Original Article
Influence of magnolol on GAD67 and GAD65 in the hippocampus of rats with epilepsy-depression comorbidity

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Abstract: Objective: The aim of this study was to investigate the protective effects of magnolol on model rats with epilepsy-depression comorbidity, examining its influences on expression of glutamate decarboxylase 67 (GAD67) and GAD65 in the hippocampus of rats. Methods: Lithium chloride-pilocarpine was used to establish the rat model of epilepsy-depression comorbidity. At 6 weeks after modeling, the rats were randomly divided into 6 groups: control group, model group, citalopram group, and high- (H), medium- (M), and low-dose (L) magnolol groups. The drug was intragastrically administrated for 4 consecutive weeks, once a day. Depression behaviors were monitored using forced swimming tests, tail suspension tests, and saccharin preference tests. A 24-hour video surveillance system was used to monitor the times of epileptic seizures in rats. Expression of GAD67 and GAD65 messenger ribonucleic acid (mRNA) in the rat hippocampus was detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Expression of GAD67 and GAD65 proteins in the rat hippocampus was detected via Western blotting. Results: Compared with the control group, the cumulative immobility time in forced swimming tests in the model group was significantly prolonged ($P < 0.01$), tail-suspension immobility time was significantly increased ($P < 0.01$), consumption of sugar water was significantly reduced ($P < 0.01$), and times of epileptic seizure were significantly increased ($P < 0.01$). Compared with the model group, the cumulative immobility time in forced swimming tests in the citalopram group and magnolol-H, magnolol-M, and magnolol-L groups was significantly shortened ($P < 0.01$). Tail-suspension immobility time was significantly decreased ($P < 0.01$), consumption of sugar water was significantly increased ($P < 0.01$), and times of epileptic seizure were significantly decreased ($P < 0.01$). Results of qRT-PCR and Western blotting showed that expression of GAD67 and GAD65 mRNAs and proteins in the hippocampus in the model group was significantly lower than in the blank control group. Compared with the model group, expression of GAD67 and GAD65 mRNAs and proteins in the citalopram group and magnolol-H, magnolol-M, and magnolol-L groups was significantly upregulated. Conclusion: Expression of GAD67 and GAD65 is involved in the pathogenesis of epilepsy-depression comorbidity. Magnolol can upregulate expression of GAD67 and GAD65 in the hippocampus of rats with epilepsy-depression comorbidity, reduce the times of epileptic seizures, and improve depression behavior.

Keywords: Magnolol, epilepsy-depression comorbidity, GAD67, GAD65

Introduction

Epilepsy is a chronic recurrent transient brain dysfunction syndrome. It is characterized by paroxysmal loss of consciousness caused by abnormal discharge of cerebral neuron [1]. Clinical studies have shown that epilepsy has a close relationship with depression. Incidence rates of depression in patients with epilepsy are up to 30%. The quality of life of patients will be seriously affected. Suicidal tendencies may be caused when suffering from depression [2, 3].

Epilepsy will occur when the excitatory-inhibitory balance in the brain is broken. Gamma-aminobutyric acid (GABA) is a central nervous system inhibitory neurotransmitter which regulates important physiological processes, such as neuronal excitability and abnormal discharge. Glutamate decarboxylase (GAD) is an important rate-limiting enzyme in the synthesis

of GABA, thus it plays a key role in occurrence and development of epilepsy [4]. GAD includes two subtypes, GAD65 and GAD67. They play different roles in GABA signaling pathways. GAD65 plays a role in the enhancement of GABAergic neuronal activity, while GAD67 plays a role in stress processes of GABAergic neurons [5, 6].

Studies have proven that magnolia officinalis possesses extensive efficacy, including dispersing abdominal distention, clearing humidity, and abating fever, as well as promoting circulation and removing stasis [7]. It has been found in chemical studies that there are a variety of phenolic compounds in magnolia officinalis. Magnolol, honokiol, and isomaltol are the major active ingredients displaying therapeutic effects. Magnolol has shown anti-bacterial, anti-tumor, and anti-oxidative effects [8, 9]. Some studies have also found that magnolol has certain protective effects on the nervous and cardiovascular system [10, 11].

In this study, the rat model of epilepsy-depression comorbidity was established using lithium chloride-pilocarpine. After modeling, citalopram and magnolol, in different doses, were administered. Next, depression behaviors were monitored via forced swimming tests and saccharin preference tests. Times of epileptic seizures were also monitored using the 24-hour video surveillance system. GAD67 and GAD65 messenger ribonucleic acid (mRNA) and protein expression in the rat hippocampus was detected via quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blotting, aiming to explore the protective effects of magnolol on model rats with epilepsy-depression comorbidity, examining its influences on GAD67 and GAD65 expression.

Materials and methods

Materials

GAD67, GAD65, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibodies, along with horseradish peroxidase (HRP)-labeled secondary antibodies (Proteintech, Wuhan, China), bicinchoninic acid (BCA) protein concentration assay kit (Beyotime Biotechnology, Nantong, China), TRIzol kit, RT kit, RT-PCR kit (Invitrogen, Carlsbad, CA, USA), primer synthesis (TaKaRa, Dalian, China), lithium chloride, pilocarpine, and citalopram (Sigma, Louis, MO,

USA) were obtained and used. A total of 80 special pathogen-free male Wistar rats, aged 6-8 weeks old and weighing 160-230 g, were cultured for 1 week under a temperature of 24-26°C and humidity of 45-50%. This study was pre-approved by the Ethics Committee.

Modeling, grouping, and drug administration

First, the rats were divided into the normal control group (n = 10) and modeling group (n = 70). Rats in modeling group were intraperitoneally injected with lithium chloride (130 mg/kg), then intraperitoneally injected with pilocarpine hydrochloride (40 mg/kg) after 24 hours. When there was a persistent epileptic state for 2 hours and the epilepsy grade was above grade 4, the rats were enrolled into the experiment. After modeling, a total of 50 rats with epilepsy-depression comorbidity were obtained [12-14]. Rats in the modeling group were randomly divided into model group (n = 10), citalopram group (n = 10), and high- (H), medium- (M), and low-dose (L) magnolol groups (n = 10 in each group). Corresponding drugs were intragastrically administrated in the blank control group and model group (2 mL normal saline/d), citalopram group (10 mg/kg/d), magnolol-H, magnolol-M, and magnolol-L groups (40, 20 and 10 mg/kg/d) once a day for 4 consecutive weeks. After drug administration, the body function of the rats was examined. The rats were executed to obtain the hippocampus.

Forced swimming tests

The rats were placed in a 30 cm-deep swimming tank at 23-25°C. The cumulative immobility time of rats in each group, within 5 minutes, was observed and video-recorded.

Tail suspension tests

The rats were suspended upside down and fixed to the horizontal bar at 2 cm away from the tail. The head was 5-6 cm above the horizontal plane, the view of adjacent rats was separated, and the cumulative immobility time, within 6 minutes, was recorded.

Sucrose consumption tests

According to the methods of Moller M, consumption of sucrose water in each group, within 24 hours, was observed and recorded. Drinking amount of sucrose water = amount of

Table 1. qRT-PCR primer sequences

Gene	Primer	Primer sequence
GAD65	Forward	5'-TGCGAGTTCTGGAAGACAATG-3'
	Reverse	5'-ATGCGGAAGAAGTTGACCTTATC-3'
GAD67	Forward	5'-CTAACCATCTCGCAAGCAACT-3'
	Reverse	5'-CCATCCATCATCCATCCATTCAG-3'
GAPDH	Forward	5'-ACGGCAAGTTCAACGGCACAG-3'
	Reverse	5'-GAAGACGCCAGTAGACTCCACGAC-3'

Table 2. Influence of magnolol on forced swimming immobility times of rats with epilepsy-depression comorbidity ($\bar{x} \pm s$, n = 10)

Group	Dose	Cumulative immobility time in forced swimming test/s
Control (normal saline)	2 mL	52.47 ± 11.26
Model (normal saline)	2 mL	127.30 ± 20.32**
Citalopram	10 mg/kg	74.22 ± 15.61##
Magnolol-H	40 mg/kg	63.83 ± 17.90##
Magnolol-M	20 mg/kg	78.29 ± 19.44##
Magnolol-L	10 mg/kg	90.34 ± 18.26##

Note: **$P < 0.01$ vs. control group, ##$P < 0.01$ vs. model group.

sucrose water before measurement - amount of sucrose water after measurement [15].

Changes in times of epileptic seizures

Intensities of epileptic seizures were graded according to the Racine method: grade 0 (no epileptic seizure), grade 1 (rhythmic twitches of mouth, ears or facial muscles), grade 2 (nodding accompanied with severer facial muscle twitches and clonus), grade 3 (forelimb clonus, but not accompanied with straightening), grade 4 (forelimb clonus accompanied with straightening), and grade 5 (systematic tonic epileptic seizure) [16].

Detection of GAD67 and GAD65 mRNA expression in the rat hippocampus via qRT-PCR

After 50 mg of the hippocampus was taken from rats in each group, total RNA was extracted, according to instructions for TRIzol. It was reversely transcribed into complementary deoxyribonucleic acid (cDNA) using Oligo dT. PCR was then performed with cDNA obtained as the template. Primers are shown in **Table 1**. Reaction conditions were as follows: 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minutes, for a total of 40 cycles. With GAPDH as a control gene, the cycle threshold (Ct) value

was output from the instrument. Relative gene expression in different groups was calculated using $2^{-\Delta\Delta Ct}$.

Analysis of GAD67 and GAD65 protein expression in the rat hippocampus via Western blotting

After 100 mg of the hippocampus was taken from rats in each group, tissues were lysed with RIPA cell lysis buffer. Protein was extracted and protein concentrations were detected using the BCA method. A total of 40 µg total proteins were taken from each group, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Protein was then transferred onto a membrane, sealed with 5% skim milk powder for 2 hours, and added with GAD67, GAD65, and GAPDH antibodies (diluted at 1:3000) in a refrigerator at 4°C overnight. After the membrane was washed with tris-buffered saline with Tween-20 (TBST) 3 times, the HRP-labeled secondary antibody (diluted at 1:5000) was added for incubation at room temperature for 2 hours. The membrane was washed again with TBST 3 times, followed by ECL image development and scanning.

Statistical processing

Measurement data are expressed as mean ± standard deviation. One-way analysis of variance was used for intergroup comparisons. Chi-squared test was adopted for enumeration data. SPSS 13.0 software (International Business Machines Corporation, New York, USA) was used for all data processing. $P < 0.05$ suggests that differences are statistically significant.

Results

Influence of magnolol on forced swimming immobility times of rats with epilepsy-depression comorbidity

Forced swimming immobility times in the model group were significantly increased, compared with the blank control group ($P < 0.01$). These times were significantly decreased in magnolol groups in different doses and the citalopram group, compared with the model group ($P < 0.01$) (**Table 2**).

Table 3. Influence of magnolol on tail-suspension immobility times of rats with epilepsy-depression comorbidity ($\overline{x}$ ± s, n = 10)
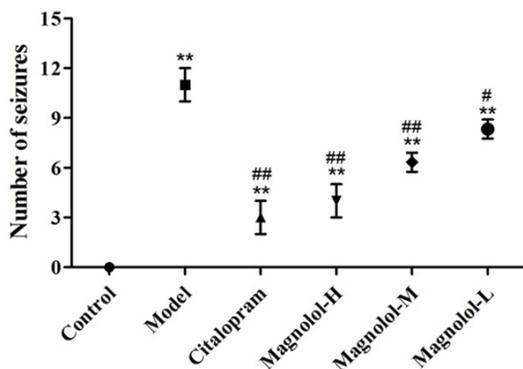
Group	Dose	Tail-suspension immobility time/s
Control (normal saline)	2 mL	90.72 ± 23.55
Model (normal saline)	2 mL	209.38 ± 20.43**
Citalopram	10 mg/kg	105.67 ± 28.64##
Magnolol-H	40 mg/kg	108.09 ± 27.58##
Magnolol-M	20 mg/kg	126.33 ± 30.35##
Magnolol-L	10 mg/kg	150.70 ± 32.98##

Note: **$P < 0.01$ vs. control group, ##$P < 0.01$ vs. model group.

Table 4. Influence of magnolol on saccharin preference of rats with epilepsy-depression comorbidity ($\overline{x}$ ± s, n = 10)

Group	Dose	Consumption of sucrose water/mL
Control (normal saline)	2 mL	44.95 ± 9.35
Model (normal saline)	2 mL	25.37 ± 5.13**
Citalopram	10 mg/kg	39.24 ± 7.84##
Magnolol-H	40 mg/kg	40.22 ± 9.98##
Magnolol-M	20 mg/kg	35.53 ± 8.45##
Magnolol-L	10 mg/kg	30.02 ± 5.39##

Note: **$P < 0.01$ vs. control group, ##$P < 0.01$ vs. model group.

Figure 1. Influence of magnolol on times of epileptic seizure of rats with epilepsy-depression comorbidity. **$P < 0.01$ vs. control group, ##$P < 0.01$ vs. model group.

Influence of magnolol on tail-suspension immobility times of rats with epilepsy-depression comorbidity

Tail-suspension immobility times in the model group were significantly increased, compared with the blank control group ($P < 0.01$). These times were significantly decreased in magnolol groups in different doses and the citalopram group, compared with the model group ($P < 0.01$) (**Table 3**).

Influence of magnolol on saccharin preference of rats with epilepsy-depression comorbidity

The base value of saccharin preference in the model group was obviously decreased, compared with that in the blank control group ($P < 0.01$). It was obviously increased in magnolol groups in different doses and the citalopram group, compared with the model group ($P < 0.01$) (**Table 4**).

Influence of magnolol on times of epileptic seizures of rats with epilepsy-depression comorbidity

Times of epileptic seizures of rats in the model group were increased, compared with the blank control group, displaying statistically significant differences ($P < 0.01$). They obviously declined in magnolol groups in different doses and the citalopram group, compared with the model group, showing statistically significant differences ($P < 0.01$) (**Figure 1**).
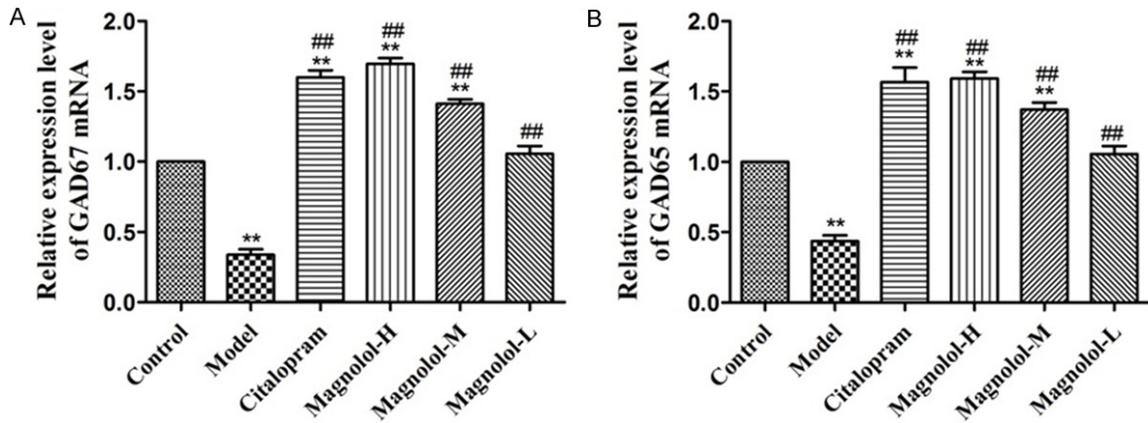
Influence of magnolol on GAD67 and GAD65 mRNA expression in the hippocampus of rats with epilepsy-depression comorbidity

Results of qRT-PCR (**Figure 2**) revealed that, compared with the blank control group, GAD67 and GAD65 mRNA expression in the rat hippocampus was remarkably deceased in the model group ($P < 0.01$). Expression was remarkably increased in magnolol-H and magnolol-M groups and the citalopram group. Moreover, GAD-67 and GAD65 mRNA expression in the rat hippocampus in magnolol groups in different doses and the citalopram group were significantly increased, compared with the model group, showing statistically significant differences ($P < 0.01$).
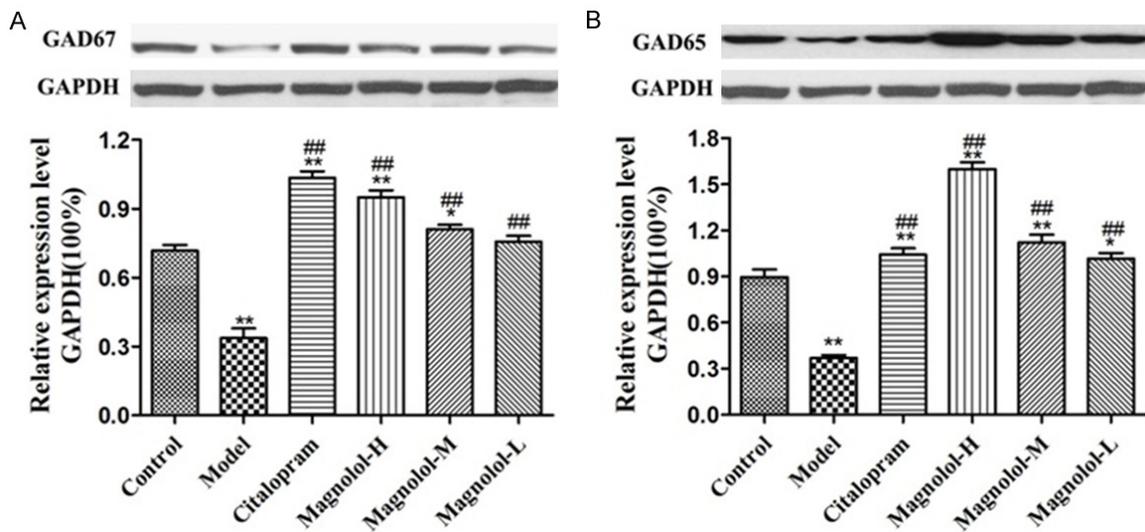
Influence of magnolol on GAD67 and GAD65 protein expression in the hippocampus of rats with epilepsy-depression comorbidity

GAD67 and GAD65 protein expression in the rat hippocampus was detected via Western blotting. Results (**Figure 3**) manifested that GAD67 and GAD65 protein expression in the

Figure 2. Influence of magnolol on GAD67 and GAD65 mRNA expression in the hippocampus of rats with epilepsy-depression comorbidity detected via qRT-PCR. **$P < 0.01$ vs. blank control group, ##$P < 0.01$ vs. model group.

Figure 3. Influence of magnolol on GAD67 and GAD65 protein expression in the hippocampus of rats with epilepsy-depression comorbidity detected via Western blotting. **$P < 0.01$ vs. blank control group, ##$P < 0.01$ vs. model group.

rat hippocampus was significantly deceased in the model group, compared with the blank control group ($P < 0.01$). Expression was significantly increased in magnolol groups in different doses and the citalopram group, compared with the model group, showing statistically significant differences ($P < 0.01$).

Discussion

The pathogenesis of epilepsy is an abnormal discharge of brain neurons. Recurrent nervous system diseases occur, in which temporal lobe epilepsy is the most common in clinic. It is also the most difficult disease to address with drug therapy. Studies have demonstrated that epi-

lepsy has a close correlation with depression, with incidence rates of depression in epilepsy patients higher than 30% [17]. Depression not only seriously affects the quality of life of patients, but also leads to suicidal tendencies in epilepsy patients [18, 19]. Drug therapy has failed to obtain significant efficacy in epilepsy patients with depression. Depression can last throughout the course of epilepsy, seriously affecting the efficacy and control of epilepsy [20].

At present, the establishment of animal models of epilepsy plays a crucial role in the study of epilepsy. These include the pentylenetetrazole-induced epilepsy model and kainic acid chronic

kindling model [21]. The lithium chloride-pilocarpine kindling model is the most commonly-used model of temporal lobe epilepsy. Studies have demonstrated that the lithium chloride-pilocarpine model is in accord with human temporal lobe epilepsy behavior, electroencephalograms, and pathological changes, thus it is an ideal model in the study of epilepsy [22]. The hippocampus, as a key structure, plays an important role in refractory temporal lobe epilepsy. Hippocampal lesions are considered as the pathological basis of epileptic seizures. The hippocampus is rich in fiber connection, which can ensure afferent and efferent glutamatergic neural projection. Lesions among synapses in the hippocampus and the release of neurotransmitters can result in epilepsy [23, 24]. GAD65 and GAD67, two subtypes of GAD, have significant differences in molecular weight, protein domain, distribution in cells, and correlation with the cofactor pyridoxal phosphate, indicating that they play different roles in regulating GABA signaling pathways [25]. Currently, basic and clinical research on epilepsy has demonstrated that GAD65 and GAD67 are closely related to the onset of epilepsy [26], but no consistent conclusions have been made on changes in GAD expression after epileptic seizures. Some studies have found that GAD expression is weakened after epileptic seizures, while some argue that the GAD content is increased after epileptic seizures [27]. It is speculated that the possible reasons for such differences are different animal models or different types of epileptic seizures [28].

In this study, the rat model of epilepsy-depression comorbidity was established using lithium chloride-pilocarpine. After modeling, citalopram and magnolol, in different doses, were administered. Depression behaviors were monitored via forced swimming tests, tail suspension tests, and saccharin preference tests. It was found that, in the model group, the immobility time in forced swimming test was significantly increased, tail-suspension immobility time was also significantly increased, and the base value of saccharin preference was obviously decreased, compared with the blank control group. In magnolol groups in different doses and the citalopram group, the immobility time in forced swimming tests was significantly decreased, tail-suspension immobility time was also significantly decreased, and the base value of saccharin preference was obviously increased, compared with the model group.

The times of epileptic seizures were monitored using a 24-hour video surveillance system. Results revealed that the times of epileptic seizures of rats in the model group were increased, compared with those in the blank control group. They obviously declined in magnolol groups in different doses and the citalopram group, compared with the model group. In addition, GAD67 and GAD65 mRNA and protein expression in the rat hippocampus was detected via qRT-PCR and Western blotting. Results showed that GAD67 and GAD65 mRNA and protein expression in the rat hippocampus remarkably declined in the model group, compared with the blank control group. Expression was also obviously increased in the rat hippocampus in magnolol groups in different doses and the citalopram group, compared with the model group.

Conclusion

In conclusion, the present study demonstrates that expression of GAD67 and GAD65 plays key roles in occurrence and development of epilepsy-depression comorbidity. Magnolol can upregulate expression of GAD67 and GAD65 in the hippocampus of rats with epilepsy-depression comorbidity, reduce the times of epileptic seizures, and improve the depression behavior of rats.

Disclosure of conflict of interest

None.

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