Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Anti-epileptic effect of 16-O-acetyldigitoxigenin via suppressing mTOR signaling pathway

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Received February 27, 2019; **Accepted** June 19, 2019; **Published** June 30, 2019

Doi: http://dx.doi.org/10.14715/cmb/2019.65.5.10

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Abstract: Epilepsy is a common chronic disease of the central nervous system that can last for years or even decades, causing serious adverse effects on the body, mind, and psychology of patients. Traditional antiepileptic drugs can effectively control seizures, but because of large individual differences, serious adverse reactions, narrow therapeutic window and other shortcomings, more effective, new treatment drugs are looked for. Streptocaulon griffithii is a plant of Asclepiadaceae. 16-O-acetyldigitoxigenin (ACE) is a strong cardiac glycoside isolated from methanol extract of Streptocaulon griffithii. The aim of this study was to investigate the antiepileptic effect of ACE on Pilocarpine (Pilo) induced epilepsy in mice, and to explore the effect of mTOR signaling pathway on its antiepileptic effect. The results showed that ACE had antiepileptic and neuroprotective effects on Pilo induced epilepsy mice. ACE attenuates Pilo induced seizures by inhibiting the activation of p-mTOR/p-70S6K pathway, and inhibits Pilocarpine induced brain damage by inhibiting mTOR signaling pathway. These results suggest that ACE has a promising future in the treatment of epilepsy and other nervous system diseases.

Key words: Streptocaulon juventas; Anti-epileptic; 16-O-acetyldigitoxigenin; mTOR.

Introduction

Epilepsy is a kind of brain dysfunction caused by the specific discharge of neurons in the brain and there are conscious, motor, sensory, mental or autonomic functional disorders in clinic. It can be divided into big attack, small attack and local attack (1). The pathogenesis of epilepsy is complex, and has not been fully elucidated so far. But the imbalance of excitatory and inhibitory neurotransmitters, gene mutation of ion channel, abnormal function of glial cells, abnormal synaptic connection, heredity caused genetic mutation, chromosomal abnormality and mitochondrial mutations are closely related to seizures (2-5). Neurophysiology between epilepsy and emotional disorders is receiving increasing attention. The current medical treatment level cannot completely cure the disease, mostly to control. 70% to 80% of the patients take chemical drugs can effectively control epileptic seizures, 20% to 30% of patients will evolve into refractory epilepsy. The disease serious harm to the patient's health and has a great burden on the family and society (6).

Common drugs for epilepsy include carbamazepine, phenobarbital and so on. However, with the prolongation of antiepileptic drug use, not only the effective rate of clinical treatment will decrease, but also many adverse reactions will appear in patients, which will eventually lead to a decrease in compliance. In view of this, it is urgent to find more effective and new therapeutic drugs in order to improve the clinical curative effect. Therefore, the analysis of herbaceous plant extracts and their antiepileptic components not only provides scientific proof for the development of traditional Chinese medicine, but also provides a reference for the clinical study of epilepsy (7). Nowadays, Chinese medicine has obtained good curative effect on epilepsy, such as Dingxian Pill, Zhixian Pill, Shenpu decoction and so on, which have proved good antiepileptic effect in laboratory and clinic (8,9).

Streptocaulon griffithii (Streptocaulon juventas) is the dry root of Morian saddle of Asclepiadaceae, also known as south Radix Sophorae Flavescentis, Streptocaulon griffithii Hook, Rhizoma huyinensis, ground Radix Sophorae Flavescentis, Xiaoanxiao and so on (10). Streptocaulon griffithii is mainly used in traditional medicine to treat dysentery, damp-heat diarrhea, heart-stomach-qi pain, cold and fever, falling down, venomous snake bite and sores (11). Modern pharmacological studies show that Streptocaulon griffithii also has anti-tumor effect (12). The composition of Streptocaulon griffithii is complex, and contains various components such as cardiac glycoside, Terpene, alkaloid, lignan and phenolic acid, among which cardiac glycoside is the main component. In recent years, the study of the chemical constituents of the cardiac glycoside in the root of Streptocaulon griffithii is more and more in recent years. 16-O-acetyldigitoxigenin (ACE, Fig.1) is a strong cardiac glycoside isolated from methanol extract of Streptocaulon griffithii (13). The aglycone has the same parent structure, and the sugar group is composed of a single or plurality of monosaccharide groups. The aim of this study was to investigate the antiepileptic effect of ACE on pilocarpine (Pilo) induced epilepsy in mice, and to explore the effect of mTOR signaling

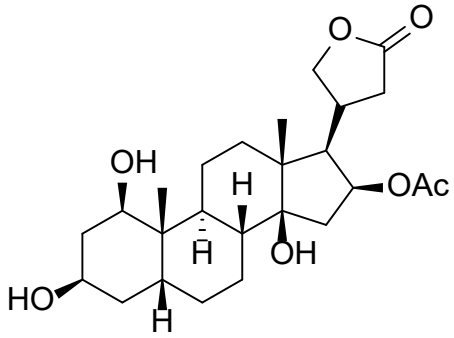

Figure 1. The structure of 16-*O*-acetyldigitoxigenin.

pathway on its antiepileptic effect. The study provide theoretical basis for the development of new safe epilepsy treatment drugs, and provide new ideas for clinical treatment of epilepsy.

Materials and Methods

Materials and reagents

16-O-acetyldigitoxigenin (presented by Yin Jun, School of traditional Chinese Medicine, Shenyang Pharmaceutical University); Pilocarpine (J&K Scientific, China); Leucine (Macklin Biochemical Technology Co., Ltd, China); Caspase-3 Activity Assay Kit (Cell Signaling Technology, USA); Pierce TM BCA protein assay kit (Thermo Fisher Scientific, USA); Polyvinylidene fluoride membrane (Millipore, USA); Trizol reagent (Invitrogen, USA); ELISA reagent (ScienCell, USA); TUNEL kit (Roche, Germany); Rabbit monoclonal antibody against Caspase-3, IL-1β and TNF-α antibody (Santa Cruz, CA, USA); Goat anti rabbit IgG (horseradish peroxidase labeled) (Proteinech Group, USA); PVDF membrane and ECL luminescence kit (Abcam, UK); Other reagents are imported or domestic analytical pure.

Experimental animal

Healthy ICR male mice (Experimental Animal Research Center of Wuhan University, license No. 201803007) were with body weight of 20 to 25 g. The mice were fed in separate cages and placed in a quiet, warm and humidity, well-ventilated environment, drinking water freely and feeding reasonably. The experiment began after 1 week of adaptation to the environment.

Experimental grouping and preparation of epileptic model induced by Pilo

Male adult ICR mice weighing 20 to 25 g were randomly divided into three groups: pilocarpine (Pilo, n = 30) induced epilepsy model group, drug group treated with ACE (Pilo ACE, n = 30). Epileptic mice treated with ACE and leucine (Pilo + ACE + leucine, n = 6).

Mice were induced epilepsy 13 times by peritoneal injection of 60mg/kg Pilo every other day. Daily intraperitoneal injection of 1.8mg/kg ACE was used to treat Pilo induced epilepsy for four weeks. In order to study the role of the mTOR pathway in the protective effect of ACE on epilepsy, 1 mg/kg of leucine (a mTOR agonist) was injected intraperitoneally. The severity, duration and latency of seizures were recorded 60 minutes after drug treatment.

Determination of Caspase-3 activity

The hippocampal tissue was collected and the activity of Caspase-3 was measured according to the steps of Caspase-3 assay kit .

Culture of the hippocampal neurons

Within 24 hours, the mouse brain was cut off in the ice, the hippocampus was isolated and washed with DMEM/F12 medium. After trypsin digestion, the hippocampal samples were filtered by 200 mesh sieve. After the supernatant was removed, the hippocampal neurons were resuspended in DMEM/F12 medium, inoculated in 24-well plate and cultured with 5% CO_2 at 37 ℃. The culture medium was replaced every three days and the morphology of the cells was observed daily. ACE (2 mmol/L) was preincubated with neurons for 2 hours before being treated with Pilo (0.5mmol/L). The expression of p-mTOR and p-70S6 K protein was detected by western blotting assay after treatment with Pilo for 6 hours. After 12 hours, the IL-1 and TNF-1 mRNA levels and concentration were measured by real-time PCR and ELISA kits, and TUNEL assay was performed after 24 hours to determine neuronal apoptosis. The concentrations of leucine and rapamycin were both 0.2mmol / L at the subsequent experiments.

Detection of protein in hippocampal tissue in mice with Pilo induced epilepsy by Western blot

After washing with ice PBS buffer, hippocampal neurons were obtained and RIPA cell lysate was used for lysis. Pierce TM BCA protein assay kit was used to detect the protein concentration of cell lysates. The protein in each sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. After being sealed with 5%BSA for 1 hour at room temperature, the specificity of p-mTOR, mTOR, p-70S6K and 70S6K was added to incubate overnight in a shaking bed at 4 ℃. The membrane was washed with TBST (pH 7.6) for 3 times with each time of 5 min, followed by the addition of horseradish peroxidase (HRP)-labeled second antibody and incubated at room temperature for 2 h. The enhanced chemiluminescence (ECL) kit was used to detect chemiluminescence. All the experiments were repeated three times.

Real-time PCR Detection of IL-1β and TNF-α expression in hippocampus of Pilo induced Epilepsy mice

Trizol reagent was used for isolation of total RNA from hippocampal tissues or neurons. The concentration and purity of total RNA were measured by UV spectrophotometer and oligo dT primer was used to reverse to cDNA. Real-time PCR was performed with the target gene of IL-1 and TNF-1, and the expression of IL-1 and TNF-1 was detected using specific primers from the ABI PRISM 7900HT sequence detection system. All primers were synthesized and purified by Sangon Biotech (Shanghai, China). The experiment was repeated three times.

Determination of IL-1β and TNF-α in hippocampus of Pilo induced Epilepsy mice by ELISA

According to the steps of ELISA kit, the absorptivity

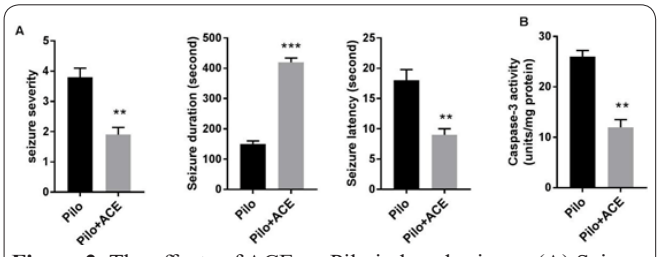

Figure 2. The effects of ACE on Pilo induced seizure. (A) Seizure severity, latency and duration were determined by the treatment of ACE. (B) The caspase -3 activity of hippocampus was measured after ACE treatment. n = 6. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Pilo induced mice.

of IL-1b and TNF-α was measured at the wavelength of 450nm and 630nm by the enzyme standard instrument, and the secretion of IL-1b and TNF-α was calculated. The experiment was repeated three times.

TUNEL analysis

Routine paraffin sections were performed to prepare samples for analysis of apoptosis. The apoptosis of hippocampal neurons was measured according to the steps of TUNEL kit.

Statistical method

All the data of this study are statistically analyzed by GraphpadPrism7.0 statistical software. The measurement data were expressed as mean ± standard deviation (x ± s), and single factor analysis of variance (ANOVA) was used for comparison. Student-Newman-Keuls test (SNK) was used for post-comparison. P<0.05 indicated that the difference was statistically significant.

Results

Pilo induced epileptic seizure

The epileptic mouse model was successfully established by injection of Pilo. The severity and duration of epileptic seizures in mice treated with ACE decreased significantly while the latency of epileptic seizures increased significantly (Fig. 2A). Hippocampal bodies were isolated from mice. The results showed that caspase-3 activity was significantly decreased after ACE treatment with dosage of 1.8mg/kg (Fig. 2B).

ACE down-regulates the expression of p-mTOR and p-70S6K in hippocampal neurons

The effect of ACE on the expression of p-70S6KG, p-mTOR, IL-1β and TNF-α in vitro was studied. According to the results in vivo, Pilo induced the increase of p-70S6KG, p-mTOR, IL-1β and TNF-α, but the expression of IL-1β and TNF-α decreased after ACE treatment (Fig. 3A). The apoptosis rate of mouse hippocampal neurons induced by Pilo was also decreased (Fig. 3B).

mTOR agonist inhibits the protective effect of ACE on hippocampal neurons

Hippocampal neurons were precultured with leucine and ACE for 2 hours, then treated with Pilo. As shown in figure 4A, ACE significantly reduced the expression of IL-1β and TNF-α mRNA, as well as the concentration of IL-1β and TNF-α in Pilo induced hippocampal neurons. The expression of IL-1β and TNF-α mRNA

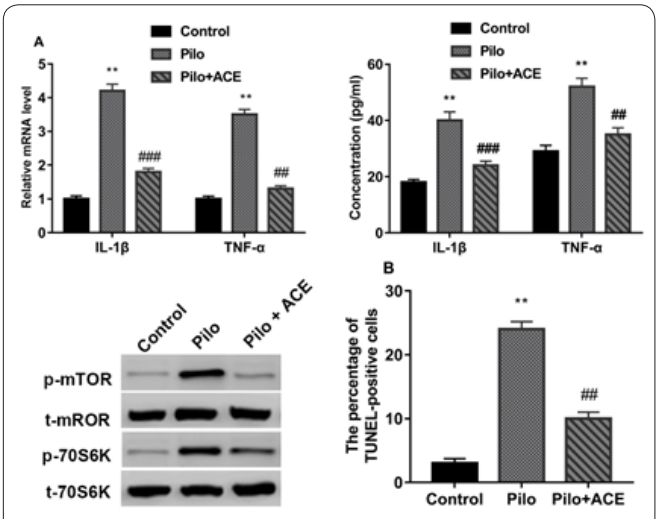

Figure 3. The effects of ACE on p-mTOR, p-70S6 K, IL-1β, and TNF-α expression and cell apoptosis in hippocampal neurons exposed to PTZ. (A) The protein expression levels of p-mTOR/T-mTOR and p-70S6 K/T-mTOR, relative mRNA expression levels of IL-1β and TNF- α, and IL-1β and TNF- α concentrations were measured in Pilo induced hippocampal neurons with or without the pre-incubation of ACE. (B) The neurons apoptosis was also detected. Each experiment repeated three times. $^{**}P < 0.01$, vs. control. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, vs. Pilo.

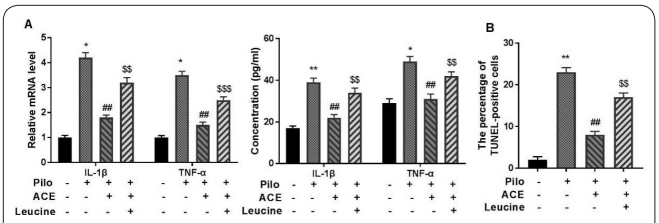

Figure 4. The effects of mTOR agonist leucine on IL-1β, and TNF-α expression and cell apoptosis in Pilo induced hippocampal neurons with the treatment ACE. (A) The relative mRNA expression levels of IL-1βand TNF-α and IL-1βand TNF-α concentrations were measured in hippocampal neurons with different treatments. (B) The neurons apoptosis was also detected. Each experiment repeated three times. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. control. $^{\#\#}P < 0.01$, vs. Pilo, $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, vs. Pilo+ ACE.

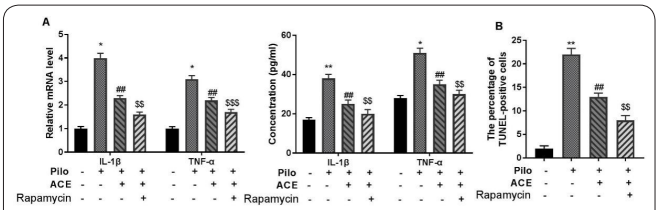

Figure 5. The effects of mTOR inhibitor rapamycin on IL-1β, and TNF-α expression and cell apoptosis in Pilo induced hippocampal neurons. (A) The relative mRNA expression levels of IL-1β and TNF- α and IL-1β and TNF- a concentrations were measured in hippocampal neurons with different treatments. (B) The neurons apoptosis was also detected. Each experiment repeated three times. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. control. $^{\#\#}P < 0.01$, vs. Pilo, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$, vs. Pilo+ ACE.

and their total concentration in hippocampal neurons were increased by leucine treatment, and the protective effect of ACE on Pilo induced apoptosis was eliminated by leucine incubation (Fig. 4B).

mTOR inhibitor protects hippocampal neurons from Pilo induced damage

Hippocampal neurons were treated with rapamy-

cin, a mTOR inhibitor, for 2 hours. The expression of IL-1β and TNF-α and apoptosis were evaluated. ACE played the same role as rapamycin, and decreased the expression of IL-1β and TNF-α mRNA in hippocampal neurons induced by Pilo (Fig. 5A). Meanwhile, Pilo induced neuronal apoptosis was also inhibited (Fig. 5B).

Discussion

Epilepsy is a chronic central system disease caused by repeated and abnormal discharges of brain neurons, which is characterized by transient brain dysfunction. Repeated abnormal discharges of epileptic neurons are closely associated with abnormal neurotransmitters, ion channels, genetic and immune. The pathogenesis of epilepsy is complex and has not been fully elucidated so far. Streptocaulon griffithii is a plant of Asclepiadaceae. 16-O-acetyldigitoxigenin (ACE) is a strong cardiac glycoside isolated from methanol extract of Streptocaulon griffithii. The aim of this study was to investigate the antiepileptic effect of ACE on Pilocarpine (Pilo) induced epilepsy in mice, and to explore the effect of mTOR signaling pathway on its antiepileptic effect. Pilocarpine (Pilo) is a M type acetylcholine receptor agonist and its mechanism of inducing epilepsy is achieved by stimulating M receptor (14). Pilo-induced epilepsy models have been widely used in the research of epilepsy pathogenesis and antiepileptic drugs (15). In this experiment the acute seizures were induced by intraperitoneal injection of pilocarpine (Pilo). The severity and duration of epileptic seizures in mice treated with ACE remarkably decreased and the latency of epileptic seizures increased significantly. Hippocampal bodies were isolated from mice and the results showed that caspase-3 activity was also decreased after ACE treatment.

MTOR is a serine / threonine protein kinase involved in many physiological processes and diseases, including cell growth, apoptosis, autophagy, protein synthesis and mRNA transcription (16, 17). P70S6K is the downstream kinase of PIP3 and phosphoinositol-dependent kinase-1 in the PI3 kinase pathway (18). The mTOR signaling pathway affects multiple neurological disorders and brain damage, such as Alzheimer's disease, Parkinson's disease, epilepsy, stroke and trauma (19-21), and is an important component of the cell. It is reported that the mTOR signaling pathway is activated in various epileptic models. The mTOR signaling pathway inhibitors improve the histological structural change induced by epilepsy, including abnormal neuronal enlargement and hippocampal neuron death in hereditary epilepsy, sprouting of mossy fibers in regenerated and acquired epilepsy (22). In the present study, we also observed that the expression of p-mTOR and p-70S6K in hippocampus of epileptic mice induced by Pilo was up-regulated at protein level. There is growing evidence that over-activation of the mTOR pathway leads to neuronal hyperstimulation and is involved in seizures and epileptogenesis (23, 24). The phosphorylation levels of mTOR and 70S6K proteins in ACE treated epilepsy mice were significantly decreased suggesting that the mTOR pathway might be involved in the antiepileptic effect of ACE. The cytokines IL-1β and TNF-α were mainly produced by glial cells and a small part by neurons, which were overexpressed in the experimental model of epileptic seizures (25). Disorders of IL-1β and TNF-α are usually associated with the development of autoimmune diseases. IL-1β and TNF-α may be involved in the pathophysiological process of epilepsy by inhibiting the activity of glutamine synthetase, enhancing the activity of excitatory amino acids and attenuating the abnormal epileptic discharge induced by inhibitory postsynaptic potential. It was found that mTOR inhibitor decreased the expression of these cytokines in hippocampal neurons induced by Pilo. ACE treatment also contributes to down-regulation of cytokine expression, which is reversed by mTOR agonists. These data suggest that ACE inhibits brain damage induced by Pilo through inhibiting the mTOR signaling pathway.

In conclusion, our experiment proved that ACE has antiepileptic and neuroprotective effects on Pilo induced epilepsy mice. ACE attenuates the epileptic seizure induced by Pilo through inhibiting the activation of p-mTOR/p-70S6K signaling pathway and mediates the expression of inflammatory cytokines. These results suggest that ACE has a promising future in the treatment of epilepsy and other neurological diseases. More knowledge of its molecular mechanisms needs to be addressed in future research.

Acknowledgements
None.

Conflict of Interest
There are no conflict of interest in this study.

Author's contribution
All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its content. The study was conceived and designed by Wenjing Tu; Wenjing Tu and Sheng Qian collected and analysed the data; Wenjing Tu wrote the text and all authors have read and approved the text prior to publication.

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