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ORIGINAL ARTICLE

Onjisaponin B Attenuates Glutamate Release via Inhibition of Calmodulin-Dependent Protein Kinase II and Connexin 43 Pathways in Rat Astrocytes Subjected to Oxygen and Glucose Deprivation

Yingqi Mao[1,2], Yuyang Chen[2], Xiaoyun Pan[3], Lingfeng Gao[3], Lidan Wang[4], Da Li[5], Tao Qiu[2]

[1]Faculty of Chinese Medicine, Macau University of Science and Technology, Macau, [2]Department of Neurology, Zhejiang Provincial Hospital of Chinese Medicine, [3]First Clinical Medical College, Zhejiang Chinese Medical University, [4]Department of Gynecology, Zhejiang Xiaoshan Hospital, [5]Department of Endocrinology, The First People's Hospital of Xiaoshan District, Hangzhou, China

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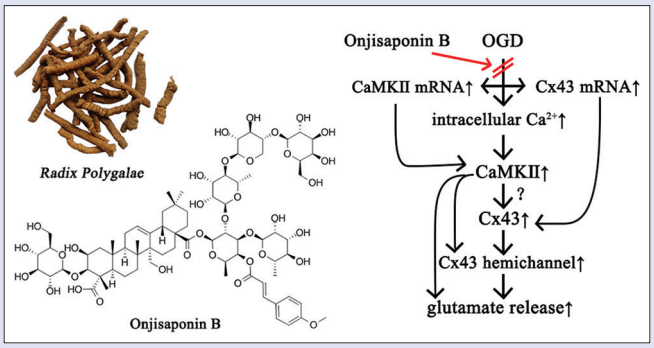
ABSTRACT

Background: Post-stroke depression (PSD) is one of the most common complications of stroke. Many studies have confirmed that PSD is associated with the abnormal release of glutamic acid (Glu) via the Ca^{2+}/calmodulin (CaM)/CaM-dependent protein kinase II (CaMKII) and connexin 43 (Cx43) hemichannel pathways. Onjisaponin B, a traditional Chinese medicine, has been discovered to have neuroprotective effects. **Objectives:** In this study, we aimed to investigate the effects and underlying mechanisms of onjisaponin B on the release of glutamate in rat astrocytes subjected to oxygen and glucose deprivation (OGD). **Materials and Methods:** The ischemic and anoxic cell model was established in rat astrocytes through OGD injury. Rat astrocytes were randomly divided into control, model, 10 μM onjisaponin B, and 20 μM onjisaponin B groups. Cell viability was assessed by acridine orange/ethidium bromide bilabel assay. The intracellular Ca^{2+} level was measured with Fluo-3-AM as the fluorescence indicator. Western blot analysis and quantitative polymerase chain reaction were used to detect the expressions of Cx43 and CaMKII in the samples. The glutamate level of the extracellular fluid was determined using high-performance liquid chromatography-mass spectrometry. **Results:** The intracellular Ca^{2+} levels and the mRNA and protein expression of CaMKII and Cx43 in the onjisaponin B group were significantly lower than that of the model group. The glutamate level in the extracellular fluid was significantly reduced by onjisaponin B in comparison to that in the model group. Moreover, onjisaponin B attenuated the inhibitory effect of OGD on the cell viability of astrocytes. **Conclusion:** The results of this study suggest that onjisaponin B reduced the release of glutamate via inhibition of the Ca^{2+}/CaMKII and Cx43 pathways in the rat astrocytes, thus inhibiting the downstream glutamic pathway and reinforcing the cell viability of astrocyte.

Key words: Astrocyte, CaMKII, Cx43, glutamate, onjisaponin B, oxygen and glucose deprivation

SUMMARY

- Onjisaponin B attenuates the inhibitory effect of oxygen and glucose deprivation and increases cell viability of astrocytes
- Onjisaponin B decreases the glutamate release from the astrocytes subjected to OGD
- Onjisaponin B inhibits the Ca^{2+}/CaMKII/Glu and Cx43/Glu pathways in astrocytes subjected to OGD.



Abbreviations used: PSD: Post-stroke depression; CaM: Calmodulin; CaMKII: CaM-dependent protein kinase II; Cx43: Connexin 43; OGD: Oxygen and glucose deprivation; Glu: Glutamic acid; RAPO: *Radix Polygalae*; CSLM: Confocal scanning laser microscope; SDS-PAGE: SDS polyacrylamide gel electrophoresis; PVDF: Polyvinylidene difluoride; HPLC-MS: High-performance liquid chromatography-tandem mass spectrometer; MRM: Multiple reaction monitoring.

Correspondence:

Dr. Tao Qiu,
Zhejiang Provincial Hospital of Chinese Medicine, Hangzhou 310006, China.
E-mail: qiutao2002002@163.com

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INTRODUCTION

Post-stroke depression (PSD) is one of the most common complications after stroke that affects around one-third of the patients with stroke.[1,2] It is an independent risk factor for functional recovery and recurrence after stroke. It causes functional impairment, poor activities of daily living, and poor cognitive and social function, which puts heavy burden on the patients, families, and society.[3-5]

So far, the pathogenesis of PSD has not been fully understood, and there is no evidence supporting the use of a specific biomarker for the diagnosis of PSD.[6-8] Currently, many studies have revealed that PSD is associated with the abnormal release of glutamic acid (Glu) and that astrocytes

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can release Glu, as well as participate in the glutamate metabolism of the nervous system.[9-12] Some studies have confirmed that the release of Glu is regulated via Ca^{2+}/CaM/CaMKII pathway.[13-15] Moreover, other studies have shown that connexin 43 (Cx43) regulates the glutamatergic nervous system[16,17] and that calmodulin (CaM) regulates gap junction channel and hemichannel of Cx43 in a Ca^{2+}-dependent manner.[18,19]

Radix polygalae (RAPO), the dried root of *Polygala tenuifolia* Wild. (Polygalaceae), is a typical and widely used traditional Chinese medicine. It is used in the treatment of dementia, insomnia, depression, and so on. Previous studies have mainly focused on the nootropic activity of RAPO;[20] however, through clinical practice, we have been able to find that RAPO exhibits beneficial effects against PSD.[21-24] Onjisaponin B, one of the primary ingredients of RAPO, has been confirmed to have a neuroprotective effect.[20,25]

Therefore, in this study, we hypothesized that onjisaponin B is responsible for the antidepressant effect in PSD and we also hypothesized that it does so by regulating the Ca^{2+}/CaMKII/Glu and Cx43/Glu pathways. To prove this hypothesis, we established an ischemic and anoxic cellular model (using rat astrocytes) through oxygen and glucose deprivation (OGD) injury.

MATERIALS AND METHODS

Reagents, drugs, and equipments

Acridine orange (AO)/ethidium bromide (EB) staining kit was purchased from Sangon Biotech (Shanghai) Co., Ltd.(China). Fluo-3-AM was purchased from Shanghai Yuanye Bio-Technology Co., Ltd.(China). Cx43 antibody (#3512) and CaMKII antibody (#3362) were purchased from CST (Gojan-Dong, Namdong-Gu, Incheon, Korea). Dulbecco's modified Eagle's medium (DMEM)/F12 medium and 10% fetal calf serum were purchased from Sigma-Aldrich (Irvine, United Kingdom). Penicillin and streptomycin were purchased from Shandong Xinhua Pharmaceutical Co., Ltd. (Zibo, Shandong, China). High Purity Total RNA Rapid Extraction Kit was purchased from Generay Biotech (Shanghai) Co., Ltd. (China). Reverse Transcription Kit was supplied by Thermo Fisher Scientific Co., Ltd. (Shanghai, China). Furthermore, quantitative polymerase chain reaction (qPCR) reagent was obtained from Bio-Rad Laboratories (Hercules, California, USA). PCR primer synthesis was purchased from Yingjie Jieji (Shanghai) Trading Co., Ltd (China). Onjisaponin B (purity >98%) was purchased from Chengdu Must Biotechnology Company Ltd.(Chengdu, China). High-performance liquid chromatography (HPLC) was purchased from Waters (Milford, Massachusetts, USA). Tandem mass spectrometer (MS) was purchased from Quattro Co., Ltd. (Hongkong, China). Gel Imager was supplied by Bio-Rad Co., Ltd. A high-precision spectrophotometer was obtained from BioDrop (Cambridge, UK).

Animals

Specific pathogen-free (SPF) male Sprague–Dawley (SD) rats (24 h old) were purchased from Shanghai Slack Lab Animal Co., Ltd. (Shanghai, China). The rats were fed in the SPF grade house at a constant temperature (25°C) and light cycles (12 h light and 12 h dark). The experimental studies on the rats were performed according to the guidelines of the Zhejiang Chinese Medicine University Animal Research Committee. All experimental protocols were approved by the Zhejiang Chinese Medicine University Biosafety and Animal Research Committees.

Cell culture

Brains from newly born SD rats were removed under sterile conditions. Meninges and blood vessels were cleaned in the Hanks solution. The cerebral cortical astrocytes were separated after digestion with 0.25% trypsin. The resultant cell suspension was filtered by using a sifter with a mesh size of 75 μm and then centrifuged at 800 rpm for 5 min and resuspended in DMEM/F12 medium. The suspension was allowed to sit for 20 min at room temperature and pipetted into culture flasks pretreated with polylysine to remove fibroblasts. The cells were incubated at 37°C in 5% carbon dioxide incubator with DMEM/F12 medium, supplemented with 10% fetal calf serum, and 100 U/mL of penicillin and streptomycin. The medium was changed every 3-4 days. The culture was passaged when the cells grew to a 80% confluency, with constant shaking (220 rpm) in a shaking water bath at 37°C for 12 h and removed the cells above astrocytes before passage.

Experimental grouping and the hypoxia and hypoglycemia stimulation

After 21 days of culture, the astrocytes were randomly divided into control, model, and onjisaponin B (10 and 20 μM) groups. All the cells except the control group cells were washed twice with a nitrogen-saturated sugar-free DMEM. The model group was added to a sugar-free DMEM and cultured in a nitrogen-filled culture tank (37°C, 5% CO_2). The onjisaponin B group was cultured in a culture tank filled with nitrogen (37°C, 5% CO_2) by adding onjisaponin B (10 and 20 μM). The cells were harvested at 2 and 4 h for relevant analysis.

Acridine orange/ethidium bromide staining test

At each collection time point, AO/EB Staining Kit (Sangon Biotech, Shanghai, China) was used to stain the astrocytes. Then observed and toke pictures with a fluorescence microscope.

Measurement of intracellular Ca^{2+} levels

At each collection time point, the cultured astrocytes were washed with phosphate-buffered saline (PBS), and the cells were incubated with Fluo-3-AM for 40 min, then washed thrice with PBS. The confocal scanning laser microscope was used to observe and photograph the fluorescence intensity of Ca^{2+}. The mean gray value calculated via Image J software can semi-quantitatively represent the concentration of intracellular Ca^{2+}.

Western blot analysis

At each collection time point, cells were harvested, added to RIPA lysate, and kept on ice for 30 min. After centrifuging the cell lysate 13000 rpm at 4°C for 10 min, the supernatant was added to a 5 mL sample buffer and boiled at 100°C for 10 min. Proteins were measured using the bicinchoninic acid method. Proteins from each group were separated on a 4%–20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then, the separated bands were transferred onto polyvinylidene difluoride membrane and blocked using a solution containing TBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl and 0.05% Tween 20) and 5% nonfat dried milk at room temperature for 2 h. Then, they were incubated overnight with Cx43 antibody (1:1000) at 4°C. After washing with TBST buffer, the membrane was incubated with peroxidase-labeled anti-rabbit antibody (1:3000 dilution) at room temperature for 2 h. The membrane was exposed to electrochemiluminescence reagent and imaged via a gel imaging system by exposing it to chemical light-sensitive mode. The gray-scale scanning analysis quantification was completed using Image J software.

The quantitative polymerase chain reaction detection system

The mRNA sequence of the Cx43 and CaMKII gene was retrieved from the GenBank, and the primers were designed using Primer 5.0

software. They were verified through experiments and used for real-time PCR [Table 1].

The total RNA of tissue was extracted using a High Purity Total RNA Rapid Extraction Kit (Generay Biotech, Shanghai, China). The mRNA expressions of Cx43 and CaMKII were detected by the RT-qPCR method. The cycle threshold (Ct) was detected by monitoring the changes in the fluorescence intensity signal using fluorescence quantitative PCR instrument (ABI). The amounts of 2xSYBR Green SuperMix, reverse transcription product of cDNA with PCR H_2O, upstream primer, and downstream primer were 10, 8, 1, and 1 μL, respectively. Reaction conditions were as follows: 95°C pre-denaturation for 3 min, 95°C denaturation for 15 s, 59°C annealing for 15 s, 72°C amplification for 30 s for a total of 40 cycles. In this study, the quantitative analysis of fluorescence PCR results was conducted by $2^{-\triangle\triangle Ct}$ method.

High-performance liquid chromatography-mass spectrometry

At each time point, the cell culture medium was extracted, centrifuged for 4 min, and the supernatant collected. HPLC-MS was used to measure the glutamate concentration in the extracellular fluid. Chromatographic separation was performed using an Acquity UPLCTM BEH C_{18} column (150 mm × 2.1 mm, 1.7 μm). The chromatographic separation conditions were as follows: Mobile phase A: Methanol, B: 10 mmol/L ammonium acetate aqueous solution (containing 0.1% formic acid); flow rate: 0.1 mL/min (gradient elution); injection volume: 1 μL; column temperature: 35°C. Mass spectrometry conditions: Electrospray ion source; positive ion ionization mode; scanning method: Multiple reaction monitoring; ion source voltage 3300 V; ion source temperature 110°C.

Statistical analysis

All data were analyzed by SPSS 19.0 and presented as mean ± standard error of the mean ($\bar{x}$±s). The differences considered to be statistically significant are based on a criterion of $P < 0.05$. One-way analysis of variance was used for the comparison of the multiple samples. The least-square difference *t*-test was used for comparison between groups.

RESULTS

Effects of onjisaponin B on the cell viability of astrocytes

The cell viability of astrocytes was assessed by the AO/EB bilabel assay. The live and injured cells are permeable to both AO and EB; therefore, the cells appear orange after fluorescence excitation. However, only EB can enter the dead cells; therefore, it appears red after fluorescence excitation. The results are expressed as the rate of injured or dead cells (orange or red cells/total cells). As shown in Figure 1, at 2 h and 4 h time points, OGD caused a significant increase in the rate of injured

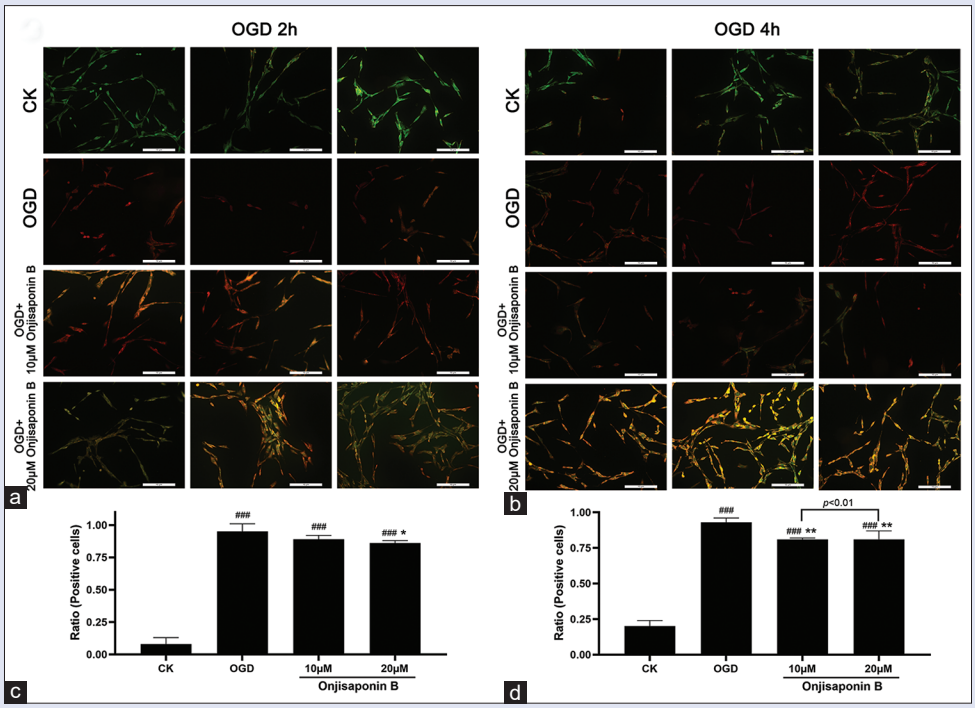

Figure 1: Effects of onjisaponin B on the cell viability of astrocytes at OGD 2 h (a and c) and OGD 4 h (b and d). The cell viability of astrocytes was assessed by the AO/EB bi-label assay ($n = 3$). The results were expressed as the rate of positive cells (injured or died cells)/total cells. CK: Control group; OGD: Model group; 10 μM onjisaponin B: OGD + 10 μM onjisaponin B group; 20 μM onjisaponin B: OGD + 20 μM onjisaponin B group. ###$P < 0.001$ versus CK; *$P < 0.05$ versus OGD; **$P < 0.01$ versus OGD. OGD: Oxygen and glucose deprivation; AO: Acridine orange; EB: Ethidium bromide

Table 1: Primer sequence

Gene	Gene ID	Upstream sequence	Downstream sequence
Cx43	2697	TCGCCTATGTCTCCTCC	GGTCCACGATGGCTAAT
Calmodulin -dependent protein kinase II	818	ATGATGGCGTGAAGGAA	CAGGTGGATGTGAGGGTT
GAPDH	2597	CGGATTTGGTCGTATTG	GAAGATGGTGATGGGATT

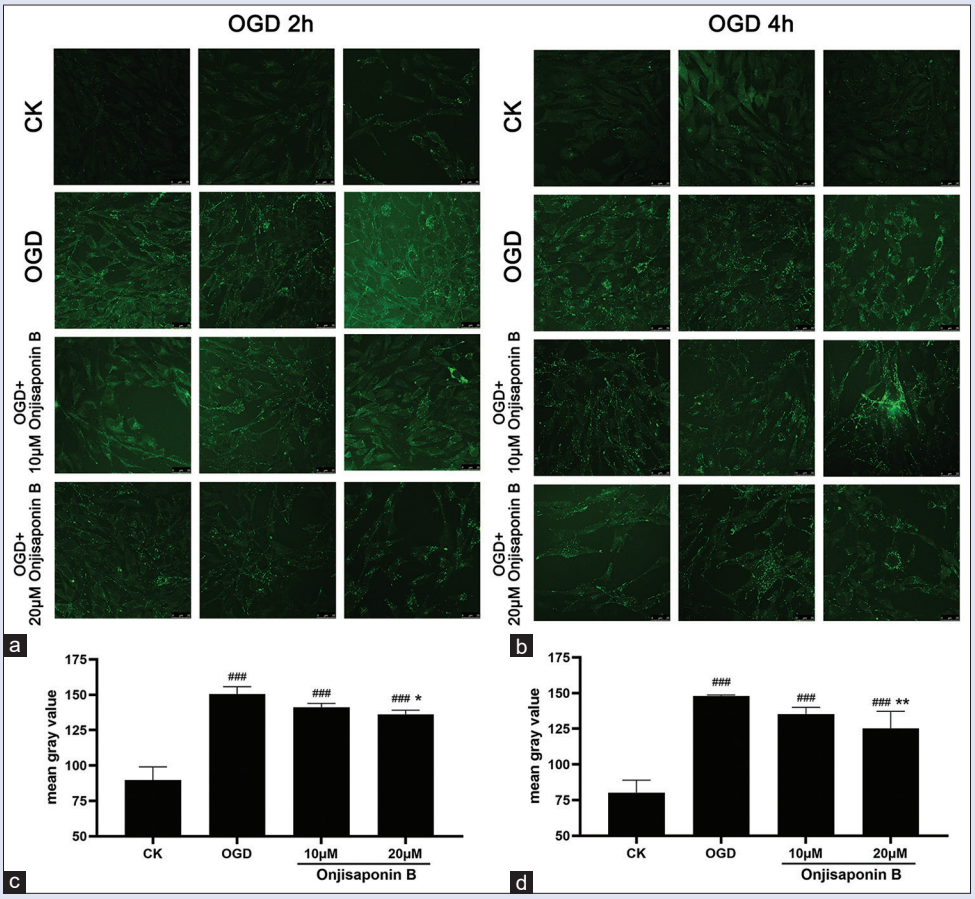

Figure 2: Effects of onjisaponin B on the intracellular Ca^{2+} of astrocytes at OGD 2 h (a and c) and OGD 4 h (b and d). The level of intracellular Ca^{2+} was assessed by Fluo-3-AM plus Confocal Scanning Laser Microscope ($n = 3$) and measured by fluorescence intensity. CK: Control group; OGD: Model group; 10 μM onjisaponin B: OGD + 10 μM onjisaponin B group; 20 μM onjisaponin B: OGD + 20 μM onjisaponin B group. $^{###}P < 0.001$ versus CK; $^{*}P < 0.05$ versus OGD; $^{**}P < 0.01$ versus OGD. OGD: Oxygen and glucose deprivation

or dead cells from 0.08 ± 0.05 to 0.95 ± 0.06 ($P < 0.001$) and from 0.20 ± 0.04 to 0.93 ± 0.03 ($P < 0.001$), respectively. Onjisaponin B (20 μM) significantly attenuated this inhibitory effect at 2 h (from 0.95 ± 0.06 to 0.86 ± 0.02, $P < 0.05$) and 4 h of incubation with OGD (from 0.93 ± 0.03 to 0.81 ± 0.01, $P < 0.01$). Onjisaponin B (10 μM) reinforced the cell viability at 4 h of incubation with OGD (from 0.93 ± 0.03 to 0.81 ± 0.06, $P < 0.01$).

Effects of onjisaponin B on the Ca^{2+}/CaMKII/Glu and Cx43/Glu pathways

The level of intracellular Ca^{2+} was measured by fluorescence intensity. The expression of CaMK protein and Cx43 protein of astrocytes was measured by Western blot analysis, and the expression of CaMKII mRNA and Cx43 mRNA was evaluated by qPCR. The extracellular glutamate concentration was assessed by HPLC-MS.

As shown in Figure 2, at 2 h and 4 h time points, the level of intracellular Ca^{2+} remarkably increased compared with those of the control group. However, onjisaponin B (20 μM) reduced the intracellular Ca^{2+} levels from 150.58 ± 5.06 to 136.17 ± 2.97 ($P < 0.05$) at 2 h and from 148.03 ± 0.68 to 125.16 ± 12.06 ($P < 0.01$) at 4 h time point, respectively.

Compared with the control group, OGD markedly increased the concentrations of CaMKII and Cx43 proteins at 2 h and 4 h of incubation with OGD. At 2 h, onjisaponin B (10 and 20 μM) significantly decreased the levels of Cx43 protein compared with the model group, from 1.1477 ± 0.0668 to 0.9420 ± 0.0461 ($P < 0.01$) and to 0.9014 ± 0.0639 ($P < 0.01$). At 4 h, onjisaponin B (10 and 20 μM) significantly decreased the levels of Cx43 protein (from 0.5627 ± 0.0277 to 0.5114 ± 0.0039, $P < 0.05$ and to 0.5176 ± 0.0122, $P < 0.05$), respectively. As shown in Figure 3b, the rate of CaMKII protein was also decreased by onjisaponin B (10 μM) from 0.6359 ± 0.0409 to 0.5529 ± 0.0196 ($P < 0.05$) at 4 h of incubation with OGD.

The expression of mRNA showed a similar trend [Figure 3a-d]. Compared with the control group, OGD treatment increased the mRNA expression of CaMKII and Cx43. Onjisaponin B (10 μM) significantly decreased the levels of Cx43 mRNA from 4.96 ± 0.56 to 3.44 ± 0.20 ($P < 0.01$) at 4 h, whereas onjisaponin B (20 μM) decreased the levels of both CaMKII and Cx43 mRNA from 4.83 ± 0.18 to 3.84 ± 0.52 ($P < 0.05$) and from 4.96 ± 0.56 to 3.34 ± 0.23 ($P < 0.01$), respectively, at 4 h [Figure 3c].

The concentration of extracellular glutamate in the model group was significantly higher than that in the control group after 2 h and 4 h of OGD ($P < 0.001$) [Figure 3e]. However, onjisaponin B (10 and 20 μM) reduced the extracellular concentration of glutamate at 2 h from 80.47 ± 2.45 to 74.85 ± 1.81 μg/mL ($P < 0.01$) and to 73.61 ± 1.47 μg/mL ($P < 0.001$), respectively, compared with the model group. At 4 h, onjisaponin B (10 and 20 μM) also reduced the extracellular concentration of glutamate compared with the model group (from

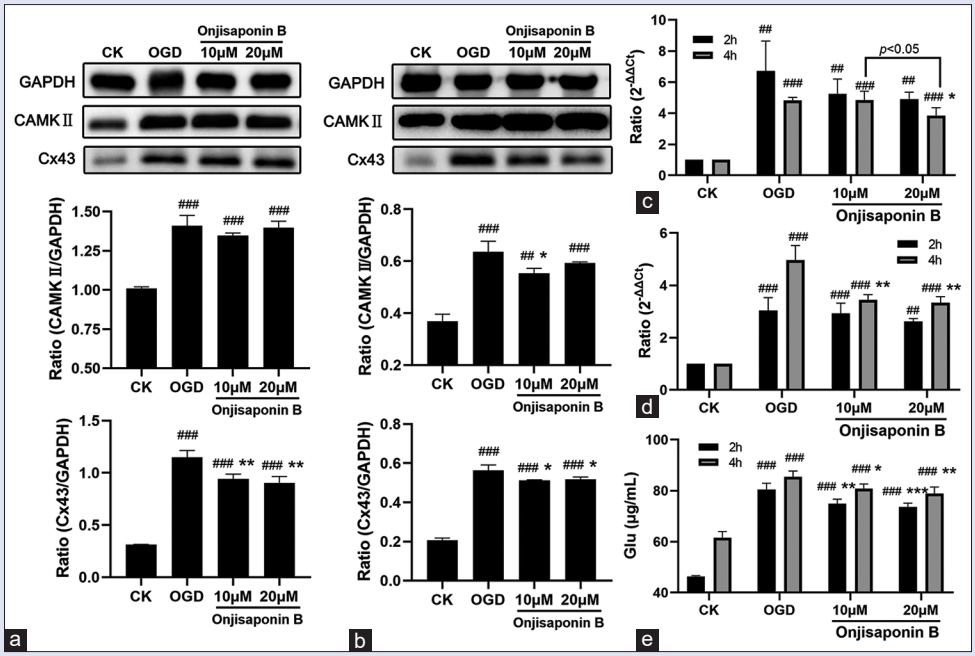

Figure 3: Effects of onjisaponin B on the expression of CaMKII and Cx43 protein of astrocytes at OGD 2 h (a) and OGD 4 h (b). The level of protein was shown by western blots ($n = 3$). The relative expression level of protein was determined with GAPDH densitometric intensity as the control. Effects of onjisaponin B on the expression of CaMKII mRNA (c) and Cx43 mRNA (d) of astrocytes at OGD 2 h and OGD 4 h. The level of mRNA was detected via qPCR ($n = 3$). The relative expression level of mRNA was determined with GAPDH by $^{2-\Delta\Delta Ct}$. (e) Effects of onjisaponin B on glutamate release of astrocytes at OGD 2 h and OGD 4 h. The level of Glu was determined via HPLC-MS. CK: Control group; OGD: Model group; 10 μM onjisaponin B: OGD + 10 μM onjisaponin B group; 20 μM onjisaponin B: OGD + 20 μM onjisaponin B group. $^{\#\#}P < 0.01$ versus CK; $^{\#\#\#}P < 0.001$ versus CK; $^{*}P < 0.05$ versus OGD; $^{**}P < 0.01$ versus OGD; $^{***}P < 0.001$ versus OGD. OGD: Oxygen and glucose deprivation; CaMKII: Calmodulin -dependent protein kinase II; HPLC-MS: High-performance liquid chromatography-tandem mass spectrometer

85.43 ± 2.29 to 80.85 ± 1.77 μg/mL, $P < 0.05$ and to 78.89 ± 2.56 μg/mL, $P < 0.01$).

These results show that onjisaponin B may reduce the release of glutamate from astrocytes by inhibiting the Ca^{2+}/CaMKII/Glu and Cx43/Glu pathways.

DISCUSSION

Astrocytes, the most abundant brain cells, play a very crucial role as neurotransmitters. They are involved in glutamate metabolism, synaptic transmission, regulating ion balance, supporting central nervous system energy metabolism, maintaining brain function stability, and protecting neurons.[26] Astrocytes have a wide gap junction for material exchanges. Cx43 is the primary structural basis constituting gap junction in mammals.[27] As a structural protein regulating the glial network, Cx43 enhances the connection and communication between adjacent cells. It plays an important role in the electrical signal transduction and nutrient metabolism in neurons.[28] The Cx43 gap junction channel and hemichannel were motivated by Ca^{2+}/CaM. The Cx43 hemichannel is closed under normal conditions; however, overexpression of intracellular Ca^{2+}/CaM may open the Cx43 hemichannel and increase the release of small ions and molecules with signaling potential such as glutamate and ATP.[18,29,30]

According to previous studies, ischemia and hypoxia dephosphorylates Cx43, causing conformational changes of the protein which in turn causes functional changes. However, changes in the total amount of Cx43 protein are controversial. Several studies have demonstrated that the changes in the expression of Cx43 are related to different ischemic models and different degrees of cerebral ischemic injury; therefore, the expression of Cx43 protein is different.[17,31] The results of this study revealed that the expression of the Cx43 mRNA and protein in the astrocytes induced by OGD was stronger than that in the control group. After incubation of astrocytes with onjisaponin B, the expression of the Cx43 mRNA and protein was significantly lowered than that in the model group. After OGD, the Cx43 hemichannel dysfunction may cause the abnormal release of glutamate. However, onjisaponin B to some degree reduced this effect of OGD.

Glutamate is the most important excitatory transmitter in the central nervous system and a potential neurotoxin that can damage nerves through excitotoxicity. Moreover, excessive stimulation of the glutamate receptors in the postsynaptic membrane has been revealed to continuously depolarize neurons, thus interfering with neuronal function.[15,32-34] The release of glutamate is calcium-dependent. Many studies have confirmed that with the overload of Ca^{2+}, the levels and activity of CaM and CaMKII in the brain tissue also significantly increase, which might cause the release of glutamate and the symptoms of cerebral ischemia increase after perfusion.[35,36] Moreover, researchers have also shown that hypoglycemia and hypoxia stimulation can induce astrocytes to release glutamate through the Cx43 hemichannel.[37,38] Over-release of glutamate can cause depression.[12,13] The levels of glutamate in the peripheral blood and cerebrospinal fluid of patients with depression are significantly increased and are positively correlated with the severity of depression.[9,11,39]

A recent study reported that in addition to regulating the level of glutamate in the extracellular fluid, astrocytes can also be transported by glutamate reuptake transporter, Ca^{2+} dependent glutamate release coupled with exocytosis, the Cx43 hemichannel, and other pathways actively release glutamate.[40] Similarly, in this study, we found that the concentration of extracellular glutamate was significantly increased after

stimulation of glucose-deficient hypoxia since the concentration of the intracellular Ca^{2+} and CaMKII mRNA and protein were increased. The concentration of extracellular glutamate at 4 h of OGD exposure was higher than at 2 h of OGD exposure. Subsequently, after onjisaponin B intervention, the Ca^{2+}/CaMKII/Glu and Cx43/Glu pathways were significantly inhibited than that in the model group.

CONCLUSION

In summary, it can be said that onjisaponin B acts on astrocytes by attenuating the level of intracellular Ca^{2+} levels and the levels of CaMKII and Cx43 mRNA and protein, thus reducing the release of glutamate from astrocyte and further inhibiting the downstream pathway.

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Conflicts of interest

There are no conflicts of interest.

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