European Review for Medical and Pharmacological Sciences 2018; 22: 6896-6904

Nrf2-ARE signaling pathway regulates the expressions of A1R and ENT1 in the brain of epileptic rats

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Abstract. – OBJECTIVE: To explore the behavioral changes and the expressions of the A1 receptor (A1R) and balanced nucleoside transporter-1 (ENT1) in the brain of epileptic rats after activating the NF-E2-related factor 2 (Nrf2)-ARE signaling pathway.

MATERIALS AND METHODS: Adult male Sprague-Dawley (SD) rats were randomly divided into normal control group, epilepsy group, and t-butylhydroquinone (tBHQ) group, with 10 rats in each group. Lithium-pilocarpine induced epilepsy model in rats was established. The first epileptic latency and seizure frequency within 1 hour were observed in each group using the Racine scoring system. HE (Hematoxylin and Eosin) staining was used to observe the pathological lesions in the brain tissue of each group. The expressions of A1R, ENT1, and relative genes in Nrf2-ARE pathway in rat hippocampus was detected by immunohistochemistry and Western blot.

RESULTS: Compared with rats in epileptic group, the first seizure latency was prolonged and the seizure frequency decreased in tBHQ group ($p<0.05$). The degree of brain lesions in tHBQ group was lighter than that of epilepsy group. ENT1 expression in rat hippocampus of epileptic group was significantly upregulated than that of normal control group and tBHQ group. Besides, the protein levels of A1R, Nrf2, HO-1, and ARE in rat hippocampus of epilepsy group markedly decreased compared with those of normal control group. However, protein expressions of A1R, Nrf2, HO-1, and ARE proteins in rat hippocampus of tBHQ group were markedly upregulated.

CONCLUSIONS: Activation of the Nrf2-ARE signaling pathway can reduce the pathological damage of rat hippocampal neurons, prolong the latency of seizures, and reduce the degree of epileptic seizure in rats.

Key Words:
Epilepsy, Nrf2-ARE, A1R, ENT1.

Introduction

Epilepsy is one of the most common diseases of the nervous system[1]. Due to its impact on the physical and mental health of affected patients, epilepsy has become a public health issue worthy of the whole society's attention[2,3]. However, the specific pathogenesis of epilepsy has not been fully elucidated. Therefore, investigating its pathogenic mechanism and designing the specific antiepileptic drugs have become the focus and hot topics in the field of neuroscience research. Recently, Matute and Cavaliere[4] summarized the previous researches and the latest developments, and proposed that adenosine-mediated glial interactions are pathogenic factors in epilepsy.

Adenosine is one of the important neuromodulators with endogenous antiepileptic effects in the central nervous system (CNS)[5,6]. Adenosine exerts brain protection and antiepileptic effects through its combination with various adenosine receptors[7]. At present, four adenosine receptors (ARs) have been studied and confirmed, namely A1 receptor (A1R), A2a receptor (A2aR), A2b receptor (A2bR), and A3 receptor (A3R). These ARs all belong to the G protein-coupled receptor family[8], which could bind to the corresponding G protein *in vivo* and act on adenylate cyclase and ion channels

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to exert their protection functions. A1Rs in the adenosine receptor family are widely present in CNS, especially in the cerebral cortex and hippocampus. As an important antiepileptic and brain protection system in CNS[9], the adenosine system exerts its biological functions mainly acting through the coupling of A1R and its corresponding G protein[10]. A1Rs can reduce the release of the excitatory amino acid Glu (glutamic acid) in the brain to reduce its excitatory neurotoxicity[11]. It has long been found that the balanced nucleoside transporter-1 (ENT1) is widely distributed in human brain tissues, including amygdala, caudate nucleus, hippocampus, subthalamic nucleus, and thalamus. Meanwhile, ENT1 is also widely expressed in hippocampal pyramidal cells and dentate gyrus granule cells of rat brain tissue, so as A1Rs[12]. ENT1 is also widely expressed in astrocytes[13]. The uptake of adenosine by astrocytes mainly depends on the nucleoside transporter[14]. Astrocytes are the main source of extracellular adenosine through exocytosis[15]. To regulate adenosine levels and affect the release of glutamate, astrocytes uptake glutamate in the synaptic cleft, thereby avoiding excessive excitation of neurons[16]. Relative researches have confirmed that glutamate uptake by astrocytes is mainly regulated by excitatory amino acid transporter 2 (EAAT2). ENT1 knockdown reduces uptake of amino acids by astrocytes through downregulating EAAT2[17]. Under basic conditions, inhibiting the function of ENT1 can lead to an increased level of extracellular adenosine. On the contrary, enhancing the activity of ENT1 can increase the translocation of adenosine into cells[18].

NF-E2-related factor 2 (Nrf2) is a key factor in the cellular regulation of oxidative stress. Some studies have found that Nrf2 mediates and activates the transcription of a variety of phase II detoxification enzymes and antioxidant genes through the Nrf2-ARE signaling pathway. Hence, Nrf2 is capable of reducing cellular damage caused by oxidative stress and maintaining the homeostasis of the oxidative-antioxidant system in the body. Reports have confirmed that activation of Nrf2-ARE signaling pathway can increase the expressions of total and nuclear Nrf2. The disruption of Nrf2-ARE signaling pathway increases the body's susceptibility to oxidative stress and toxic substances. Upregulated neuronal expressions of HO-1 or NQO1 can protect against oxidative stress and excitotoxicity effects. Previous investigations[19,20] have showed that activation of the Nrf2-ARE signaling pathway has strong neuroprotective effects on neurological diseases, including the Parkinson's disease, cerebral hemorrhage, cerebral infarction, and brain trauma. Wang et al[21] have indicated that activation of Nrf2-ARE signaling pathway improves oxidative stress and cognitive impairment caused by seizures in animal models.

These above results indicated that the A1R, ENT1, and Nrf2-ARE pathways all play important roles in the development of epilepsy. However, whether the Nrf2-ARE pathway could regulate A1R and ENT1 is less studied. Therefore, we first established the rat epilepsy model to activate the Nrf2-ARE pathway. We detected the expressions of A1R and ENT1 in the rat hippocampus and explored the role of Nrf2-ARE signaling pathway in the regulation of A1R and ENT1 in the brain tissue of epileptic rats.

Methods and Materials

Experimental Animals

Thirty healthy male Sprague-Dawley (SD) rats weighing approximately 180-220 g were housed in separate cages and maintained in an environment with constant temperature (24±2°C). Rats were given standard diets and purified drinking water. The Animal Ethics Committee of Lanzhou University Animal Center approved this study.

Establishment of a Rat Model of Epilepsy

Rats were randomly divided into 3 groups: normal control group, epileptic group, and tBHQ group, with 10 rats in each group. Rats in epileptic group and tBHQ group were intraperitoneally injected with lithium chloride (127 mg/kg), followed by intraperitoneal injection of atropine sulfate (1 mg/kg) 18-20 h later. After 30 min, rats were intraperitoneally injected with pilocarpine (50 mg/kg). Behavioral changes of the rats were observed and scored according to the Racine score[21]. Rats with grade IV-V seizures were included in the experiment while those did not reach grade IV-V seizures were additionally intraperitoneally injected with pilocarpine (10 mg/kg).

Rats in normal control group were administered with isodose saline instead of lithium chloride and pilocarpine. The behavioral changes in rats were also observed.

Rats in tBHQ group were fed with chow diet containing 1% tBHQ (w/w) on the third day before epilepsy was established. All other operations

and feeding conditions were consistent with epilepsy group. Behavioral changes were observed after the treatment.

HE (Hematoxylin and Eosin) Staining

After rats were sacrificed, the brain tissues were harvested and fixed in 10% neutral formaldehyde to prepare paraffin sections. Subsequently, paraffin-embedded brain tissue was dehydrated in gradient alcohol, stained with hematoxylin-eosin, sealed with neutral resin, and photographed. The images were collected under an optical microscope (Carl Zeiss, Jena, Germany).

Immunohistochemically Staining

After decapitation, rat brain tissue underwent gradient dehydration in 20% and 30% sucrose sequentially. Paraffin sections were prepared after dewaxing and gradient alcohol dehydration. A1R antibody and ENT1 antibody were added for immunohistochemical staining, and images were collected under an optical microscope.

Brain Tissue Immunofluorescence

Frozen sections of brain tissue were prepared, placed in 0.01 M sodium citrate solution and heated in a microwave oven. After cooling, the slices were placed in antigen-repair solution (EDTA) and incubated in a 37°C incubator for 20-30 minutes (Beyotime, Shanghai, China). Then, the slices were incubated with the A1R antibody and ENT1 antibody at 4°C overnight. Slices were incubated with secondary antibody in the dark on the next day. 50% glycerol was used as a backup and the images were collected under a laser confocal scanning microscope.

Western Blot

Different groups of rat brain tissue were taken and lysed by radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China). The brain homogenates were prepared using a homogenizer (all operations were performed on ice), and bicinchoninic acid (BCA; Pierce, Rockford, IL, USA) was used for protein quantitation. Then, electrophoresis was performed and the proteins were transferred to the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blockage, the antibody incubation was performed. Protein bands were detected by chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA). The software was used to statistically analyze the gray values of the proteins and the corresponding internal reference proteins.

Statistical Analysis

The data obtained were statistically analyzed using Statistical Product and Service Solutions (SPSS) version 18.0 (SPSS Inc., Chicago, IL, USA) and expressed as mean ± SD. Rat behavioral results (latency and episodes), immunohistochemistry, and Western blot results were analyzed by One-way ANOVA, followed by Post-Hoc Test (Least Significant Difference). Difference between groups was considered statistically significant at $p<0.05$.

Results

The First Seizure Latency and Seizure Frequency in Each Group of Rats

Compared with epilepsy group, the first seizure latency of seizures was prolonged in tBHQ group, and the number of epileptic seizures decreased as well (Figure 1A, 1B). Intraperitoneal injection of the Nrf2 activator tBHQ significantly prolonged the first seizure latency and reduced seizure frequency in epileptic rats.

Observation of Pathological Changes in Brain Tissue of Normal and Epileptic Rats by HE Staining

Hippocampal neurons in the normal rats were dense and well-arranged. In addition, neurons were abundant in cytoplasm and lightly stained while the nucleus was centered, and the nucleoli were clear (Figure 1C). However, hippocampal neurons in epileptic group were dark red with shrinking nucleus. The neurons showed expansion of intercellular spaces and structurally disordered. Part of the nerve cell lysis and nuclei lysis were also observed (Figure 1D). Although the hippocampus region also displayed part of the nerve cell degeneration and necrosis in tBHQ group, the degree was lighter than that of the epilepsy group (Figure 1E).

Immunohistochemistry and Immunofluorescence Detection of A1R Expression in the Hippocampus

Immunohistochemistry showed that in the hippocampus of rats, the A1Rs-positive cells were mainly nerve cells. The amount of AIRs-positive cells in epilepsy group were significantly reduced compared to normal control group. Meanwhile, the color of the positive stained cells was shallow (Figure 2A, 2B). Besides, the number of A1R-positive cells in tBHQ

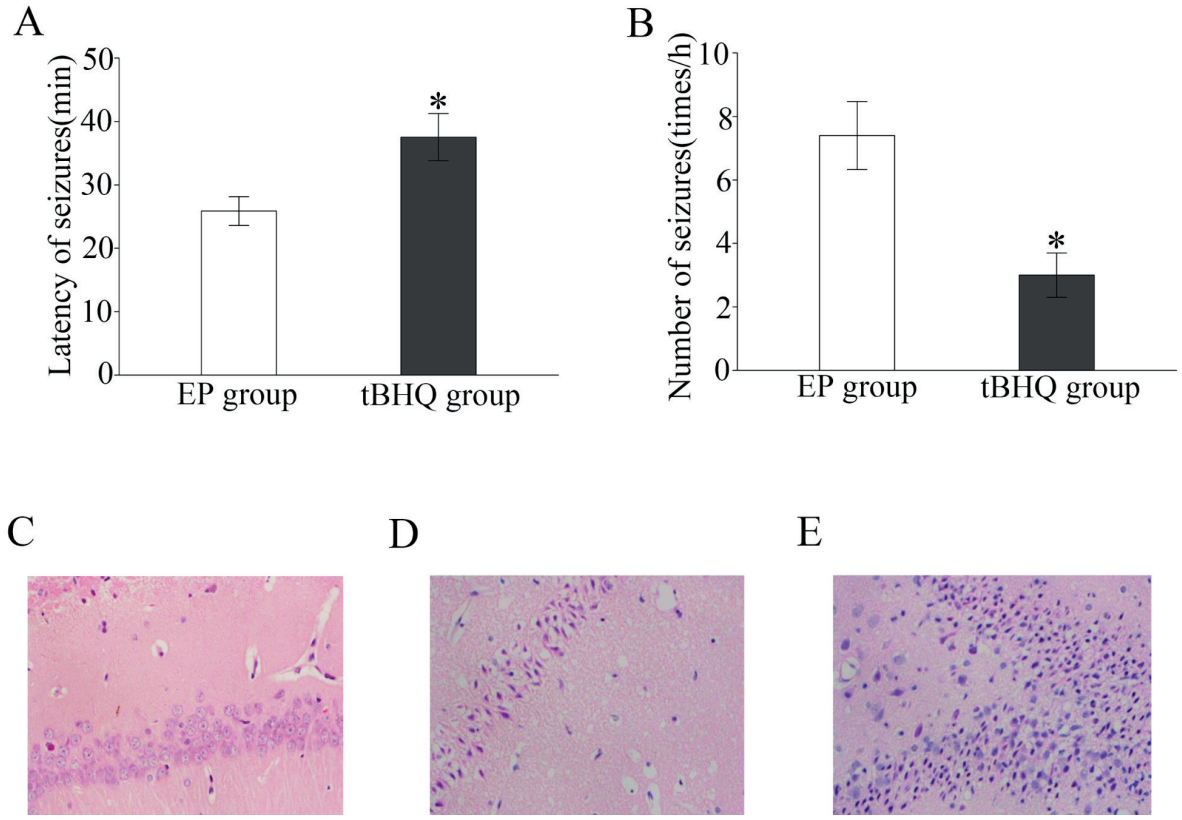

Figure 1. Pathological changes, first seizure latency, and seizure frequency of rats in each group. ***A,*** Comparison of the first seizure latency of rats in each group. ***B,*** Comparison of seizure frequency in each group. ***C,*** Pathological changes in hippocampus in each group. ***D,*** Pathological changes in hippocampus of epileptic rats. ***E,*** The pathological changes of hippocampus in tBHQ rats *: Significant difference compared with epilepsy group (p<0.05)

group increased compared with epilepsy group, while the coloring was deepened (Figure 2B, 2C). Immunofluorescence results also showed that A1R expression in epilepsy group decreased compared to normal control group (Figure 2D, 2E), while A1R expression in the tBHQ group increased (Figure 2F). In addition, the difference in the number of positive cells was statistically significant (p<0.05) (Figure 2G).

Immunohistochemical Detection of ENT1 Expression in the Hippocampus

Immunohistochemistry results showed that the ENT1-positive cells in epilepsy group significantly increased than the control group, as shown in Figure 3A and B. However, the number of ENT1-positive cells in tBHQ group decreased than normal control group (Figure 3B and 3C). Immunofluorescent labeling showed that ENT1 expression was upregulated in rat hippocampus of epileptic group (Figure 3D, 3E), while ENT1 expression was lower in tBHO group than epileptic group (Figure 3F). Besides, the difference in the number of positive cells was statistically significant (p<0.05) (Figure 3G)

Western Blotting Detection of Protein Expression

The expressions of A1R and ENT1 in rat hippocampus of normal control group were detected (Figure 4A). However, A1R expression was significantly downregulated and ENT1 expression was upregulated in epileptic and tBHQ groups. In tBHQ group, A1R expression was markedly increased than that in epilepsy group, whereas ETN1 expression was downregulated (Figure 4A, 4B).

The protein expressions of Nrf2, HO-1, and ARE in hippocampus of epileptic rats were significantly downregulated compared with normal control group (p<0.05). After tBHQ treatment, protein expressions of Nrf2, HO-1, and ARE in the hippocampus of epileptic rats were significantly upregulated (Figure 4C, 4D).

Discussion

Epilepsy is one of the most common diseases of the nervous system. It is a clinical syndrome in which high-synchronized abnormal discharges of brain neurons are caused by multiple causes[22]. It is characterized by episodic, transient, repetitive, and stereotypic clinical features. It is estimated that epilepsy accounts for 0.75% of the global disease burden, and most of epilepsy cases are in low- and middle-income countries. Epilepsy patients generally need lifelong treatment. Antiepileptic drugs are the most commonly applied in the epilepsy treatment[23,24]. The currently used antiepileptic drugs mainly suppress epileptic seizures by inhibiting nerve hyperactivity. However, about 30% of epilepsy patients still cannot control the recurrence of epilepsy even after taking antiepileptic drugs. Therefore, it is still very important to study new treatment options for patients with refractory epilepsy[25-27].

Adenosine exists in all cells and is involved in controlling the function of each tissue and organ. The elevation of adenosine levels plays an important role in preventing cell damage and preventing organ dysfunction. Adenosine also plays an

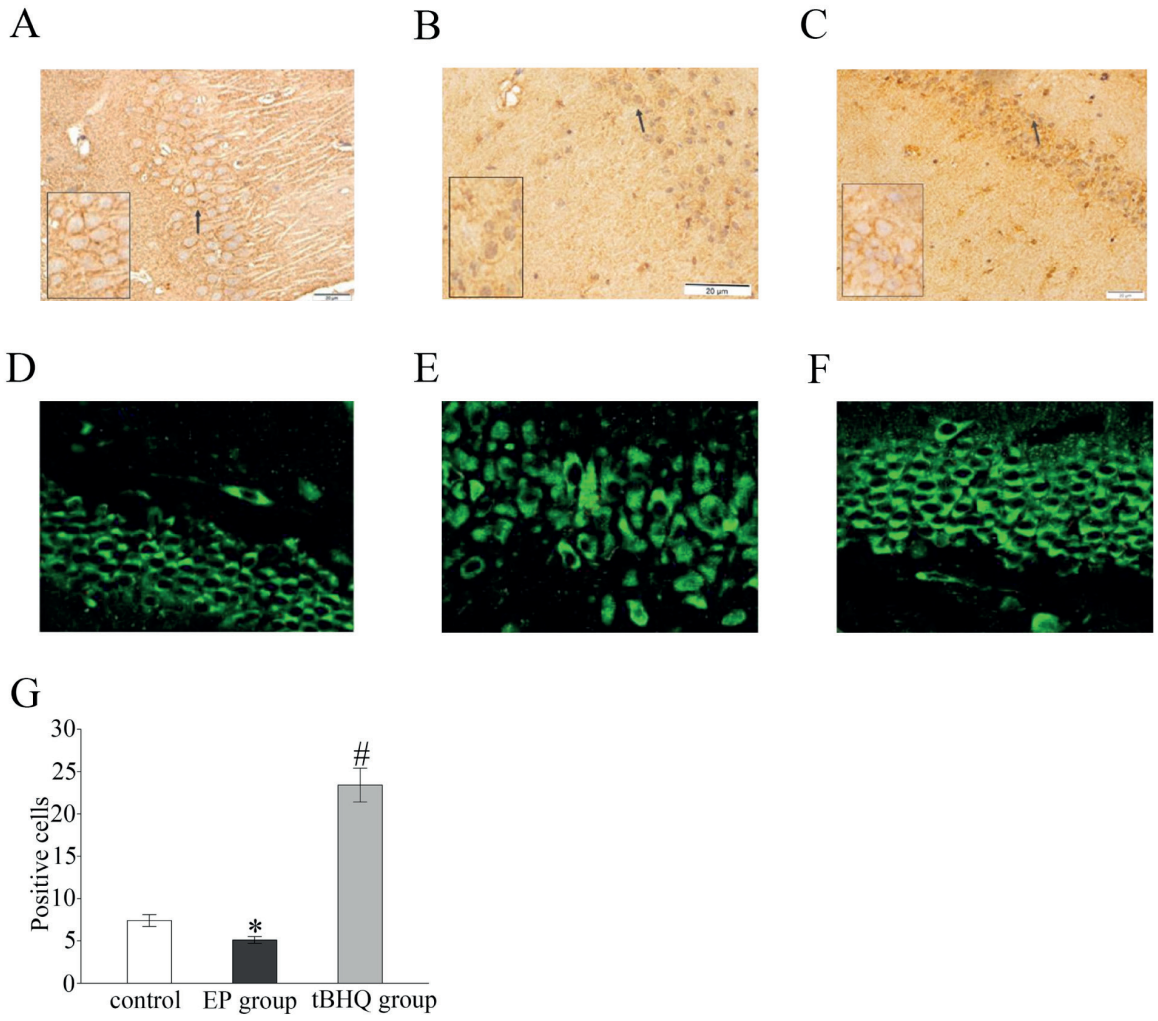

Figure 2. The results of A1R immunohistochemistry in hippocampus of rats in each group (magnification 200×). ***A,*** Immunohistochemical detection of A1R expression in hippocampus of control rats. ***B,*** Immunohistochemical detection of A1R expression in hippocampus of epileptic rats. ***C,*** Immunohistochemical detection of A1R expression in hippocampus of tBHQ rats. ***D,*** Detection of A1R in hippocampal tissue of control rats by Immunofluorescence labeling. ***E,*** Detection of A1R in hippocampus of epileptic rats by immunofluorescence labeling. ***F,*** Detection of A1R in hippocampal tissue of tBHQ rats by immunofluorescence labeling. ***G,*** A comparison of A1R positive cells in hippocampus of rats in each group. *: Compared with normal control group, the difference was statistically significant ($p<0.05$); #: Compared with epilepsy group, the difference was statistically significant ($p<0.05$).

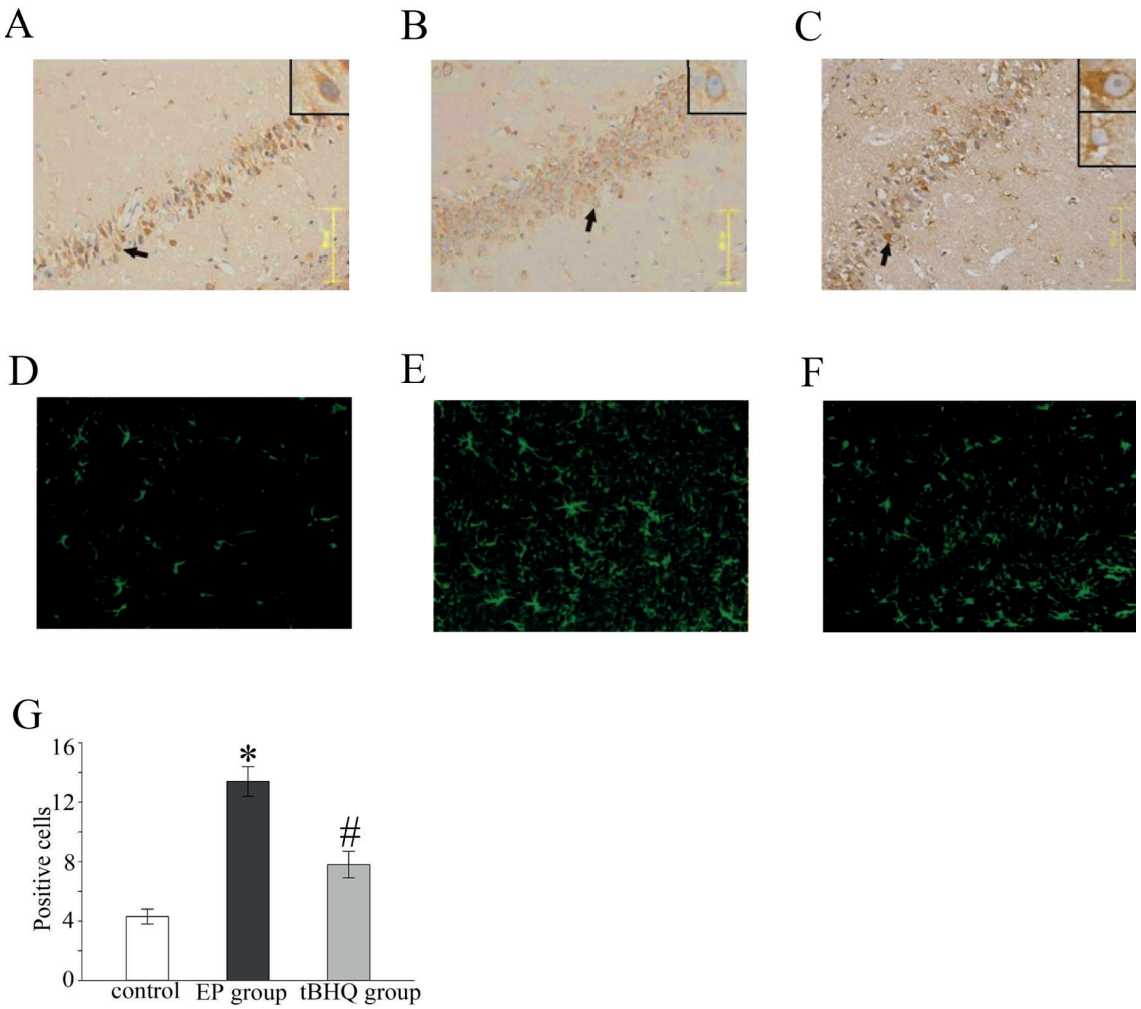

Figure 3. ENT1 immunohistochemistry results in hippocampus of rats in each group (magnification 200×). A, Immunohistochemical detection of ENT1 expression in hippocampus of control rats. B, Immunohistochemical detection of ENT1 expression in hippocampus of epileptic rats. C, Immunohistochemical detection of ENT1 expression in hippocampus of tBHQ rats. D, Immunofluorescence labeling to detect the expression of ENT1 in hippocampus of control rats. E, Fluorescent labeling for detection of ENT1 expression in hippocampus of epileptic rats. F, Detection of ENT1 expression in hippocampus of tBHQ rats by immunofluorescence labeling. G, Comparison of the number of ENT1 positive cells in hippocampus of rats in each group. *: Compared with normal control group, the difference was statistically significant ($p<0.05$); #: Compared with epilepsy group, the difference was statistically significant ($p<0.05$).

important role in the regulation of physiological processes, such as managing sleep and wakefulness states, affecting the release of postsynaptic receptors involved in neurotransmitters (such as glutamate, acetylcholine, dethyroxine, serotonin, dopamine, etc.)[28]. The adenosine system has an inhibitory effect on the brain, so adenosine has been considered as an endogenous anticonvulsant[29]. The anticonvulsant effect of adenosine is mainly mediated by two high-affinity receptors, A1R and adenosine A2a receptor[30]. A1Rs prevent the release of glutamate by inhibiting the activation of voltage-gated Ca^{2+} channels and K^{+} channels, and prevent neuronal depolarization[31]. The adenosine A2a receptor is mainly expressed in the thalamus and can promote excitatory synaptic transmission, counteracting the effect of A1Rs in inhibiting glutamate release[32]. The adenosine system is therefore considered an "endogenous antiepileptic system"[33].

We observed changes of A1Rs in rat epilepsy model. The results found that A1R expression was upregulated after successful modeling. Immunohistochemistry showed that compared with normal control group, A1R-positive cells significantly increased and a large number of nerve

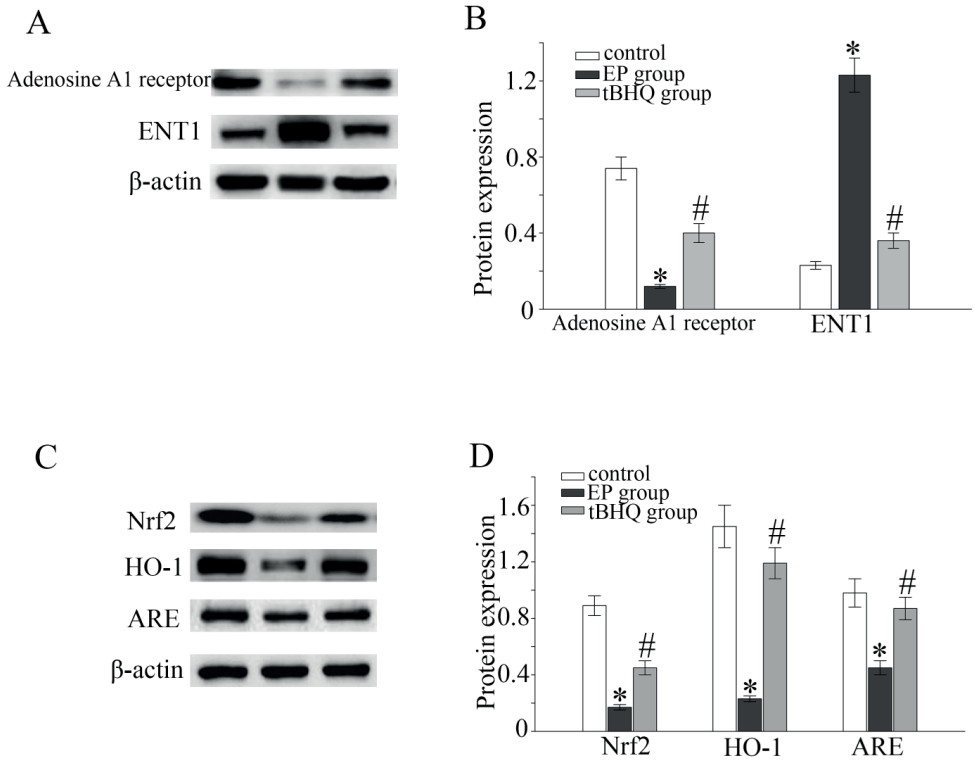

Figure 4. Protein expression in hippocampus of rats in each group. ***A,*** Expressions of A1R and ENT1 in hippocampus of rats in each group. ***B,*** Comparison of A1R and ENT1 expression levels in hippocampus of rats in each group. ***C,*** Expressions of Nrf2, HO-1 and ARE in hippocampus of rats in each group. ***D,*** Comparison of expression levels of Nrf2, HO-1, and ARE in hippocampus of rats in each group of D. *: Compared with normal control group, the difference was statistically significant ($p<0.05$); #: Compared with epilepsy group, the difference was statistically significant ($p<0.05$).

cells underwent apoptosis and necrosis in epilepsy group. In the normal brain, homeostasis of A1R and adenosine A2a receptor maintains the inhibitory effect of the adenosine system. Other scholars found that the expressions of A1Rs were significantly upregulated after 24 hours of epilepsy. They pointed out that A1R expression was significantly upregulated as an adaptive response after an acute attack. Some reports have pointed out that the decreased expressions of A1Rs reflect the degeneration of neurons and increased levels of extracellular adenosine and A1Rs.

Nucleoside transporters play an important role in sleep-wakefulness, drug and alcohol addiction, nociception, and analgesia. In addition, there have been reports[34,35] on the use of inhibitors of nucleoside transporters in tumors and cardiovascular diseases. In mammals, nucleoside transporters have two major classes of equilibrium and concentration types, and they are composed of SLC29A and SLC28A, respectively. The balanced nucleoside transporters encoded by the SLC29A family can be divided into ENT1, ENT2, ENT3, and ENT4[36]. Both ENT1 and ENT2 can regulate the transfer of adenosine from intracellular to extracellular or from extracellular to intracellular. The final direction of transfer entirely depends on intracellular and extracellular adenosine concentrations. This work found that the protein content of ENT1 significantly increased in epileptic rats. According to other researchers[37], mRNA level of ENT1 was expressed in pyramidal neurons and dentate gyrus granule cells in the hippocampal CA1 region of the adult human brain.

NF-E2-related factor 2 (NF-E2-related factor 2) is an important member of the transcription factor leucine zipper transcriptional activator (CNC) and plays an important role in cellular defense and other stress injuries[38]. Nrf2 is an essential regulator of the induction of phase II detoxification gene expression. We found that the expressions of relative genes in Nrf2-ARE pathway in the brain tissue of epileptic rats were downregulated. After activation of Nrf2-ARE pathway by activators,

the pathological changes in the brain of epileptic rats were reduced, and the expression of adenosine receptor A1 increased, while the expression of ENT1 was downregulated. The Nrf2-ARE pathway may be involved in the regulation of A1R and ENT1 in the course of epilepsy. Therefore, the Nrf2-ARE pathway may serve as a new potential antiepileptic target. In other words, its selective activator can be used as a potential new antiepileptic drug. Our study provides novel directions for developing antiepileptic drugs, so as to improve the clinical outcomes of epilepsy patients.

Conclusions

We showed that the activation of the Nrf2-ARE signaling pathway can reduce the pathological damage of rat hippocampal neurons, prolong the latency of seizures, and reduce the degree of epileptic seizure in rats. The role of the Nrf2-ARE pathway in alleviating epilepsy is related to the increased A1R expression and decreased ENT1 expression.

Funding Acknowledgements

The National Natural Science Foundation of China (81501116), and Cuiying Scientific and Technological Innovation Program of Lanzhou University Second Hospital (CY2017-MS04).

Conflict of Interest

The Authors declare that they have no conflict of interest.

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