Original Article

Effects of gastrodin on amino acids after cerebral ischemia-reperfusion injury in rat striatum

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The aim of this study was to explore the effect of gastrodin on the level of amino acids in the striatum in the rats of cerebral ischemia-reperfusion injury. 30 male SD rats were randomly divided into 3 groups: the group of pseudo-operation (normal control group, NC group), the group of cerebral ischemia-reperfusion injury (CIRI group), and the group of cerebral ischemia-reperfusion injury treated with gastrodin (G group). Cerebral ischemia-reperfusion injury was induced through middle cerebral artery occlusion (MCAO). 10 minutes before the operation, the rats in the G group were injected with gastrodin (50mg/kg) intraperitoneally once. The rats in the CIRI and NC group were injected with the same volume of 10% propylene glycol normal saline intraperitoneally once. The levels of glutamic acid (Glu), aspartic acid (Asp), γ-aminobutyric acid (GABA), taurine (Tau) in striatum in the rats of the 3 groups were measured with the method of microdialysis-HPLC techniques. The ratio of Glu to GABA was calculated. The volume of cerebral infarction was quantified. This study showed that gastrodin can decrease the volume of cerebral infarction, ameliorate the cerebral injury in the rats of cerebral ischemia-reperfusion. The mechanisms might be that gastrodin can improve the level of amino acids in striatum.

Key Words: gastrodin, cerebral ischemia-reperfusion injury, inhibitory amino acids, excitatory amino acids

Introduction

Cerebral vascular diseases are one of the six main diseases which cause death or disability. Theoretically, it can be classified into cerebral hemorrhage and cerebral ischemia disease, and the latter is more common. As we know, ischemia brain damage is resulted from insufficient supply of blood in one or more brain vessels. During the course of brain ischemia, the concentration of neurotransmitter varies. More and more evidence suggests that the concentration of neurotransmitter as amino acids in ischemia central region may be greatly increased. Studies have showed that the excessive release of excitatory amino acids (EAA), such as glutamic acid (Glu) or aspartic acid (Asp), is the principal pathological mechanism in ischemia brain damage.

EAA exist largely in the nerve cell synapse and secondly in various nerve cell soma and glial cell kytoplasm, among which glutamic acid (Glu) and aspartic acid (Asp) have the highest content in brain. In nerve cell, there are abundant enzyme systems to synthesize these EAA. The majority of Glu released from presynaptic ending enter into the glial cell nearby. A small portion of them are ingested and the remains are combined with postsynaptic receptor. Glu is the main excitatory neurotransmitter in central nerve system. Glu takes part in the rapid excitatory synaptic transmission and plays an important role in maintaining the normal signaling pathway of nerve cell. However, in pathologic case, excitatory amino acids have an impairing effect on nerve.

It has been reported that the antagonist of Glu receptor can improve cerebral ischemia injury.[1] While excitatory amino acids are being excessively released, inhibitory amino acid, like γ-aminobutyric acid (GABA), taurine (Tau) also released during the course of cerebral ischemia. It has also been reported that inhibitory amino acids can decrease cerebral ischemia injury and can counteract the toxicity of excitatory amino acids.[2] It is believed that the imbalance between excitatory amino acids and inhibitory amino acids is one of the reasons for ischemic brain damage.[3] In the present study, we applied cerebral micro-dialysis in combination with HPLC technology to analyze the real time dynamic change process of cerebral amino acids in the rats of cerebral ischemia-reperfusion injury.

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The aims of this study were (1) to investigate the effect of gastrodin on the level of amino acids in striatum in the rats of cerebral ischemia-reperfusion injury. (2) to develop a new therapy for ischemic cerebral diseases.

Materials and methods

Animals

30 healthy and pure male SD rats (10 month old, weight 300g-350g) were purchased from animal Experimental Animal Center of Zhejiang Academy of Medical Sciences.

Drugs and reagents

O-phthalaldehyde, standard substance of amino acid (Glu, Asp, γ-GABA and Tau), mercaptoethanol, and potassium dihydrogen phosphate, were all purchased from Sigma Company. Methanol was purchased from Siyou Bio-Medical Technology Company, Tianjin, China. Gastrodin was purchased from Bali Moer Company, Ltd, Jilin, China.

Equipment

HPLC (Shimatzu LC-10ATVP system).

Study design

SD rats were anesthetized with ethylcarbamate (1.25g/kg, i.p.) and fixed in stereotaxic apparatus. The tectums of the rats were exposed. Dialysis tubes were posed on the right side of striatum (bregma forward AP=+0.2, side opening L=3.5, under cortical depth H=-5.8) according to the rats' cerebral stereotaxic atlas. Three small screws were fixed on the skull surrounding the tube, so that the tube does not move or get loosen along with the normal movement of the animal. After the operation, the rats were injected with penicillinto to prevent infection. The rats were recovered under 25～30℃ for 5 days until the normal action and diet were regained. No negative operation reaction, such as cachexia, should occur from the appearance.

Five days after the dialysis tube implanted, the model of cerebral ischemia-reperfusion injury was made through middle cerebral artery occlusion (MCAO) according to the method reported by Zea-Longa etc.[4] Amino acids level were measured in striatum with cerebral micro-dialysis-HPLC technology.

30 male SD rats were randomly divided into 3 groups: the group of pseudo-operation (normal control group, NC group), the group of cerebral ischemia-reperfusion injury (CIRI group), and the group of cerebral ischemia-reperfusion injury treated with gastrodin (G group). Cerebral ischemia-reperfusion injury was induced through middle cerebral artery occlusion (MCAO). 10 min before the operation, the rats in the G group were injected with gastrodin (50mg/kg) intraperitoneally once. The rats in the CIRI group were injected with the same volume of 10% propylene glycol normal saline intraperitoneally once.

Collection of micro-dialysis liquid

Opened dialysis reperfusion system, adjusted the constant flow pump to control the flow velocity at 2.0μL/min. The micro-dialysis probe was connected directly with cerebrospinal fluid liquid reperfusion system. Artificial cerebrospinal fluid (ACSF, pH7.4) included 126mM NaCl, 27.5mM $NaHCO_3$, 2.4mM KCl, 5mM KH_2PO_4, 5mM Na_2HPO_4, 0.5mM Na_2SO_4, 0.82mM $MgCl_2$•$6H_2O$, 1.1mM $CaCl_2$•$2H_2O$, 5mM glucose.

The rats implanted with dialysis tube were anesthetized with chloral hydrate (400mg/kg i.p.). Micro-dialysis probes were carefully inserted into the tube and fixed. After the flowing liquid were balanced for 120min, liquid were collected every 15min. The first two samples were used for self-comparison. Then the rats were suffered from MCAO. During this period, dialysate were collected every 15 min for four times. After 60 min of ischemia, the following reperfusion was taken. 8 tubes of dialyzate were collected until 120 min after the reperfusion. Then two tubes of dialyzate were collected. Before of every collection the lipuid was balanced for 120 min. The dialyzate liquid collected were immediately stored at -20℃. Amino acids level was analysed with HPLC after one week.

4 hours after ischemia reperfusion, neuroethology changes of the rats were observed according to the four-division system reported by Zea-Longa etc. Amino acids data of the rats with 2 scores of neuroethology were accepted in this experiment.

Amino acids analysis of dialyzate by HPLC

HPLC setting: In this experiment, HPLC and fluorescence detector were combined to quantitatively analyze the levels of amino acids in dialyzate. Excitation Wave length (Ex) =357nm. Emission Wave length (Ex) =455nm. Mobile phase preparation: HPLC reversed-phase separation was applied in the experiment. Buffered water solution was used as mobile phase. Properly amount of organic agents, such as methanol, was used to improve the separation. Derivation reaction: Orthophthaladehyde/amino acids (OPA/AA) ratio was prepared for derivation agents: 5 mL OPA, 125μL methanol, 20μL mercapto ethanol and 1mL sodium tetraborate (0.1mol/L) were well mixed. The derivation agents were stored in shade, and were used after 24 hours' aging. 20μL amino acid standard substance solution or micro-dialysis sample liquid were taken for detection, and then 20μL sodium hydrogen carbonate (0.05 mol/L) and 10μL derivation agent were added and mixed well. The mixture were settled for 1 min and measured immediately.

Standard curves of four sorts of amino acids

Different concentrations of amino acid standard substance (diluted with 0.05mol/L sodium bicarbonate to get different concentration grades of 40μmol/L, 20μmol/L, 10μmol/L, 5μmol/L, 2.5μmol/L, 1.25μmol/L and 0.625μmol/L) were made. The response values of various amino acid derivatives at different concentrations were obtained. The curve of response value vs. amino acid concentration was made.

Cerebral infarct volume was measured by TTC dyeing method. After suffered from 60 min of cerebral ischemia and 24hours of reperfusion, the rats were sacrificed. The brains of the rats were harvested immediately and immerged into pre-cooled normal saline. And then the brains were cooled at -20℃ for 10 min, so that the brains were hardened slightly. Coronal cerebral slices (2 mm thickness) were made. After stained with 2%TTC for 30 min at

37℃, the slices were fixed with 10% formalin over night. Total infarct volume=total infarct area × thickness of cerebral slice. Post-dropsy correction infarct volume=infarct volume × cerebral hemisphere volume on opposite side/hemisphere on the same side. Infarct rate (%) =total post-dropsy correction infarct volume/total cerebral hemisphere volume.

Statistical analyses

All the data were statistically analyzed as Mean ± SD($\bar{x}$ ±*s*). Student's t determination statistic analysis was carried out after single-factor variance analysis. *p* values <0.05 was considered as significant.

Results

Effect of gastrodin on infarct volume

After 60 min MCAO and 24 hours reperfusion, the cerebral infarct rate on the ischemia side in the rats of CIRI group was 32%, and in the rats of G group was 27%. The cerebral infarct rate in the G group was significantly lower than that in CIRI group ($p<0.05$) (Fig 1).

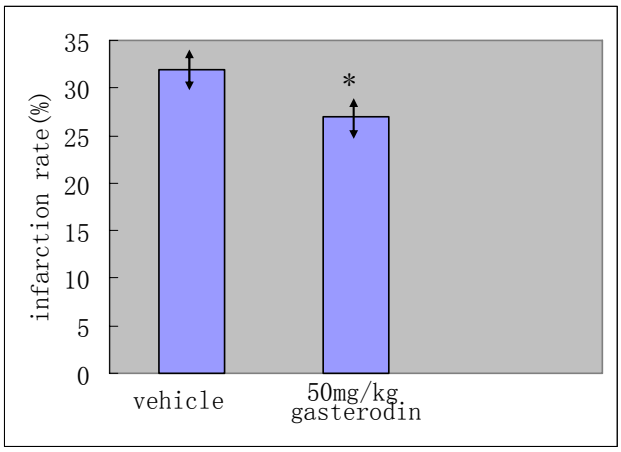

Figure 1. Effects of gastrodin on brain infarct volume induced by 60min MCAO followed by 24 hours reperfusion. When 50mg/kg of gastrodin was administrated 10 min prior to MCA occlusion, significant attenuation of infarct was observed. n=6. Data are presented as mean±SD.*$p<0.05$ vs. vehicle group (CIRI group).

Effect of gastrodin on amino acids level

Under normal circumstances, the contents of four sorts of amino acids in striatum were ASP: 0.897±0.17μM; Glu: 2.405±0.42μM; GABA: 0.677±0.073μM; Tau: 5.186±0.685μM. After MCAO, the level of the four sorts of amino acids were increased quickly, especially the level of glu. Within the period of 30～60min ischemia, the level of the four sorts of amino acids reached the maximum (ASP was 893% of that before ischemia; Glu was1413% of that before ischemia; GABA was 904% of that before ischemia and Tau was 548% of that before ischemia). After reperfusion, amino acids level droped rapidly. 60 min after the reperfusion, the lowering speed began to slow down until 120 min after the reperfusion. Except Tau, other three sorts of amino acids still remained at higher level ($p<0.05$).

In this experiment, gastrodin (50mg/kg) which could remarkable reduce the cerebral infarct volume were used to treat cerebral ischemia-reperfusion injury. The effect of gastrodin on amino acids in striatum in the rats of cerebral ischemia-reperfusion injury was investigated. The results showed that gastrodin had no significantly influence on amino acids levels in normal striatum before ischemia. Within 15 min after ischemia, the contents of the four sorts of amino acids in striatum in G group were significantly lower than those in CIRI group during the same period. The results showed that the concentration of Asp was 1.62±0.22μM, Glu 5.718±0.440μM, GABA 1.529±0.255μM and Tau 7.80±1.338μM, and they all got reduced compared with CIRI group, which were Asp 58.3% ($p<0.05$), Glu 48.1% ($p<0.01$), GABA 38.6% ($p<0.01$) and Tau 12.8% ($p<0.01$). Compared with CIRI group, the amino acids' peak values dued to ischemia were also sharply reduced in G group and the highest values were reduced by 38.5% (Asp), 41.2% (Glu), 23% (GABA) and 39.1% (Tau) respectively. After the reperfusion began, the amino acids level in striatum in the rats of G group began to decrease also. 120 min after the reperfusion, the levels of the four sorts of amino acids basically recovered back to normal levels, which had no significantly difference compared with the level before ischemia ($p>0.05$) (Fig 2).

Effects of gastrodin on the ratio of Glu to GABA in striatum

The ratio of Glu to GABA reflects the balance between the level of cerebral excitatory amino acids and inhibitory amino acids in brain, which is stable in normal situation. The result of this experiment showed that the ration of Glu to GABA in cerebral striatum was 3.38 before ischemia. After the occurring of ischemia, the ration of Glu to GABA rose and reached to the maximal level at 5.52 after 60 min ischemia, which was about a 63.31% increase compared with the ratio before ischemia. The ratio gradually decreased afterwards and reached to 4.363 at 120min after ischemia, which was a 29% increase compared with the ratio before ischemia. The ration of Glu to GABA in G group was remarkably reduced. The ratio was 3.68 after 15 min of ischemia, and the maximal ratio was 4.31, which was reduced by 21.8% compared with the ratio in CIRI group. After 120 min of reperfusion, Glu/GABA ratio resumed to 3.48, which had no significantly difference compared with the normal control group. The results provided the evidences that gastrodin could inhibit Glu level more than GABA under the circumstance of cerebral ischemia (Fig 3).

Discussion

Gastrodia elata B1 is the tuber of arethusa herbage plant. Gastrodin is the most important and effective ingredient extracted from Chinese natural herbal gastrodia elata B1. Its molecular formula is C13H1807 and molecular weight is 286.25. However, it is still not clear about the mechanism how gastrodin ameliorate cerebral ischemia-reperfusion injury. In the present study, cerebral microdialysis in combination with HPLC technology was used to analyze the real time dynamic change of cerebral amino acids in striatum in the process of cerebral ischemia-reperfusion injury, in order to explore the action mechanism of gastrodin on cerebral ischemia-reperfusion injury.

In this experiment, dynamic change of the four sorts of amino acids levels in striatum were observed in the rats of

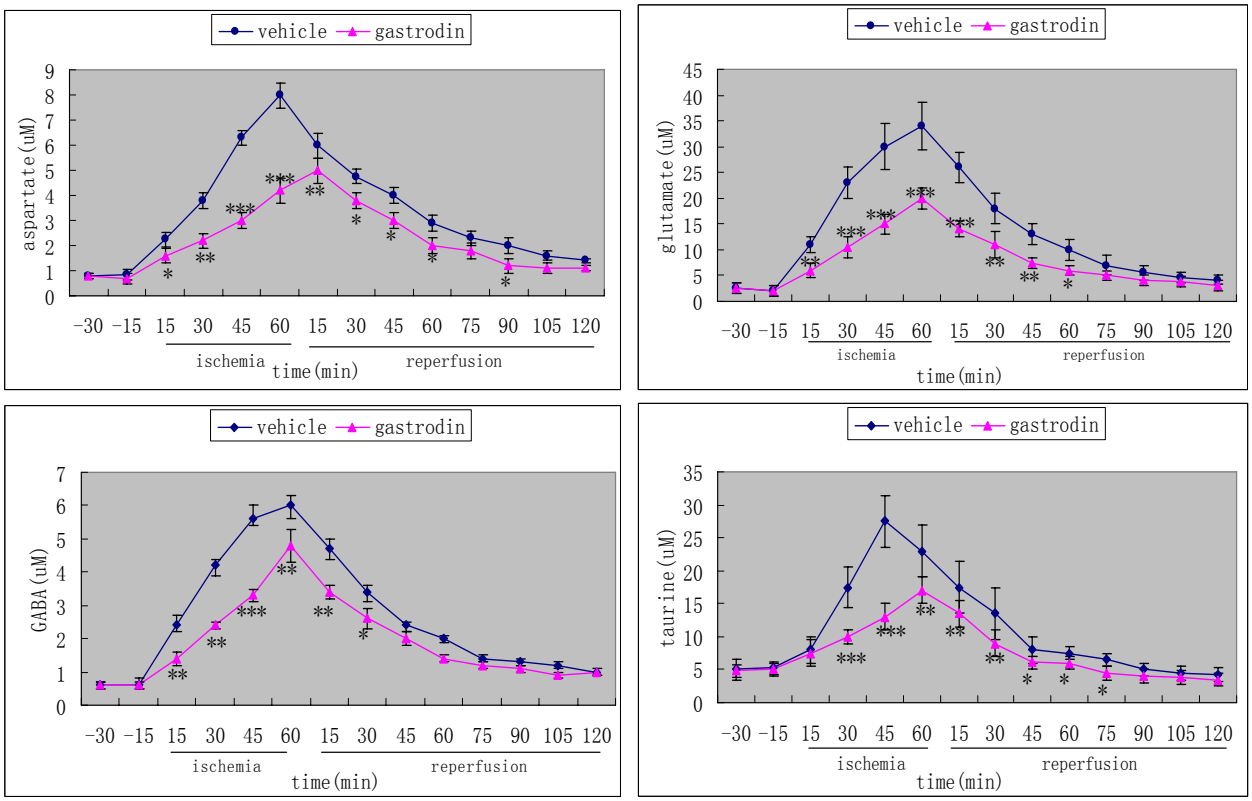

Figure 2. Effects of gastrodin(50mg/kg) on ischemia/ reperfusion-evoked changes of amino acids in striatum. Line plots showed the concentrations (μM) of amino acids before, during and after 60 min of middle cerebral artery occlusion. Gastrodin was administrated 10 min prior to the onset of ischemia. The data are presented in comparison with those from control animals administrated with vehicle (CIRI group). n=4. The data are presented as means±SD. **p*<0.05; ***p*<0.01; ****p*<0.001 vs. vehicle group (CIRI group).

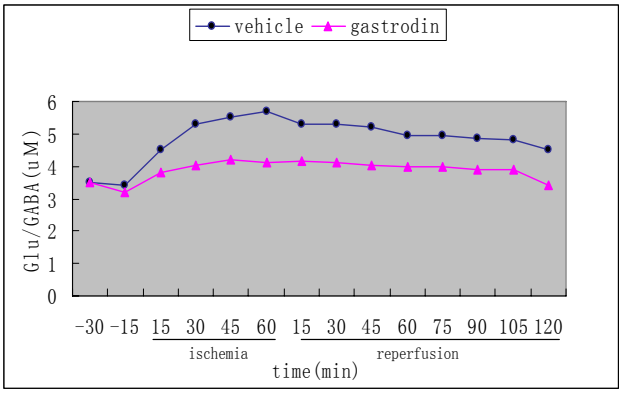

Figure 3. Effects of gastrodin (50mg/kg) on ischemia-reperfusionevoked changes of the ration of Glu to GABA in striatum.

cerebral ischemia reperfusion . It was found that ischemia could cause surprisingly increased amino acids level in center ischemia area. The level began to decrease gradually after reperfusion and resumed gradually to the normal level after 24 hours reperfusion. The change process was in coincidence with what the literatures reported. Gastrodin could notably inhibit the rise of the four sorts of amino acids levels in striatum in the process of ischemia.

The release of cerebral excitatory amino acids is considered as a key reason for the damage of ischemic nerve cell. Though, ischemia also causes a great deal of inhibitory amino acids to release, the release of excitatory amino acids is more remarkable.

Many scholars believed that the unbalance between the excitatory amino acids and inhibitory amino acids is related to the ischemic cerebral damage. In this experiment we found that the ration of Glu to GABA was increased sharply after ischemia and always remained at higher level, though declined to a certain degree after reperfusion. The excitatory and inhibitory amino acids were decreased to different degree in striatum in the rats of cerebral ischemia-reperfusion injury. However, the action of excitatory amino acids, for example, glutamate, was more remarkable. During the whole process of ischemia-reperfusion, the Glu/GABA ratio in G group was much lower than that in CIRI group. The results indicated that gastrodin could significantly inhibit the release of cerebral amino acids, especially the excitatory amino acids in ischemia process.

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