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Original Article

Inhibition of synaptophysin ubiquitination may improve the intelligent drop due to high glucose and hypoxia

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Abstract: Synaptophysin (SYP) is a synaptic vesicle membrane protein and involved in the release of neurotransmitters, synaptic plasticity, and formation and recycling of synaptic vesicles. SYP may serve as a specific marker of synaptic proteins. Our results showed high glucose and hypoxia could significantly impair the intelligence and the SYP level was positively related to the degree of intelligence in animals. Ubiquitination may influence the SYP expression. In our study, an ubiquitination inhibitor (MG-132) was injected intraperitoneally. Results showed this could increase SYP expression in the brain to improve the intelligent drop due to high glucose and hypoxia in animals. SYP ubiquitination is closely associated with E3 ubiquitin ligase, a mammalian homologue of seven in absentia. In our study, lentivirus expressing siah shRNA was injected into the cerebral ventricle to down-regulate siah expression, and results showed the SYP expression in the brain increased markedly. In addition, we also found that VitK3 could increase the SYP expression in the brain to improve the intelligent drop due to high glucose and hypoxia.

Keywords: Synaptophysin, shRNA, ubiquitination, high glucose, hypoxia

Introduction

Diabetes mellitus (DM) can cause extensive changes in neuronal structure, neurotransmitters, neurophysiology and circulation. Chronic disorder of glucose metabolism may induce a series of changes in brain physiology, including cognition impairment, brain signal transduction and synaptic plasticity [1, 2]. Some investigators have found that DM patients usually present impaired cognition, and the MMSE score, proportion of errors in response to questions, number of words learned, the ability to master words that have been learned, transient and delayed memory of DM patients were markedly different from those of healthy controls [3-7]. DM can deteriorate the cognition impairment, AD and vascular dementia [8-10]. Epidemiological studies have shown that high blood glucose [11] and insulin resistance [12] are associated with the impaired cognition in the elderly. Studies on the electroencephalogram also reveal the diffuse abnormality of brain function in DM patients [13, 14]. Ryan et al [15] found that DM chronically affected the central nervous system, and the cognition impairment in old patients with DM was more evident than that in young patients with DM. A variety of studies have demonstrated that the occurrence of cognition disorder is closely related to the aging, and to control the glucose metabolism may be effective to improve the cognition in these patients [16]. The long-term depression (LTD) hippocampus is related to the learning and memory and also impaired in the old rats with DM [17]. Thus, increasing patients with DM pay attention to the cognition disorder due to the aging.

High blood glucose is not the only contributor of cognition impairment in DM patients, and there are multiple pathological changes in DM patients. The cognition impairment in DM patients is also attributed to multiple factors [18]. More than 75% of DM patients may develop obstructive sleep apnea (OSA) [19]. Several studies have revealed that OSA is closely associated with glucose tolerance and insulin resistance [20, 21]. The pathophysiology of OSA includes hypoxia and change in the sleep archi-

tecture. Hypoxia is closely related to insulin resistance, which has been confirmed in several trials [22, 23]. The disordered sleep architecture may reduce the sensitivity to insulin and impair the glucose metabolism, which have been studied extensively [21, 24]. Hypoxia is an important risk factor of cognition disorder, and the more severe the hypoxia and the higher the frequency of hypoxia, the more severe the cognition impairment is. Available experiments have confirmed that short-term hypoxia may cause obvious reduction in memory [25]. In this study, we aimed to investigate the influence of high blood glucose and hypoxia on the intelligence of animals and explore the modalities for the improvement of cognition in DM patients.

SYP is one of specific markers of synaptic vesicles, and the regulation of SYP expression might be associated with learning and memory [26]. Thus, SYP was selected as a target protein in this study. There is evidence showing that SYP ubiquitination is closely associated with E3 ubiquitin ligase siah [17]. In this study, we explored the relationship between siah and SYP expression in the brain of animals undergoing high blood glucose and hypoxia, investigated the drugs which may alter the SYP expression in the brain and tested the influence of these drugs on the intelligence of these animals.

Materials and methods

Reagent

SIAH-1 (N-15): sc-5505: SANTA CRUZ BIOTECHNOLOGY, INC. Synaptophysin antibody: Sigma No. S5768. STREPTOZOTOCIN MIXED ANOMERS (STZ): Sigma No. S0130. Vitamin K3: M5625-Menadione: Sigma No. 58275.

Animal modeling and treatments

This study was performed in the Central Laboratory of Shanghai 10th People's Hospital and according to the Guide for the Care and Use of Laboratory Animals. In the experiments, measures were taken to minimize the suffering and number of animals.

DM animal model: Female Kunming mice aged 2-3 months were generally feed for 2 weeks and then received food deprivation for longer than 12 h (without water deprivation). STZ was dissolved in 0.1% mM citrate buffer (pH = 4.2) and injected at 60 mg/kg/d intraperitoneally once daily for consecutive 3 days. DM was defined when the fasting blood glucose level was > 16.7.

Hypoxia treatment in DM animals: DM mice were placed in a chamber which was ventilated continuously with 8% 02 for 12 h and then air for 12 h for a total of 7 days.

MG132 was dissolved in DMSO and injected intraperitoneally at 0.5 mg/kg into DM mice undergoing hypoxia for consecutive 3 days. Vitamin K3 was dissolved in DMSO and intraperitoneally injected at 2 mg/kg into DM mice undergoing hypoxia for 3 consecutive days. In control group, DMSO of equal volume was injected intraperitoneally for 3 consecutive days.

Water maze test

Animals were placed in water with the head toward wall at one quadrant, and the time of animals staying on the platform was recorded (s). The animals were trained several times and then guided to the platform when the time was longer than 60 s. Thereafter, animals were allowed to stay on the platform for 10 s.

The animals were taken out of the water and dried. If necessary, animals were dried under a lamp for 5 min. Then, these animals were placed back into cages. Each animal was trained 4 times every day with an interval of 15-20 min between trainings which was done for 5 days.

After the final acquired training, the platform was removed on the second day, and exploration training was performed for 60 s. Animals were placed at the opposite quadrant in which animals were trained. Then, the time to enter the quadrant whether the platform located and the number of entering this quadrant were recorded as indicators for the evaluation of spatial memory.

Immunohistochemistry

Frozen sections of the hippocampus were prepared from animals undergoing high blood glucose and hypoxia. Brain sections were placed in 6-well plates and blocked with 3% hydrogen peroxide for 10 min. After removal of hydrogen peroxide, sections were washed with PBS twice.

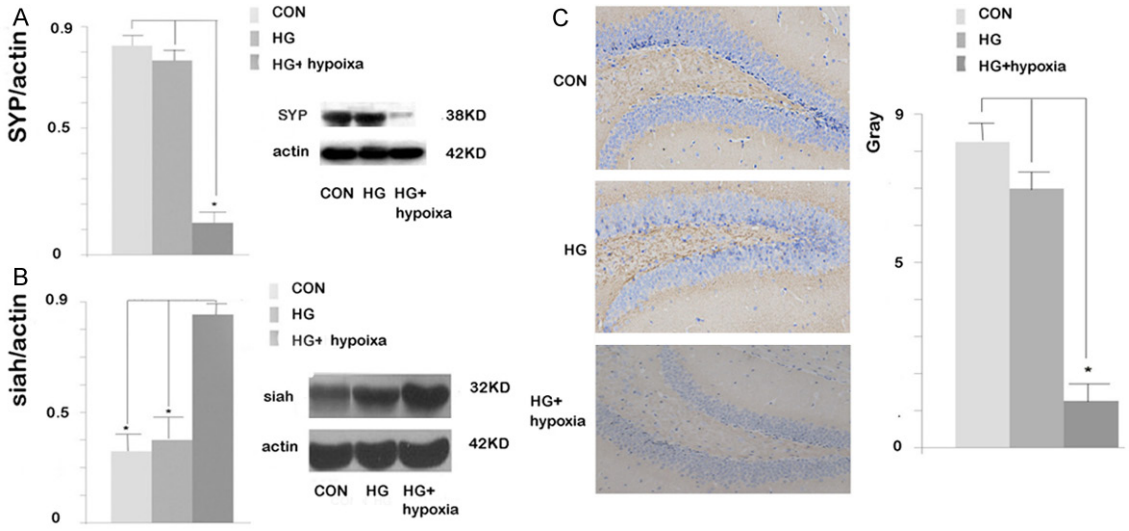

Figure 1. The expression of SYP and siah in blank control group, DM group and DM + hypoxia group. Hippocampus was collected from mice in blank control group, DM group and DM + hypoxia group, and western blot assay was performed to detect the SYP expression (A) and siah expression (B): the SYP expression in DM + hypoxia group was markedl lower than that in other two groups ($P < 0.01$); the siah expression in DM + hypoxia group was markedl higher than that in other two groups ($P < 0.05$). Immunofluorescene staining of SYP in the hippocampus of blank control group, DM group and DM + hypoxia group, and results showed the SYP expression in DM + hypoxia group was markedly lower than that in other two groups (C).

Then, these sections were incubated with primary antibody at 4°C overnight. Following washing in PBS twice, sections were treated with fluorescence conjugated secondary antibody at room temperature for 2 h. Visualization was done for 10 min. After washing in PBS twice, sections were transferred onto slides and dried. Dehydration was done with 70% ethanol for 30 min, 95% ethanol for 3 min, 100% ethanol for 3 min, 100% ethanol for 2 min, xylene for 2 min and xylene for 3-5 min.

Immunofluorescence staining

Brain sections were placed in 6-well plates and blocked in goat serum for 20-40 min. Following removal of serum, sections were washed in PBS twice and then treated with primary antibody in PBS at 4°C overnight. Following washing in PBS twice, sections were treated with fluorescence conjugated secondary antibody at room temperature for 2 h. After washing in PBS twice, sections were transferred onto slides and dried, followed by addition of anti-quencher. Sections were observed under a fluorescence microscope after mounting. And photomicrographs were captured using an Olympus microscope.

Western blot assay

In brief, total protein was extracted from the hippocampus and protein concentration was determined with BCA method. Then, 50 μg of protein was used for electrophoresis at 80 V for 2 h and transferred onto PVDF membrane at 20 mA for 1 h. The membrane was blocked in blocking medium for 1 h. Following treatment with primary antibody (1:1000) at 4°C overnight, the membrane was washed in PBST thrice at room temperature. Then, the membrane was treated with secondary antibody (1:1000) at room temperature for 2 h, followed by washing in PBST thrice at room temperature. This membrane was scanned, and protein expression was determined. The protein bands were scanned with the Odyssey color infrared fluorescence imaging system (LI-COR Company, USA). The expression of target proteins was normalized to that of β-actin.

Construction of lentivirus

The siah shRNA was designed, and finally two encoding sequences (CTGCATCCAACAATGACTTGGACTGACCGGTCTGCATCCACCAATGACGTGGGTTATATTCAAGCACCAAGTCATTGTTGGATGCAGTTTTTGAATTCACTG and GTCCATTACCACC-

CTGCAAGGACTGACCGGTGTCCATTACTACCCTGCCAGGGTTATATTCAAGCACCTTGCAGGGTGGTAATGGACTTTTTGAATTCACTG) and one non-encoding sequence (GCTGTTAATACAGGAAACAGTACTGACCGGTGCTGTTAATCCAGGAAAAAGTGTTATATTCAAGCAACTGTTTCCTGTATTAACAGCTTTTTGAATTCACTG) were selected. These shRNAs were digested with EcoR I and AgeI, and positive colonies were selected for extraction of cDNA after connection and transduction. Then, these cDNA was transfected into 293FT cells and lentivirus expressing siah shRNA was prepared. This lentivirus was used to infect N2A cells. Western blot assay and RT-PCR showed 3 shRNA could reduce the siah expression by about 75%.

Intraventricular injection

Mice were intraperitoneally anesthetized with 3% chloral hydrate at 40 mg /kg) and then fixed in a stereotaxic instrument. After preparation of skin, a midline incision was made at the head and the anterior fontanelle was exposed. The needle was inserted into 2.2 mm at a site 0.67 mm before anterior fontanelle and 0.6 mm lateral to anterior fontanelle. Then, 3 μL MG132 (10 g/L)/siah lentivirus was slowly injected, and the needle was allowed to stay in the cerebral ventricle for 5 min. After withdraw of the needle, the wound was closed and animals were housed in a temperature controlled room. In control group, 3 μL DMSO/blank lentivirus was injected.

Statistical analysis

Immunofluorescence Images were viewed and captured using an Olympus microscope, and the Image-Pro Plus software 6.0 was used to analyze the immunofluorescence intensity. The product of intensity and area of protein bands represents the relative protein expression. All values are expressed as mean ± S.E. Differences were analyzed using either one-way or two-way ANOVA followed by Newman-Keuls post hoc testing for pairwise comparisons using SPSS. The null hypothesis was rejected when the *P* value < 0.05.

Results

Intelligent drop in animals undergoing high blood glucose and hypoxia

Mice in blank control group, high blood glucose group and high blood glucose + hypoxia group (n = 10) were subjected to test of intelligence by water maze test. Results showed the intelligence level in high blood glucose + hypoxia group was significantly lower than that in control group and high blood glucose group, but no marked difference was observed between control group and high blood glucose group. This suggests that combined high blood glucose and hypoxia may cause intelligent drop, and high blood glucose alone or transient blood glucose has no significant influence on the intelligence.

SYP expression in the brain of animals with intelligent drop

SYP is one of specific markers of synaptic vesicles and a calcium-binding protein closely related to the structure and function of synapses. The regulation of SYP expression is associated with the learning and memory [26]. In this study, Western blot assay was performed to detect the expression of SYP and siah in the hippocampus and results showed the SYP expression in high blood glucose + hypoxia group was markedly lower than that in other group (**Figure 1A**), but the siah expression high blood glucose + hypoxia group was significantly higher than that in other groups (**Figure 1B**). That is, the intelligence level was positively associated with SYP expression and negatively with siah expression. In animals, siah may influence SYP expression. In addition, immunofluorescence staining was performed for SYP in different groups, and similar findings were obtained: SYP expression in high blood glucose + hypoxia group increased markedly when compared with other groups (**Figure 1C**).

MG132 reduces SYP ubiquitination to improve intelligence

MG132 is a common inhibitor of proteasome and can enter cells to selectively inhibit proteasome. In high blood glucose + hypoxia group, mice were intraperitoneally injected with 20 μM MG132 for 3 days, and animals in control group was injected with 100 μl of DMSO. Western blot assay was performed to detect the expression of siah and SYP in the brain. Results showed MG132 could significantly increase the SYP expression in the brain when compared with control group (**Figure 2A**). In addition, immunofluorescence staining was done for SYP. Results also revealed that MG132 treatment signifi-

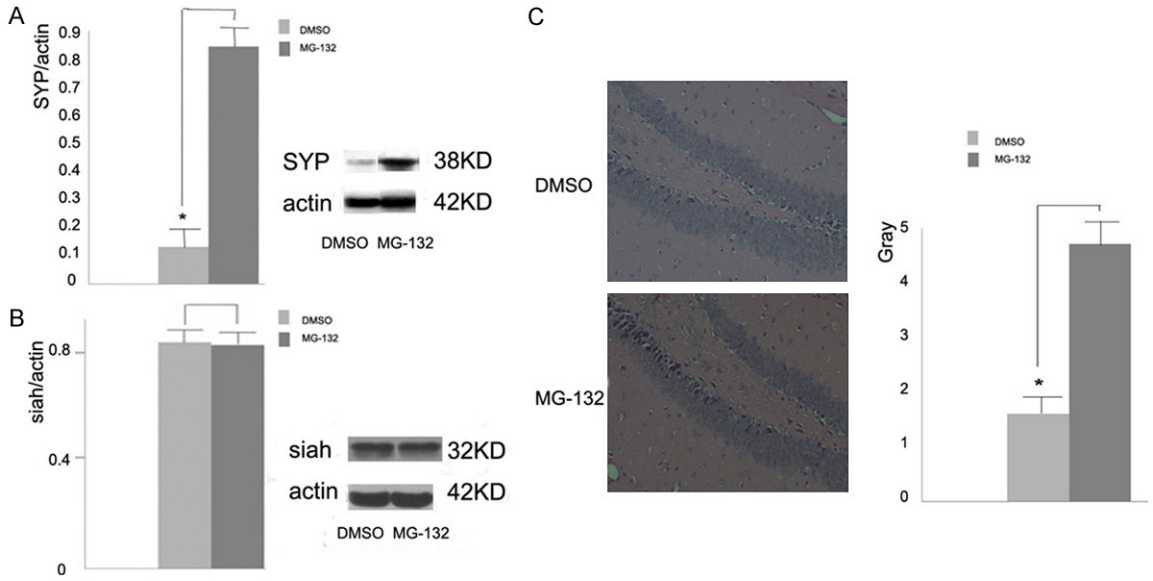

Figure 2. The expression of SYP and siah in DMSO group and MG132 group. Mice in DM + hypoxia group were divided into 2 groups: mice in experiment group were intraperitoneally injected with MG132 at 0.5 mg/kg for 3 days; mice in control group were intraperitoneally injected with DMSO of equal volume. Three days later, animals were sacrificed, and the hippocampus collected. Western blot assay was performed to detect the SYP expression (A) and siah expression (B). Results showed SYP expression in experiment group was markedly higher than that in control group ($P < 0.05$), but siah expression in experiment group was significantly lower than that in control group ($P < 0.05$). Immunofluorescence staining of SYP in the hippocampus (C) showed the SYP expression in experiment group increased markedly when compared with control group.

cantly increased the SYP expression in the brain (**Figure 2C**). Above findings suggest that ubiquitination inhibitor MG132 can increase the SYP expression via suppressing SYP ubiquitination, and further water maze test showed MG132 treatment could significantly improve the intelligence of animals when compared with DMSO group.

Siah lentivirus inhibits siah expression and promotes SYP expression

Lentivirus expressing siah shRNA was used to infect N2A cells and Western blot assay and RT-PCR confirmed that it could inhibit the siah expression by about 75% (data not shown). In high blood glucose + hypoxia group, siah shRNA lentivirus (3 μL) was intraventricularly injected; blank lentivirus (3 μL) was injected in control group. Three days later, mice were sacrificed, and brain sections were obtained. Results showed cells in the brain were infected by the lentivirus in both groups (**Figure 3A**). Protein was extracted from the hippocampus and western blot assay was used to detect the expression of siah and SYP. Results showed the siah expression in shRNA group was significantly lower than that in blank control group (**Figure 3B**), but the SYP expression in shRNA group increased dramatically (**Figure 3C**). Immunofluorescence staining of SYP also revealed that the SYP expression in shRNA group was significantly higher than that in blank control group (**Figure 3D**).

Vitamin improves the intelligence of animals

Studies have shown that VitK3 can specifically inhibit the siah activity [27]. Thus, VitK3 was used in the present study. In high glucose + hypoxia group, mice were intraperitoneally injected with vitK3 at 0.4 mg/ml for 3 days; mice in control group were treated with DMSO (100 μL). Detection of intelligence by water maze test showed vitK3 significantly improved the intelligence of animals undergoing high blood glucose and hypoxia when compared with animals treated with DMSO. Protein was extracted from the hippocampus, and western blot assay was employed to detect the expression SYP and siah. Results showed vitK3 promoted SYP expression (**Figure 4A**), but had no influence on the siah expression (**Figure 4B**). In addition, immunofluorescence staining also

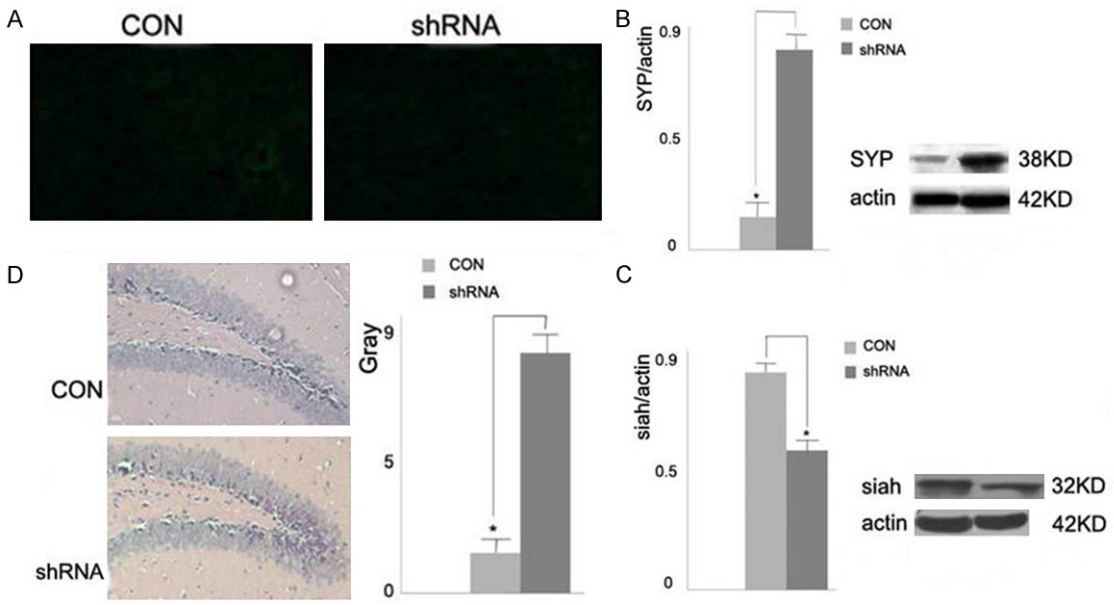

Figure 3. The expression of SYP and siah in control group (blank lentivirus) and experiment (shRNA lentivirus) group. Mice in DM + hypoxia group were divided into 2 groups: mice in experiment group were intraventricularly injected with lentivirus expressing siah shRNA (3 ul); mice in control group were intraventricularly injected with blank lentivirus. Results showed neurons were infected by lentivirus in both groups (A). Western blot assay was performed to detect the siah expression (B) and siah expression (C). Results showed siah expression in experiment group was markedly lower than that in control group ($P < 0.05$), but SYP expression in experiment group was significantly higher than that in control group ($P < 0.05$). Immunofluorescence staining of SYP in the hippocampus (D) showed the SYP expression in experiment group increased markedly when compared with control group.

revealed that vitK3 could increase the SYP expression (**Figure 4C**).

Discussion

Increasing old people pay attention to the cognition disorder due to aging. Studies have shown that DM is an independent factor causing cognition disorders among numerous risk factors of vascular diseases. This study focused on the cause and treatment of cognition impairment in DM patients. SYP is one of specific markers of synaptic vesicles and a calcium-binding protein closely related to the structure and function of synapses. SYP is expressed on the presynaptic membrane of neurons and involved in the formation and recycling of synaptic vesicles. Thus, SYP is closely associated with the synaptic plasticity. In addition, there is evidence showing that SYP is related to the formation of synapses and maintenance of synaptic stability, and the amount and distribution of SYP can indirectly reflect the density of synapses [28-33]. In addition, the regulation of SYP expression has been found to be associated with learning and memory [26] and is able to regulate the LTP in the hippocampus [34]. In the present study, results showed the SYP expression reduced in animals with intelligent drop. Thus, SYP was selected as a marker for the evaluation of cognition, and to alter the SYP expression might be able to improve the cognition.

How does DM cause cognition disorder? There is evidence showing that the oxygen partial pressure is at a very low level in the arterial wall and skin before the presence of injury to major vessels [35]. In addition, more than 75% of type 2 DM patients with obesity may develop OSA, and chronic hypoxia is a major pathophysiology of OSA [36]. Thus, hypoxia and high blood glucose are two indivisible pathological processes in DM patients. It has been confirmed that hypoxia is an important risk factor of cognition disorder. Subjects living at high altitude may develop attention and memory reduction, the recovery of which requires a long time when they come back to the normal altitude. In animal studies, findings also confirm that transient hypoxia may cause memory reduction [3]. In this study, high blood glucose and combined

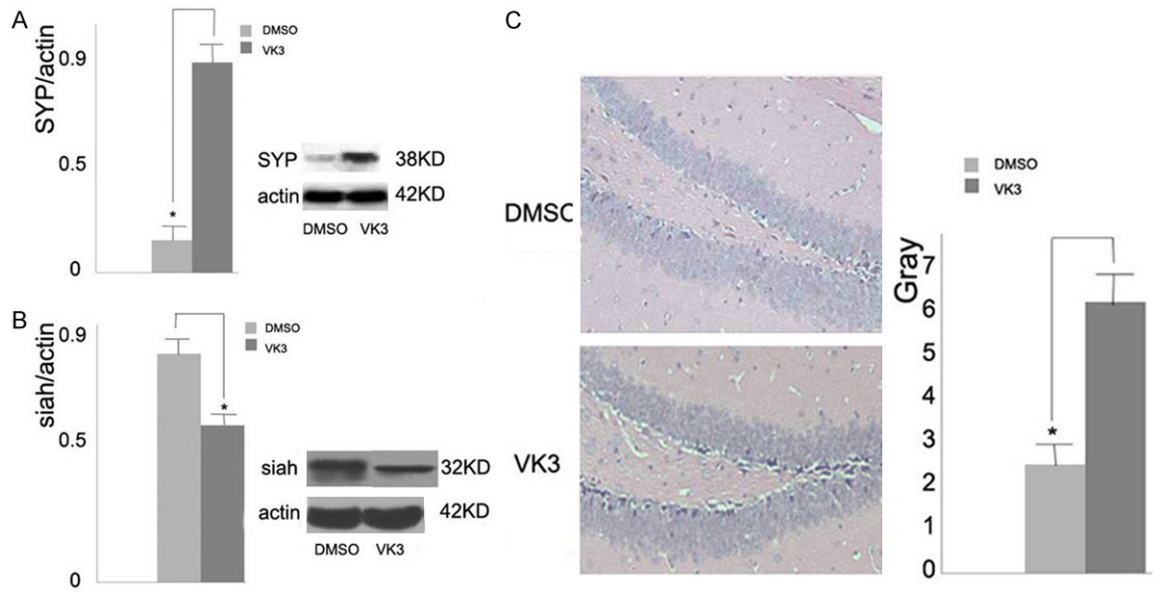

Figure 4. The expression of SYP and siah in DMSO group and vitK3 group. Mice in DM + hypoxia group were divided into 2 groups: mice in experiment group were intraperitoneally injected with vitK3 at 2 mg/kg for 3 days; mice in control group were intraperitoneally injected with DMSO of equal volume. Three days later, mice were sacrificed, and hippocampus was collected. Western blot assay was performed to detect the siah expression (A) and siah expression (B). Results showed SYP expression in vitK3 group was markedly higher than that in control group ($P < 0.05$), but siah expression in vitK3 group was significantly lower than that in control group ($P < 0.05$). Immunofluorescence staining of SYP in the hippocampus (C) showed the SYP expression in vitK3 group increased markedly when compared with control group.

high blood glucose and hypoxia were investigated, aiming to elucidate the specific cause of cognition impairment in DM patients. Water maze test showed the intelligence level in high blood glucose + hypoxia group was significantly lower than that in high blood glucose group and control group, but there was no marked difference between high blood glucose group and control group. This suggests that combined high blood glucose and hypoxia can significantly influence the intelligence of animals. Thus, we speculate that the cognition disorder in DM patients results from the interaction between high blood glucose and hypoxia.

If the intelligence of animals with high blood glucose and hypoxia is related to SYP expression, we speculate that to alter the SYP expression may be able to improve the intelligence of these animals. Thus, the SYP expression in the hippocampus was detected in this study. Results were consistent with what we expected: the SYP expression in high blood glucose + hypoxia group reduced markedly when compared with other groups, confirmed that the intelligence drop due to high blood glucose and hypoxia is associated with the SYP expression. Whether to regulate the SYP ubiquitination can improve the intelligence level is still unclear. Then, ubiquitination inhibitor MG132 was injected intraperitoneally, and SYP expression was detected. Western blot assay showed MG132 could increase the SYP expression in the hippocampus, and water maze test also revealed that MG132 could improve the intelligence of these animals. That is, ubiquitination inhibitor may inhibit the SYP ubiquitination to increase SYP expression and then improve the intelligence level.

There is evidence showing that Hela cells overexpressing siah1 have reduced SYP expression in vitro [37]. The SYP expression in cells is mainly regulated by the siah associated ubiquitination [38]. Siah protein is an E3 ubiquitin ligase and has homology to SINA (seven in absentia) in drosophila. Siah can recognize substrate and be degraded by ubiquitin-proteasome system. Siah has been found to be involved in some signaling pathway and play important roles in the regulation of cell cycle, cell differentiation, cell apoptosis, tumorigenesis and neurodegeneration. In this study, siah expression was specifically inhibited with

shRNA, and then the SYP expression in the brain was detected. Mice were intraventricularly injected with lentivirus expressing siah shRNA, and the SYP expression in the brain was detected. Three days after injection of lentivirus, western blot assay was employed to detect the protein expression of siah and SYP in the brain. Results showed mice in lentivirus group had significantly reduced siah expression, but markedly increased SYP expression, when compared with control group. These findings suggest that siah can influence the SYP expression.

In a previous study, high-throughput screening was performed in 1840 chemicals, and results showed vitK3, an adjuvant in the chemotherapy of cancers, can specifically inhibit siah activity [27]. In this study, vitK3 was used to inhibit siah-1, aiming to investigate the influence of vitK3 on the SYP expression and intelligence in animals. Results showed, in animals undergoing high blood glucose and hypoxia, vitK3 could significantly reduce the siah expression and increase the SYP expression in the brain, accompanied by improvement of intelligence, when compared with control group. The expression of siah and SYP and intelligence in vitK3 group were comparable to those in MG132 group. That is, vitK3 can inhibit the siah activity and suppress SYP ubiquitination to increase the SYP expression, which finally improve the cognition impairment due to DM. Thus, we speculate that vitK3 may be used to treat the cognition impairment in DM patients.

Conclusions

Taken together, our findings show that combined high blood glucose and hypoxia may increase the SYP ubiquitination in the hippocampus and reduce the SYP expression, leading to the cognition impairment. In addition, ubiquitination inhibitor MG-132 may inhibit the SYP ubiquitination to improve the cognition impairment due to high blood glucose and hypoxia. Moreover, treatment with lentivirus expressing siah shRNA can specifically downregulate siah expression to increase SYP expression, and vitK3 is also able to inhibit the siah-1 to improve the cognition impairment in animals with high blood glucose and hypoxia.

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Disclosure of conflict of interest

None.

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