Effect of Gastrodin on Cognitive Dysfunction in Diabetes by Inhibiting PAK2 Phosphorylation

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Abstract

Diabetes and cognitive dysfunction are highly prevalent disorders, while the underlying mechanism is still elusive. The effects of Gastrodin on central nervous system have been emphasized recently. In this study, we aim to explore the potential mechanism leading to cognitive dysfunction in diabetes and the therapeutic effect of Gastrodin. Diabetes was induced by a single injection of streptozotocin. RNA sequencing technique was used to identify the potential factors involved. Western blot and immunofluorescence were applied to detect the protein expression. Our results have shown that spatial learning was impaired and hippocampal pyramidal neurons were damaged in diabetic rats, which could be ameliorated by Gastrodin intervention. Transcriptional analysis identified differential expression genes, which were confirmed by qPCR and western blot. Furthermore, p21 activated kinase 2 (PAK2) was selected and its inhibitor could promote the survival of primary hippocampal neurons. It suggested that PAK2 pathway may be involved in cognitive dysfunction in diabetes and a therapeutic target for Gastrodin intervention.

Introduction

Cognitive dysfunction has been increasingly recognized as a common complication and comorbidity of both type 1 and type 2 diabetes[1]. Up to 20% of type 2 diabetic patients older than 60 years could have dementia[2]. Cognitive dysfunction in diabetes (CID) includes dysfunction in memory, executive function, language and spatial ability[3]. However, the underlying mechanisms between diabetes and cognitive dysfunction are largely unknown.

The hippocampus plays a key role in cognitive functions and is affected in many diseases. The hippocampal CA1 pyramidal neurons are selectively attacked during the progression of Alzheimer’s disease[4]. Diabetes could cause neuronal loss in the hippocampal CA1 region and hippocampal insulin resistance is a potential mediator of CID[5]. The decline of hippocampal neuronal function is closely related to cognitive dysfunction caused by degenerative diseases [6]. To search for the potential factors involved in diabetes-related hippocampal damage, we used RNA-seq to reveal the expression changes of significant differential genes between the hippocampus of diabetic rats and normal controls.

Gastrodia elata is a traditional Chinese herbal medicine, which has been used for the treatment of headache, dizziness, epilepsy, stroke and amnesia for a long time[7]. Gastrodin is a phenolphthalein compound and the main active ingredient of Gastrodia elata, whose therapeutical effects on central nervous system diseases has been frequently reported recently[8]. It works through regulating neurotransmitters[9], restoring vascular function[10], anti-oxidation and anti-inflammatory effects[11]. Since there is no effective treatment of CID, we aim to test the effects of Gastrodin on protecting hippocampal neurons and improving cognitive function in diabetes.

Materials And Methods

Animals

120 adult male Sprague-Dawley rats (Liaoning Changsheng Biotechnology, Liaoning, China) were randomly divided into 4 groups: NC9w (normal control, n=20); DM9w+S group (diabetes mellitus for 9 weeks, n=40); DM9w+G60 group (diabetes mellitus for 9 weeks combined with gastric administration of Gastrodin 60mg/kg/d for 6 weeks, n=30); DM9w+G120 group (diabetes mellitus for 9 weeks combined with gastric administration of Gastrodin 120mg/kg/d for 6 weeks, n=30). Type 1 diabetes was induced by a single intraperitoneal injection of streptozotocin (65 mg/kg), while control rats received the same volume of citrate buffer[10].

Primary Neuron Culture

Primary neurons were prepared from the hippocampus of P0 C57 mouse. Briefly, dissociated hippocampal cells were spread on 6-well plates coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA) and cultured in DMEM at a density of 25 X 10^5 cells/well. After 3.5 h of seeding, the medium was changed to Neurobasal medium (Gibco, Carlsbad, NM, USA) supplemented with B-27 (Gibco). Cells were cultured in a humidified incubator at 37°C with 5% CO2. Cultures were used for experiments 3 to 7
days after seeding, which were divided into NC, DM (50 mM high glucose for 48 hours), DM+G (50 mM high glucose and 30 µM Gastrodin for 24 hours) and DM+I (50 mM high glucose and 5 µM FRAX597 for 24 hours) groups.

**Morris Water Maze Test**

Morris water maze was performed as previously described[12]. All trials were performed in a quiet room with indirect lighting and the animals would be dried under a heater after each experiment. The apparatus was a circular tank with 190 cm in diameter as a swimming pool and contained water at approximately 22 ± 1 °C. Spatial learning was assessed across repeated trials for 7 days. On the first day, the animal was placed into the water for 2 min. On the second day, a circular platform of 15 cm in diameter was positioned 2 cm below the water, and the rats were first put on the platform for 30 s and then guided into the water. If the rats found the platform within 30 s, they would be remaining on it for 30 s. If not, they would be guided to the platform and staying on it for 30 s. Afterwards, the rats were put into the water facing the sidewalls subsequently from one of the four separate quadrants of the pool. If the animal failed to find the platform within 30 s, it would be guided to it and stay there for 30 s. Since the third day, the rats began to be released into the water facing the sidewalls of the pool from one of the quadrants and should find the platform within 120 s. If it succeeds, it would be allowed to stay for 30 s. And if it failed, it would be guided to the platform and stay for 30 s. On the last day, the platform was removed and the rats were released into the water facing the wall of the pool from the farthest point from the platform. In this test, if the rat recalled the position of the platform, it would swim along a shorter path to the platform on the second trial. The rats not managed to remember the platform’s position in the previous days would not be able to locate it easily. Therefore, we compared the differences among these groups to evaluate their spatial learning ability.

**Hematoxylin and Eosin (H&E) staining**

To observe the histological changes, the sections were first incubated with hematoxylin (Beyotime Institute of Biotechnology, Shanghai, China) for 5 min and then washed with 1% ethanol hydrochloride for 3 s. After rinsing with water, the sections were stained with eosin. After this, the sections were captured under a light microscope at the magnification ×400 in a blinded manner.

**RNA-sequencing**

The mRNA libraries were sequenced on the Illumina sequencing platform by Genedenovo Biotechnology Co., Ltd (Guangzhou, China). After the total RNA is extracted from the sample, for eukaryotes, the magnetic beads with Oligo (dT) are used to enrich the mRNA, and the fragmentation buffer is added to the obtained mRNA to make the fragment into a short fragment, and then the fragmented mRNA is used as a template. The first strand of cDNA was synthesized by random hexamers, and the second strand of cDNA was synthesized by adding buffer, dNTPs, RNase H and DNA polymerase I. It was purified by QiaQuick PCR kit and eluted with EB buffer. Add base A, add sequencing linker, and then recover the target size fragment by agarose gel electrophoresis, and carry out PCR amplification to complete the whole library preparation work. The constructed library was sequenced with Illumina HiSeqTM 2500. The SRA data have been uploaded to NCBI (BioProject: PRJNA759189).

**Bioinformatics analysis**

The bioinformatics analysis is mainly divided into three modules: First, the TopHat comparison is performed separately for the reads and reference genomes of each sample, and the comparison results of each sample are obtained. Then the cufflinks are used to assemble the transcripts, and the assembly of each sample is obtained. Second, multiple samples were grouped and combined using cuffmerge according to different treatments, the results of different groups were also combined by cuffmerge, and finally the expression levels of the genes in different groups were obtained. Finally, the predicted gene is analyzed by using edgeR for difference analysis, functional annotation of the differential genes and annotation of new genes.

**qPCR**

The hippocampal tissues were isolated and collected to examine the mRNA level. RNA extraction was conducted by RNAPre pure Tissue Kit reagent (TIANGEN, Beijing, China). After measurement of RNA concentration by spectrophotometer (Bio-Rad, Hercules, CA, USA), the reverse transcription reaction was performed by a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instruction. Then cDNA was used to amplify and quantify
by SYBR Green Realtime PCR Master (Solarbio, Beijing, China). The amplification parameters were 95°C for 2 min followed by 45 cycles of 95°C for 15 s and 58°C for 15 s. The measurement was conducted in triplicate. The mRNA level was normalized to the reference gene GAPDH and shown as relative expression of mRNA by 2^−ΔΔct method.

Western Blotting Analysis

The rats were anesthetized with 10% chloral hydrate administered intraperitoneally. The hippocampal tissues were rapidly dissected and immediately frozen in liquid nitrogen and stored in −80°C. Proteins were extracted from the hippocampal tissues by RIPA buffer (1:1; Beyotime) containing a 1% protease inhibitor cocktail (1:100; Cell Signaling Technology, Danvers, MA, USA) and 1% phosphatase inhibitor cocktails (1:100; Cell Signaling Technology) at 4°C. Homogenates were centrifuged at 12,000 g for 10 min, and the supernatant was collected. Protein concentration was measured using a BCA protein assay kit. The proteins (30 µg) were loaded unto SDS-PAGE gel. The gels were electrophoresed and then transferred to PVDF membranes. After that, the membranes were blocked with a blocking buffer using 5% non-fat milk for 120 min and probed with primary antibodies overnight at 4°C. They were then incubated for 2 h at room temperature with appropriate secondary mouse antibodies (1:1,000; Thermo Fisher Scientific). The following primary antibodies were used for this study: rabbit anti-phosphatidylinositol 3-kinase (PI3K) antibody (1:2000 dilution; Abcam, Cambridge, MA, USA), rabbit anti-AKT antibody (1:2,000 dilution; ABclonal, Woburn, MA, USA), rabbit anti-p-AKT antibody (1:1,000 dilution; CST), rabbit anti-PAK2 antibody (1:2000 dilutions; Abcam), rabbit anti-p-PAK2 antibody (1:1000 dilution; CST), mouse anti-GLUT4 antibody (1:2000 dilution; Santa Cruz, Dallas, TX, USA) and β-tubulin (1:2,000; Santa Cruz). The blots were developed with enhanced chemiluminescence and densitometric analysis of the film was accomplished with ImageJ software (version 1.4.3.67).

Double Immunofluorescence

The hippocampus was dissected, immersed in 4% formaldehyde, dehydrated, cleared with xylene, and embedded in paraffin blocks. Paraffin sections of 4 µm thickness were deparaffinized and hydrated through a series of graded alcohol. The tissues were incubated in citrate buffer for antigen retrieval and the slices were incubated with 5% normal goat serum. The following primary antibodies were used: rabbit anti-PI3K antibody (1:1,00 dilution; Abcam), rabbit anti-p-AKT antibody (1:25 dilution; CST) and mouse anti-GLUT4 antibody (1:20 dilution; Santa Cruz). Primary antibodies were added in a fresh blocking solution and incubated overnight at 4°C. The staining was visualized with anti-mouse and anti-rabbit Alexa Fluor 488 and 568 2nd antibodies (1:500; A11010, A11001, Invitrogen, Waltham, MA, USA). After washing in PBS, secondary antibodies were added in PBS containing 0.1% Triton X-100 to prevent nonspecific antigen binding for 2 h at room temperature. Tissue sections were viewed, and images captured on an Olympus FV1000 microscope.

CCK8 Assay

Cells (4 × 10^4/well) were seeded in 96-well culture plates and exposed to different conditions as above. Cell viability was then determined by Cell Counting Kit-8 (CCK8) (Beyotime, China) assay. After the exposure, 10 µl of CCK8 solution was added to each well, and the plates were incubated for an additional 2 h at 37°C. Cell viability was measured as the absorbance at 450 nm with a microplate reader (Bio-Rad, USA) and expressed as a percentage of the control level. The mean optical density values from six wells for each treatment were used as the index of cell viability.

Statistical processing

Statistical analysis was performed using the SPSS 17.0 statistical software package. The data of each group were expressed as mean±standard deviation ( ), and the one-way ANOVA was used to compare the sample groups.

Results

Gastrodin Intervention Improved Cognitive Function and Ameliorated Pathological Changes in Diabetic Rats
To evaluate the effects of Gastrodin intervention on cognitive function, Morris water maze test was performed. Diabetic rats exhibited significantly higher escape latency than the controls (p<0.01), which was significantly decreased after 60 mg/kg of Gastrodin intervention (Figure 1A). In the space exploration stage, the number of platform crossing and the target quadrant dwelling time were significantly decreased in the DM9w+S group than that of the NC9w group (p<0.01). 60 mg/kg of Gastrodin intervention significantly increased the target quadrant dwelling time of diabetic rats, while it had no effect on the number of platform crossings (Figure 1B-C). Interestingly, 120 mg/kg of Gastrodin intervention showed no therapeutic effect on the performance of Morris water maze test.

H&E staining showed that neurons in the CA1 pyramidal region of the hippocampus were with intact structure and clear nuclei in the NC9w group. In addition, the axon of the radiatum layer was extended and arranged neatly. In the DM9w+S group, the number of pyramidal neurons in the CA1 region of the hippocampus was noticeably decreased. Furthermore, the cytoplasm was reduced, the nucleus exhibited edema, and the axon extension of the radiatum layer was decreased and disarranged. Compared with the DM9w+S group, the number of pyramidal neurons in the hippocampal CA1 area was higher both in the DM9w+G60 and DM9w+G120 group. The cytoplasm of neurons in both groups was abundant, their nuclei were clear, and the axons of the radiation layer were arranged neatly (Figure 1D-G).

**Screening Possible Mechanisms Underlying Diabetes-induced Cognitive Dysfunction and Gastrodin Intervention by RNA-seq Analysis**

The differentially expressed genes (DEGs) were screened by FDR and log2FC under the conditions of FDR<0.05 and |log2FC| >0.1. Compared with the NC9w group, DM9w+S group had 166 upregulations of DEGs and 554 downregulations; DM9w+G60 group had 145 up-regulations and 641 downregulations; DM9w+G120 group had 128 upregulations and 382 down-regulations on DEGs. Compared with the DM9w+S group, there were 126 up-regulation and 238 down-regulation of DEGs in the DM9w+G60 group; 92 up-regulation and 74 down-regulation in the DM9w+G120 group. Compared with the DM9w+G60 group, there were 181 up-regulations and 84 down-regulations in the DM9w+G120 group (Figure 2A-B).

The properties of DEGs were described according to the internationally standardized gene function classification system Gene Ontology (GO). The molecular function, cellular component and biological progress of the genes were respectively described. GO analysis showed that the main modules of these DEGs are cellular process, transmitter transport and extracellular component changes. Pathway enrichment analysis with KEGG Pathway was applied to find significant enrichment pathways in sub-differential expression compared with the entire genetic background. The first 20 significantly enriched pathways between different groups were found, which included cell adhesion molecule pathway, phagocytic pathway, extracellular matrix receptor interaction pathway, PI3K-AKT signaling pathway, focal adhesion pathway, etc (Figure 2C). Furthermore, DEGs with fold change ≥ 5 were selected according to the behavioral changes assessed by the Morris water maze test (Table 1).
| Gene ID            | Symbol | Pathway                     | GO component       | GO function              | GO process                     |
|-------------------|--------|-----------------------------|--------------------|--------------------------|--------------------------------|
| ENSRNOG00000002070| Mrpl1  | ko03010//Ribosome            | cytoplasmic part   | nucleic acid binding     | peptide metabolic process      |
|                   |        |                             |                    |                          |                                |
| ENSRNOG00000009331| Hck    | ko04062//Chemokine signaling pathway | cellular component | transferase activity     | organelle organization          |
|                   |        |                             |                    |                          |                                |
| ENSRNOG00000010597| Slc5a7 | ko04725//Cholinergic synapse | intracellular organelle | transporter activity     | transmembrane transport        |
|                   |        |                             |                    |                          |                                |
| ENSRNOG00000012686| Pomc   | ko04916//Melanogenesis       | extracellular space | molecular function       | regulation of biological process |
|                   |        |                             |                    |                          |                                |
| ENSRNOG00000018827| Htr7   | ko04014//Ras signaling pathway | intracellular membrane-bounded organelle | signal transducer activity | single-organism cellular process |
|                   |        |                             |                    |                          |                                |
| ENSRNOG00000047864| RT1-DMa| ko04612//Antigen processing and presentation | plasma membrane | –                        | biological process             |
|                   |        |                             |                    |                          |                                |
| ENSRNOG00000048597| Pak2   | ko04010//MAPK signaling pathway | membrane-bounded vesicle | catalytic activity        | cellular protein metabolic process |
|                   |        |                             |                    |                          |                                |
| ENSRNOG0000003160 | LOC100909655 | mTOR signaling pathway | endosome | molecular function        | response to stimulus           |
|                   |        |                             |                    |                          |                                |
| ENSRNOG0000004372 | Cbln4 | –                           | cellular component | molecular function        | biological process             |
|                   |        |                             |                    |                          |                                |
| ENSRNOG0000005387 | Rbm3   | –                           | intracellular membrane-bounded organelle | heterocyclic compound binding | –                              |
|                   |        |                             |                    |                          |                                |
| ENSRNOG0000005943 | Sp8    | –                           | –                  | binding                  | single-multicellular organism process |
|                   |        |                             |                    |                          |                                |
| ENSRNOG0000011459 | Rhbdf2| –                           | endoplasmic reticulum | –                        | regulation of biological process |
|                   |        |                             |                    |                          |                                |
| ENSRNOG0000019351 | LOC100911881 | –                         | cellular component | catalytic activity        | carbohydrate metabolic process |
|                   |        |                             |                    |                          |                                |
| ENSRNOG0000031743 | Gbp2   | –                           | cell               | molecular function        | biological process             |
|                   |        |                             |                    |                          |                                |
| ENSRNOG0000047651 | ProSAP1| –                           | –                  | –                        | –                              |
|                   |        |                             |                    |                          |                                |
| ENSRNOG0000049913 | Rbm12  | –                           | –                  | molecular function        | –                              |
|                   |        |                             |                    |                          |                                |
| ENSRNOG0000052512 | Vps37c | –                           | –                  | –                        | –                              |
|                   |        |                             |                    |                          |                                |
| ENSRNOG0000053115 | Zfp82  | –                           | intracellular      | ion binding              | nitrogen compound              |
|                   |        |                             |                    |                          |                                |
PAK2, Mrp11, RagA/B and Sp8 were selected for further confirmation by qPCR test. The result showed that the expression of Mrp11, RagA and RagB in DM9w+S group was not significantly higher than that of the NC9w group. By contrast, the expression of PAK2 and Sp8 was significantly increased after diabetes induction. In addition, their expression in the DM9w+G60 and DM9w+G120 group was significantly lower than that of the DM9w+S group, which was consistent with transcriptome changes (Figure 2D-H). Because diabetic rats exhibited impaired learning ability and Gastrodin had a therapeutic effect, we reviewed the reports on cognition and found that PI3K-AKT signaling pathway was associated with insulin-mediated cognitive function. Moreover, PAK2 was closely related with PI3K-AKT insulin signaling, which downwardly resulted in reduced glucose uptake under pathological conditions through glucose transduction receptors GLUT4. It would lead to the damage of insulin-dependent neurons and compromised cognitive function. Therefore, PAK2 and PI3K/AKT/GLUT4 signaling pathway was selected for further investigation.

**Gastrodin Intervention Inhibited the Phosphorylation of PAK2 Through Regulating PI3K/AKT Pathway in Diabetic Rats**

The expression of PI3K, p-AKT and GLUT4 in the hippocampus of DM9w+S group was significantly lower than that of the NC9w group, while the expression of p-PAK2 was significantly higher. After 60 mg/kg of Gastrodin intervention, the expression of PI3K, p-AKT, and GLUT4 was significantly increased in diabetic rats, while the expression of p-PAK2 was significantly decreased. However, 120 mg/kg of Gastrodin could not restore the expression of PI3K, p-AKT, and GLUT4 in diabetic rats, except influencing the phosphorylation of p-PAK2 (Figure 3).

Double immunofluorescence staining showed that PI3K, p-AKT and GLUT4 were localized primarily in the cell body of the hippocampal neurons. The hippocampal neurons emitted intense PI3K immunofluorescence in the NC9w group, which was markedly attenuated in the DM9w+S group. After 60 mg/kg of Gastrodin intervention, the expression of PI3K was restored to a comparable intensity. Furthermore, the number of hippocampal neurons was decreased in the DM9w+S group in comparison with the NC9w and DM9w+G60 groups (Figure 4A). p-AKT and GLUT4 immunofluorescence in the Purkinje cells exhibited similar expression changes in the above groups (Figure 4B-C).

**Inhibition of PAK2 Improved the Survival of Hippocampal Neurons in vitro Through PI3K/AKT Pathway**

To investigate the mechanism of diabetes-induced neuronal death, we examined the effects of PAK2 inhibition on neurotoxicity under high-glucose exposure. CCK-8 analysis of primary hippocampal neurons showed that hyperglycemia caused a significant reduction of cell viability, which could be restored by FRAX597, a PAK2 inhibitor, in a dose-dependent manner. 5, 10 and 20 µM of FRAX597 intervention had a significant effect on the cell viability of primary neurons, while the effect of 2 µM FRAX597 was insignificant (Supplementary Figure 1). Since there was no significant difference between the concentration of 5 µM and 20 µM, 5 µM was selected for the subsequent experiments.

The expression of PI3K, p-AKT, p-PAK2 and GLUT4 in the primary neurons has shown similar changes compared with the hippocampus. The protein expression of PI3K, p-AKT and GLUT4 was significantly decreased after high glucose intervention, while p-PAK2 was significantly increased. After both 30 µM of Gastrodin and 5 µM of FRAX597 treatment, the expression of PI3K, p-AKT and GLUT4 was significantly increased and p-PAK2 was significantly decreased (Figure 5).

Double immunofluorescent staining has shown that PI3K, p-AKT and GLUT4 were distributed mainly in the cell body of the primary hippocampal neurons (Figure 6). The diminution of the above factors after high glucose exposure could be restored both by Gastrodin and PAK2 inhibitor intervention. Moreover, the number of primary hippocampal neurons exhibited a drastic reduction in the DM group. Gastrodin and PAK2 inhibition showed a protective effect on the survival of neurons.

**Discussion**

This present study has shown that diabetes could cause hippocampal neuronal damage and impairment of spatial learning. Our study is the first to screen for the key factors for underlying the therapeutic effects of Gastrodin on diabetes-induced hippocampal injury using RNA-seq. After screening the relevant enrichment pathways, we selected PAK2 and PI3K/AKT
signaling pathway for further investigation. We compared the expression of PAK2 between normal controls, diabetic rats and diabetic rats with Gastrodin intervention. We found that PI3K/AKT signaling pathway was downregulated in diabetic rats, while p-PAK2 expression was increased and GLUT4 expression decreased. After Gastrodin intervention, PI3K/AKT signaling pathway was restored, coupled with decreased p-PAK2 expression and increased GLUT4 expression. This indicated that PAK2 activation in diabetes contributed to decreased GLUT4 expression through PI3K/AKT pathway. As a result, it reduced neuronal glucose uptake and led to decreased hippocampal neuronal energy and neuronal injury. Suppressing PAK2 activity by Gastrodin intervention or PAK2 inhibitors can restore neuronal glucose uptake and protect hippocampal neurons.

CID is one of the typical central nervous system complications of DM, but its pathogenesis is far from clear[13]. In the brain, insulin signaling is involved in processes including neurogenesis, cognition, eating behavior, and glucose metabolism[14]. Previous studies have shown that insulin signaling dysfunction is an important mechanism of diabetic complications, including cognitive dysfunction[15]. However, the underlying mechanism of insulin signaling dysfunction is still unknown. The result of our bioinformatic analysis suggested that P21-activated kinase 2 (PAK2) could be an important factor of CID, which may regulate the insulin signaling function and glucose metabolism in the hippocampus.

P21-activated kinase (PAK) is an important component of glucose homeostasis in the muscle, pancreas and liver by mediating insulin signaling[16, 17]. Previous studies have found that PAK2 is involved in neuronal insulin signaling, glucose uptake and insulin resistance[18]. However, its role in neuronal insulin signaling is still unknown[19]. We found that the expression of PAK2 was elevated after diabetes, which suppressed the expression of GLUT4 through PI3K/AKT pathway. Other independent studies have also indicated that PI3K/AKT is involved in the regulation of glucose uptake, which were regulated by PAK2 in the neurons[17]. In Alzheimer's disease, impaired insulin signaling could inhibit the PI3K/AKT pathway and lead to neurodegeneration by increasing oxidative stress, apoptosis, mitochondrial dysfunction and necrosis[20].

The activity of PI3K/AKT pathway is related to the phosphorylation of AKT. Phosphorylation of AKT, a key downstream protein of PI3K, would be downregulated at high glucose level[18]. This signal transduction dysregulation caused by inhibition of AKT phosphorylation is the key to hyperglycemia induced cognitive deficits[21, 22]. Furthermore, inhibition of AKT and PI3K expression by treatment with AKTi-1/2 and wortmannin reduced insulin regulation of PAK2 and glucose uptake[23].

GLUT4, an insulin-dependent glucose transporter, is involved in the uptake of glucose by neurons[24]. This process is associated with normal cognitive formation[25]. In the neurons, an increase in neurotransmitter activity is also considered as an indirect effect of insulin-dependent glucose uptake, by which insulin regulates cognitive activity[26]. We have found that increased expression of PAK2 in the hippocampus of diabetic rats suppressed GLUT4 expression, which further reduced glucose uptake, supported by a study indicating that overexpression of PAK2 could reduce GLUT4 expression and glucose uptake[27]. Moreover, inhibition PAK2 expression restored the expression of GLUT4 and protect hippocampal neurons.

Finally, we found that Gastrodin intervention had a similar effect on CID as PAK2 inhibitor, suggesting that it could be a potential treatment for CID[28, 29]. Recently, the therapeutic effects of Gastrodin have been widely investigated. Some clinical trials have found the beneficial outcome of Gastrodin on patients with vascular dementia[8, 30]. Though many studies have found that Gastrodin treated diabetes through PI3K/AKT pathway[31, 32], our study was the first to report that the PAK2 is important for the effect of Gastrodin. Our result also found that 60 mg/kg of Gastrodin intervention had a better outcome than 120 mg/kg. This could be partly explained by the lower expression of PI3K/AKT/GLUT4 signaling pathway.

Declarations

Ethics approval

Animal procedures were reviewed and approved by the Medical Ethics Committee of Kunming Medical University, Kunming, China.

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**Consent for publication**

The authors declare that no human subjects were involved in the study.

**Data availability statement**

The sequencing data has been deposited into the Sequence Read Archive (accession: PRJNA759189). Other datasets generated during the current study are available from the corresponding author on reasonable request.

**Conflict of interest**

Author Yi-Dan Liu was employed by the company Kunming Pharmaceutical Corporation. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Author contributions**

Ying-Ying Zou, Yue-Qin Zeng and Zhi-Hong Yang designed the project and finalized the manuscript. Zhi-Hao Mu, Zhi-Min Zhao and Su-Su Yang performed most of the experiments, participated in discussion, analyzed the data, and prepared the first draft of the manuscript. Yi-Dan Liu designed and guided the use of Gastrodin. Lei Zhou, Zhong-Yi Qian, Xin-Jie Liu and Peng-Chao Zhao conducted part of experiments, participated in discussion, and analyzed the data. Ren-Bo Tang and Jia-Yin Li performed the paraffin embedding, sectioning, and H&E staining. Jing-Yao Zeng conducted the part of experiments and helped with removal of tissue samples and took care of the experimental rats. Yong-Hua Ruan revised the manuscript. Ying-Ying Zou is the corresponding author, Yue-Qin Zeng and Zhi-Hong Yang are the co-corresponding author of this manuscript.

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“Not applicable”

**Consent to participate**

“Not applicable”

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Figures
Figure 1

Gastrodin intervention ameliorated diabetes-induced cognitive dysfunction and pathological changes in the CA1 area of the hippocampus. Escape latency (A), number of platform entries (B) and time spent in target quadrant (C) of rats from the NC9w, DM9w+S, DM9w+G60 and DM9w+G120 groups in the probe trial. (D-G) H&E staining of CA1 area of hippocampus in the NC9w, DM9w+S, DM9w+G60 and DM9w+G120 groups. Black arrows indicated the nucleus of the damaged neuron and arrowheads indicated the synapse. Bar = 50 μm. *p < 0.05, **p < 0.01.
Figure 2

The transcriptomic profile of Gastrodin intervention on the hippocampus of diabetic rats and further confirmation. (A) Differentially expressed genes among NC9w, DM9w+S, DM9w+G60 and DM9w+G120 group. (B) Volcano plot of all genes based on their log2 fold-change and adjusted P-values between NC9w and DM9w+S group. Differentially expressed genes were classified at an adjusted P-value of < 0.05. (C) The KEGG pathways enriched between NC9w and DM9w+S group. The rich factor refers to the ratio of the number of DEGs to the number of total annotated genes in a certain pathway. (D-H) qPCR analysis of the mRNA expression of PAK2 (D), Mrpl1 (E), Sp8 (F), RagA (G) and RagB (H) in the hippocampus of the NC9w, DM9w+S, DM9w+G60 and DM9w+G120 groups. *p < 0.05 and **p < 0.01.
Gastrodin intervention suppressed diabetes-induced PAK2 phosphorylation and activated PI3K-AKT pathway. Western blot analysis of PI3K (A), p-PAK2 (B), p-AKT (C) and GLUT4 (D) protein expression in the hippocampus of the NC9W, DM9W+S, DM9W+G60 and DM9W+G120 groups. Bar graphs represented optical density of these factors normalized with β-actin, while p-PAK2 and p-AKT were further normalized with total-PAK2 and total-AKT respectively. ∗p < 0.05 and ∗∗p < 0.01.
Gastrodin intervention restored the expression of PI3K-AKT pathway in the hippocampal neurons of diabetic rats. Double immunofluorescence staining showed PI3K (A), GLUT4 (B) and p-AKT (C) positive neurons in the hippocampus of NC9w, DM9w+S, DM9w+G60 and DM9w+G120 groups. Note the diminution of these factors’ immunofluorescence in the neurons of the DM9w+S group as compared with the normal control. However, the immunofluorescence was restored to a level comparable to that of the normal in the DM9w+G60 group. White arrows indicated double positive cells. Bar = 50 μm.
The effects of Gastrodin intervention and PAK2 inhibition on the PI3K-AKT pathway in the primary hippocampal neurons during hyperglycemia. Western blot analysis of PI3K (A), p-AKT (B), PAK2 (C) and GLUT4 (D) protein expression in the primary hippocampal neurons of the NC, DM, DM+G and DM+I groups. Bar graphs represented optical density of these factors normalized with β-actin. *p < 0.05, **p < 0.01 and ***p < 0.001.
Gastrodin intervention and PAK2 inhibition restored the expression of PI3K-AKT pathway in the primary hippocampal neurons exposed to high glucose. Double immunofluorescence staining of PI3K (A), GLUT4 (B) and p-AKT (C) with NeuN in the primary hippocampal neurons of NC, DM, DM+G and DM+I groups. Bar = 50 μm.

Supplementary Files

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