.8 mm; lateral: 4.8 mm; ventral: 8.8 mm from skull surface) [11]. Three screws positioned over the right frontal cortex, left and right occipital cortices served as recording, reference and ground electrodes. Dental cement bound the electrodes and screws to the skull. ABS connectors coupled the electrodes and screws to stimulating and recording equipments. After surgery, the rats were treated with antibiotics for 1 week to prevent infection.

Kindling procedure

The initial after discharge threshold (ADT) was determined using an electric stimulator (SEN-7203; Nihon Kohden) 1 week after electrode implantation [12]. The stimulation parameters were performed by constant current stimulations (monophasic square-wave pulses, 60 Hz for 1 s). The stimulus intensity started at 100 μA, and thereafter increased in 40 μA increments at 5 min intervals until an after discharge of at least 3 s duration was elicited (140-340 μA). The next day animals of kindling group were kindled at 10 min intervals stimulation at 120% of their individual ADT. In the course of kindling, stimulation was delivered 40 times for the kindling group, with 20 times every day.

Tissue dissection

Following the behavioral tests, the rats and mice in each group were sacrificed by decapitation. The hippocampus was quickly harvested and was frozen in liquid nitrogen and stored at -80°C until further utilization. Bilateral hippocampus of six rats in each group was pooled from individual rats for spectrophotometry. Unilateral hippocampi of the other six rats in each group were processed into a plastic tube for real-time PCR or Western blot separately.

Primary rat hippocampal neuron cultures

All animals were purchased from the Animal Center of the Fourth Military Medical University. Studies were conducted on primary mixed hippocampal neuronal cultures prepared as described previously with slight modifications [7]. Primary hippocampal cultures were prepared from embryonic day 18 Wistar rats brains. Cells were plated onto a 35-mm Falcon cell culture dishes or glass coverslips coated with poly-L-lysine (0.05 mg/ml). Hippocampal cultures were maintained at 37°C in a 5% CO_2/95% air

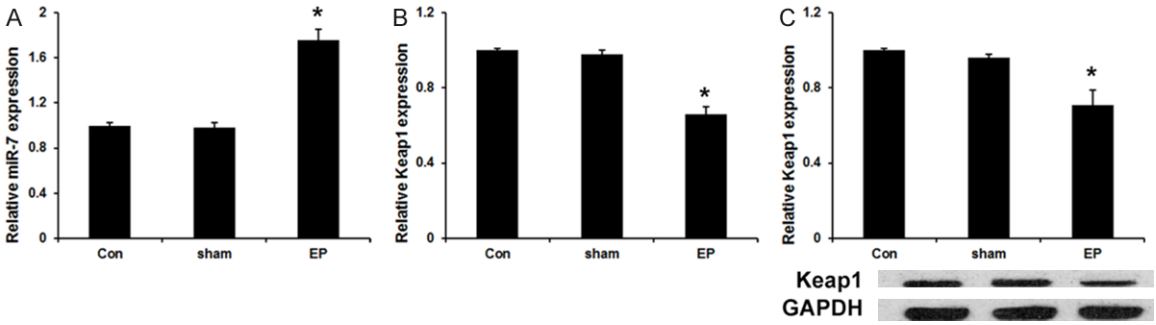

Figure 1. MiR-7 and Keap1 expression in hippocampus of rats. The 'Con', 'sham' and 'EP' in abscissa represent control, sham and kindling groups, respectively. A. The expression of miR-7 was measured by quantitative real-time PCR. B. The expression levels of Keap1 mRNA were measured by quantitative real-time PCR. C. The expression levels of Keap1 mRNA were measured by western blot. Values are expressed as mean ± SD. *P < 0.01.

atmosphere and grown in neurobasal medium (NB) supplemented with 2% B27, 0.5 mM L-glutamine, 100 units/ml penicillin and 100 g/ml streptomycin. Cultures were fed twice weekly.

Cell transfection

The miR-7 inhibitors and negative control molecules were purchased from Dharmacon (Austin, TX, USA). Cell transfection was performed using DharmFECT1 (Dharmacon) until a final concentration of 20 nM. Medium was changed after 6 h. After transfected and cultured for 48 h, cells were collected for Western blot and qRT-PCR analyses.

Luciferase reporter gene assays

The 3'-UTR of Keap1 containing the putative binding site of miR-7 was amplified and subcloned into pGL3 luciferase promoter vector (Promega, Madison, WI, USA). The vector was co-transfected with miR-7 mimics into HEK293 cells for 48 h. The cells were harvested and relative luciferase activity was detected using a dual-luciferase reporter assay kit (Promega) according to the manufacturer's instructions. All experiments were performed at least three times.

Quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from tissues and cell lines using the miRNeasy Mini Kit (Qiagen). The miRNA Q-PCR Detection Kit (GeneCopoeia) was used for quantification of miRNA levels according to the manufacturer's protocol. For quantification of PRMT1 mRNA levels, the RT reactions were conducted with the RevertAid TM H Minus First Strand cDNA Synthesis Kit (Fermentas). qRT-PCR was performed using an ABI 7900 System (Bio-Rad). RNU6B and β-actin were used as normalizing controls for miRNA and mRNA quantification, respectively. The $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative expression levels. The primers were as follows: Keap1, forward primer: 5'-CAACTTCGCTGAGCAGATTGGC-3' and reverse primer: 5'-TGATGAGGGTCACCAGTTGGCA-3'. Nrf2, forward primer: 5'-CACATCCAGTCAGAAACCAGTGG-3' and reverse primer: 5'-GGAATGTCTGCGCCAAAAGCTG-3'. HO-1 forward primer: 5'-TGTCCCAGGATTTGTCCGAG-3' and reverse primer: 5'-ACTGGGTTCTGCTTGTTTCGCT-3', NQO1 forward primer: 5'-GGGGACATGAACGTCATTCTCT-3' and reverse primer: 5'-AGTGGTGACTCCTCCCAGACAG-3', GAPDH forward primer: 5'-TGAACGGGAAGCTCACTG-3' and reverse primer: 5'-GCTTCACCACCTTCTTGATG-3').

Western blot analysis

Cells were rinsed with ice-cold PBS and lysed in PBS containing 2% SDS with protease inhibitors cocktail and phosphatase inhibitors (Roche). Cell lysates were sonicated for 20 sec, and protein concentration in the lysates was quantified using BCA Protein Assay Reagent (Thermo Scientific). Cell lysates were analyzed by Western blot analysis, using anti-Keap1 (catalog #8047, Cell-Signaling), anti-Nrf2 (catalog #EP1808Y, Abcam) or anti-β-actin (catalog #A5316, Sigma-Aldrich), followed by incubation with horseradish peroxidase-conjugated anti-rabbit (catalog #HAF008, R&D Systems) or anti-mouse antibody (catalog #HAF007, R&D Systems). ECL detection systems (SuperSignal West Femto, Pierce) were used for detection.

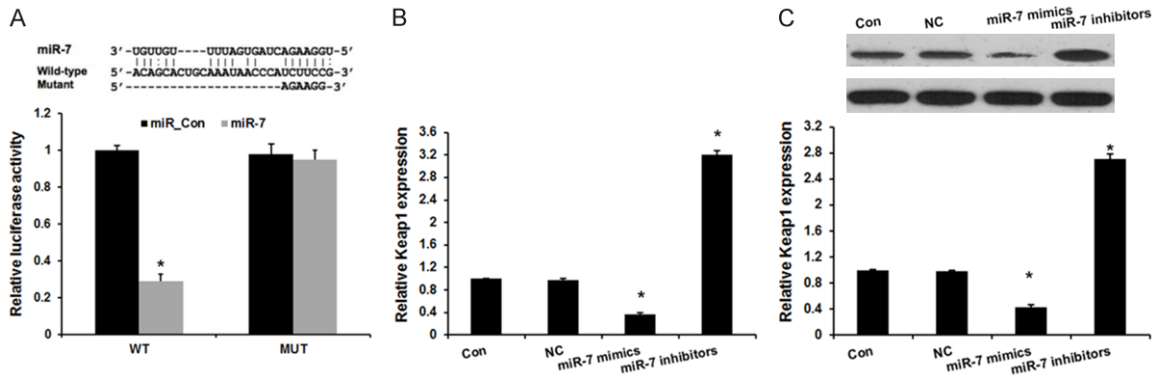

Figure 2. Keap1 is a direct target of miR-7. A. Sequence alignment of miR-7 and 3' UTR of Keap1 using mirco-RNA. org. Luciferase reporter assay with co-transfection of wild-type or mutant Keap1 and miR-7 mimics or miR-7 inhibitor or mimics-control or inhibitor-control or blank control in HEK293 cells. Error bars represent ± S.E. and *, $P < 0.01$ versus control. B. qRT-PCR analyses were performed to examine the effects of miR-7 on expression of Keap1. Error bars represent ± S.E. and *, $P < 0.01$ versus control. C. Western blotting was performed to determine Keap1 protein levels hippocampal neurons cells. Error bars represent ± S.E. and *, $P < 0.01$ versus control.

Statistical analysis

Each experiment was repeated at least three times. Data were shown as mean ± s.d and analyzed using SPSS 19.0. Statistical comparisons between groups were analyzed using Student's t-test and a two-tailed $P < 0.05$ was considered to indicate statistical significance.

Results

MiR-7 and Keap1 expression in hippocampus

We first detected the miR-7 expression in hippocampus was studied by real-time fluorescence quantitative PCR. As shown in **Figure 1A**, the expression of miR-7 is much higher in kindling groups than that in control and sham groups. Moreover, qRT-PCR assay and western blot assay revealed that the expression of Keap1 mRNA (**Figure 1B**) and protein (**Figure 1C**) is much lower in kindling groups compared with control and sham groups.

MiR-7 directly targets Keap1 in hippocampal neurons cells

Keap1 is also reported associated with epilepsy [13]. However, the relationship between miR-7 and Keap1 in hippocampal neurons cells remains unclear. In this study, the miRNA target prediction websites www.microRNA.org and TargetScan were used and identified a conserved miR-7-binding site in the 3'-UTR of Keap1 mRNA. We then cloned WT or Mut target region sequence of the Keap1 3'-UTR, which was inserted into a luciferase reporter vector (**Figure 2A**). Subsequently, these reporter vectors were cotransfected with miR-7 mimics and mimics control (mimics_con) into the HEK293 cell line. As shown in **Figure 2A**, co-transfection of miR-7 mimics suppressed the luciferase activity of the reporter containing wild-type Keap1 3' UTR sequence, but failed to inhibit that of mutated Keap1 by dual-luciferase reporter assay. These data indicate that Keap1 is one of the direct targets of miR-7.

Next, we transfected miR-7 inhibitors in hippocampal neurons cells. qRT-PCR and Western blot analysis revealed Keap1 expression were significantly increased by transfecting with miR-7 inhibitors, compared with negative control group (**Figure 2B** and **2C**). These results demonstrated that Keap1 is a direct target of miR-7 in hippocampal neurons cells.

MiR-7 regulates Nrf2 signaling pathway

Keap1 was reported to involve the regulation of the nuclear factor erythroid 2-related factor 2 (Nrf2) activity [14]. Activated Nrf2 translocates from the cytoplasm to the nucleus, and sequentially binds to ARE, which subsquently regulates the expression of a group of cytoprotective enzymes, such as heme oxygenase-1 (HO-1) and NAD(P)H: quinone oxidoreductase-1 (NQO1). These Nrf2-dependent gene products go on to protect the cells from oxidative or xenobiotic damage [11, 12]. Here, the expression of Nrf2, HO-1 and NQO1 mRNA in hippocampus was studied by real-time fluorescence quantitative PCR. As shown in **Figure 3**, Keap1 expression was reduced and Nrf2, HO-1 and NQO1

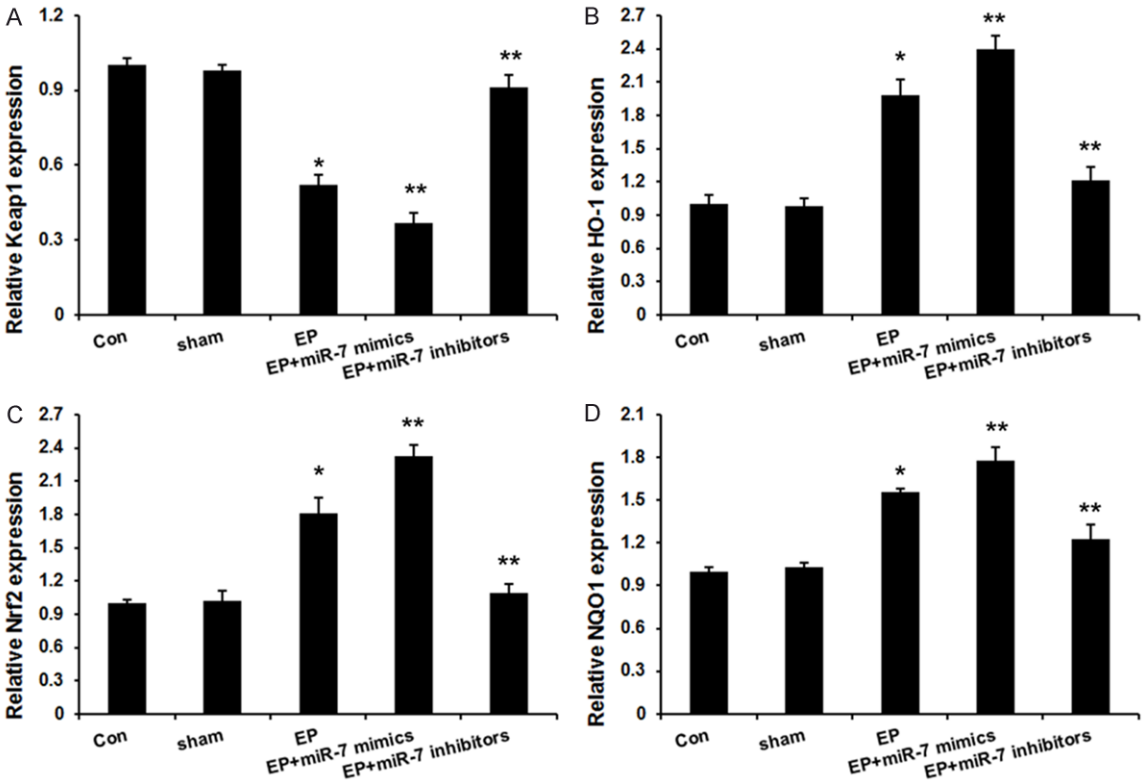

Figure 3. The graphs show Keap1 (A), HO-1 (B), Nrf2 (C), and NQO1 (D) mRNA levels in control, sham, kindling groups, EP + miR-7 mimics and EP + miR-7 inhibitors. Error bars represent ± S.E. and *, $P < 0.01$ versus control, and **, $P < 0.01$ versus EP group.

mRNA expression were increased in kindling groups, which was further induced by miR-7 mimics and reversed by miR-7 inhibitors.

Discussion

Epilepsy remains a major medical problem for which there is no effective treatment. Molecular signaling pathways that involves in epilepsy pathogenesis identify new therapeutic targets or biomarkers of intractable epilepsy. In this study, we report a higher level of miR-7 in the hippocampus of kindled rats compared with control and sham group. Moreover, Nrf2 and its two prominent transcriptional targets (HO-1 and GCLM) were significantly upregulated by miR-7 mimics and downregulated by miR-7 inhibitors, suggesting that miR-7 activates the Nrf2 pathway. In this study, we report the novel function of miR-7 involving protection from epileptic seizures via regulating the Nrf2 signaling pathway by targeting the Keap1 mRNA.

MicroRNA (miRNA), an abundant group of endogenous non-coding single strand RNAs with approximately 19~25 nucleotides, regulates the expression of genes at post-transcriptional level by translational repression or degradation of target mRNA. Previous genome-wide microRNA profiling study revealed that various miRNAs were deregulated in temporal neocortex of patients with medically intractable temporal lobe epilepsy compared with control subjects [15-17]. MiR-7 has been reported as tumor suppressors in various kinds of cancer [18-21]. Recently, Sun et al. revealed miR-7 as potential clinical biomarker for schizophrenia [22]. However, the role of miR-7 in epilepsy remains unclear. In our study, we found that the expression of miR-7 was up-regulated after seizure. Moreover, Keap1 expression was down-regulated after seizure and luciferase reporter assay revealed that Keap1 is a direct target of miR-7.

Oxidative stress has been reported as an underlying mechanism in the epilepsy pathogenesis, what's more, excessive oxidative stress contributes to neuronal degeneration in the epileptic focus [23]. Increases in reactive oxygen species occur in response to sustained neuronal electrical activity and seizures, indicating as the therapeutic strategies for the

treatment and modulation of epilepsy. Numerous studies have addressed a pivotal role of Nrf2-ARE signal pathway in protecting cells from oxidative stress [24]. Increasing evidence has demonstrated that the protective role of Nrf2-ARE signal pathway in central nervous disease is mediated by HO-1 and NQO1 [24, 25]. In normal physiological conditions, Nrf2 is mainly localized in the cytoplasm complexed with an inhibitory protein, Keap1, where Nrf2 is constantly polyubiquitinated by Cul3-containing E3 ubiquitin ligase complex and targeted for degradation via proteasome

Pathway [26]. In addition, miR-200a has been shown to target Keap1 mRNA in the human breast cancer cell lines, leading to increased Nrf2 activation [14, 27]. In this study, Keap1 expression was reduced and Nrf2, HO-1 and NQO1 mRNA expression were increased in kindling groups, which was further induced by miR-7 mimics and reversed by miR-7 inhibitors. These results indicated that miR-7 regulated Nrf2 signal pathway in the epilepsy pathogenesis.

In conclusion, our current study demonstrates a novel mechanism by which miR-7 exerts its protective effect against epileptic seizure-induced brain damage through targeting Keap1 mRNA and activating Nrf2 pathway.

Disclosure of conflict of interest

None.

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