Review Article
Resveratrol upregulates synaptophysin expression by downregulating E3 ubiquitin ligase Siah-1 and RE1 silencing transcription factor REST

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Abstract: Objective: A previous study has shown that E3 Ubiquitin Ligase Siah-1 downregulated synaptophysin expression under high glucose conditions, which may be related to cognitive impairment in diabetic patients. The present study aimed to investigate the effects of resveratrol on synaptophysin expression and explore the underling mechanisms. Methods and materials: High glucose (50 mM) and resveratrol (10 µM, 30 µM, 50 µM) were used to treat PC12 cells. Cell activity was detected by CCK-8 assay. Real-time quantitative PCR and Western blotting were used to determine the expression of E3 ubiquitin ligase Siah-1 and RE1 silencing transcription factor REST and synaptophysin. Overexpressing REST was used to infect PC12 cells, while real-time PCR and Western blotting were employed to detect levels of synaptophysin in different experimental groups. The effects of resveratrol on the ubiquitination of Synaptophysin under high glucose levels were observed by immunoprecipitation. Results: High glucose significantly increased expression of Siah-1 and REST, but decreased synaptophysin expression. However, resveratrol decreased expression of Siah-1 and REST and increased synaptophysin expression in cases of high glucose. REST overexpression downregulated synaptophysin expression. Immunoprecipitation showed that resveratrol inhibited the ubiquitination of synaptophysin under high glucose conditions. Conclusion: Resveratrol upregulates synaptophysin expression by downregulating Siah-1 and REST, providing a new way for the prevention and treatment of diabetic cognitive dysfunction.

Keywords: Siah-1, REST, synaptophysin, diabetes, cognitive dysfunction

Introduction

Diabetes mellitus (DM), one of the independent risk factors for cognitive dysfunction, has been associated with cognitive impairment because of its vascular effects. It has been reported that 26% of inpatients over 65 years are diagnosed with DM [1]. Epidemiological studies [2-4] and biological evidence [5-7] support that older patients with DM are at greater risk for all types of dementia [8]. Animal experiments have demonstrated that DM can lead to cognitive dysfunction, accompanied by reduced synaptic vesicles [9]. Change in protein profiles, including synaptophysin in cases of DM, have been found to contribute greatly to cognitive decline [10].

Resveratrol (3,5,4'-trihydroxystilbene, RSV), a natural plant antitoxin extracted from a variety of plants, such as grapes, berries, and peanuts, can regulate cell growth. There is evidence showing that resveratrol has a variety of biochemical and physiological activities, including anti-inflammatory [11-13], anti-apoptotic [14], anti-oxidative [15], anti-diabetic [16, 17], antiviral [18], neuroprotective [19-21], and cardioprotective [22] activities.

Synaptophysin (Syp) is a key protein in the small synaptic vesicles. It has been proven to be closely related to cognitive function [23, 24]. It is ubiquitously expressed in neuroendocrine cells and neurons throughout the brain. E3 ubiquitin ligase Siah1 is the main protease involved in the degradation of synaptophysin through the ubiquitin-proteasome system in neurons [25]. Furthermore, REST is a main transcriptional factor in the transcriptional regulation of synaptophysin [26, 27].
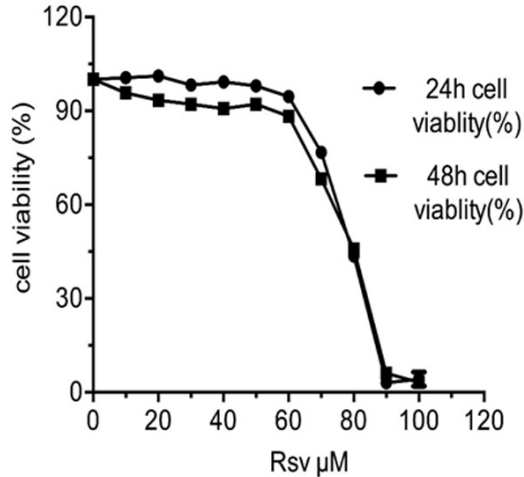
Figure 1. After high glucose exposure, PC12 cells were cultured with different concentrations (0-100 µM) of resveratrol for 24 hours and 48 hours. Resveratrol at 0-60 µM had no influence on the cell viability, but at >60 µM it significantly reduced the ability of cell proliferation, which was concentration dependent. In the following experiments, resveratrol at 10, 30, and 50 µmol/L was used to treat cells for 24 hours. Data are mean ± SD from independent experiments.

A previous study demonstrated that Siah-1 could downregulate synaptophysin expression under high glucose conditions, which may be a pathological change in diabetic cognitive dysfunction [10]. Results of the present study showed that resveratrol, at different concentrations, was able to increase synaptophysin expression by downregulating expression of Siah-1 and REST, perhaps providing a new strategy for the prevention and treatment of diabetic cognitive dysfunction.

Materials and methods

Equipment and reagents

Equipment: high-speed low temperature centrifuge (Heraeus, Hanau, Germany), real-time PCR apparatus (Thermocycler; Biometra, Göttingen, Germany), gelimage processing system (Media Cybernetics, Inc., Rockville, MD, USA), and liquid scintillation counter (Beckman Coulter, Brea CA, USA); Reagents: resveratrol (Sigma-Aldrich Co. St. Louis, MO, USA), dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co. St. Louis, MO, USA), D-glucose (Sigma-Aldrich Co. St. Louis, MO, USA), bovine serum albumin (BSA) (Sigma-Aldrich Co. St. Louis, MO, USA), Dulbecco's

Modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA), fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), primers (Shanghai Sangon Biological Engineering Company, Shanghai, China), Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Japan), and real-time quantitative PCR kit (Takara Bio Inc., Otsu, Japan).

Cell treatment

PC12 cells were cultured in DMEM containing 1% PS and 10% FBS at 37°C in a humidified environment with 5% CO_2. Neurobasal medium containing 25 mM glucose was added to the DMEM. Cells were treated with 50 mM glucose to induce neuronal DM. In resveratrol treated groups, cells in the logarithmic growth phase were treated with resveratrol at different concentrations (10 µmol/L, 30 µmol/L, and 50 µmol/L).

Cell viability assay

Cell viability was detected by CCK-8 assay. CCK-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfobenzene)-2H-tetrazole monosodium salt] can react with the electron carrier 1-methoxy-5-methylphenisinium sulfate (1-Methoxy PMS) to be reduced to formazan, which is dissolved by dehydrogenase in cells. The amount of formazan produced is proportional to the number of viable cells. Optical density (OD) was measured at 450 nm using a microplate reader to indirectly reflect the number of viable cells. Cells (100 µl; about 10,000-20,000/ml) were seeded into 96-well plates and treated with medium containing 50 mM glucose. After resveratrol treatment for 24 hours, 10 µl of CCK-8 solution was added to each well, followed by incubation for 2 hours. OD was measured at 450 nm.

Quantitative PCR

Cells were treated with TRIzol Reagent for extraction of total RNA and cDNA synthesis was performed using PrimeScript RT reagent kit (Takara Bio Inc., Otsu, Japan). Primers were designed and synthesized for amplification of rat REST, Siah-1, synaptophysin, and β-actin using real-time quantitative PCR. The standard curve was obtained and CT values were calculated. MicroRNA expression of each gene was normalized to β-actin expression. The mixture

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decreased. Compared with the Hg group, relative expression of Siah-1 in Rsv group was significantly decreased and relative expression of synaptophysin was significantly increased after effected by Rsv for 24 hours. B. C. Densitometric quantification of Siah-1 and synaptophysin in PC12 cells, which were treated as described above. Data are mean ± SD from independent experiments. *, P<0.05 (statistical significance); NC, normal control; Hg, high glucose (50 mM); Rsv, resveratrol (10 μM, 30 μM, 50 μM).

used for PCR was as follows: DNA (1 μl), SYBR FAST qPCR Master Mix (10 μl), ROX High (0.4 μl), Primer F (10 pmol/μl) (0.4 μl), Primer R (10 pmol/μl) (0.4 μl), and Total Volume (20 μl). PCR was conducted as follows: 95°C for 3 minutes, 95°C for 3 seconds, 60°C for 30 seconds, 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds, for a total of 40 cycles.

Immunoblotting

PC12 cells were treated with RIPA buffer to extract total proteins and protein concentrations were detected with the BSA method. Protein (40 μg) was mixed with loading buffer at a ratio of 4:1. After boiling for 10 minutes, the mixture was loaded for SDS-PAGE. After PVDF membranes were treated with TBST containing 5% non-fat milk, they were incubated at 4°C overnight with rabbit anti-rat polyclonal antibody against REST (1:1000; Abcam Co., Cambridge, MA, USA), rabbit anti-rat monoclonal antibody against synaptophysin (1:2000; Abcam Co., Cambridge, MA, USA), goat anti-rat polyclonal antibody against Siah-1 (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or mouse anti-rat monoclonal antibody against β-actin (1:2000; Sigma-Aldrich Co., St. Louis, MO, USA). After incubation with secondary antibodies at 37°C for 2 hours, each band was observed visually with chemiluminescence (ECL Blotting Analysis System). OD was measured by Image software and normalized to that of β-actin (Shanghai Yeasen Biotechnology Co., Ltd., Shanghai, China).

Permanent transfection of constructs

Lentivirus with REST overexpression was constructed by Shanghai Genechem Co., Ltd. The multiplicity of infection (MOI) was 75. Adherent cells were plated at 1×10⁵/well in 24-well plates. Next, 18-24 hours later, the lentivirus was transfected. The 24-well plates were inocu-

Figure 2. After high glucose exposure, resveratrol at 10, 30, and 50 μmol/L was used to treat PC12 cells for 24 hours. A. Levels of Siah-1 and synaptophysin were analyzed by immunoblotting. Compared with the NC group, relative expression of Siah-1 in Hg group was significantly increased, while relative expression of synaptophysin was significantly
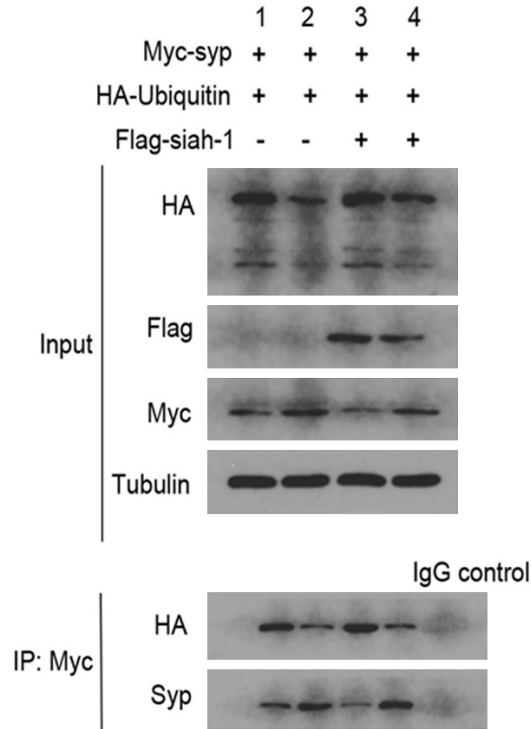
Figure 3. HEK293 cells were co-expressed with Myc-tagged synaptophysin along with HA tagged ubiquitin in the absence or presence of exogenous Siah-1. Cell lysates were subjected to immunoprecipitation with an anti-Myc antibody followed by immunoblotting with an anti-HA antibody to detect ubiquitin conjugated synaptophysin. Results showed that synaptophysin expression significantly increased in resveratrol group, but ubiquitination decreased. Exogenous Siah-1 significantly decreased synaptophysin expression and increased association of ubiquitin with synaptophysin was found by overexpression of Siah-1. Synaptophysin or HA-tagged ubiquitin was undetectable in control IP, which using mouse IgG pulled down. 1. high glucose (50 mM); 2. high glucose + Rsv (50 µM); 3. high glucose + Siah-1; 4. high glucose + Rsv (50 µM) + Siah-1.

Figure 4. A. Levels of HA and Myc in Input group were analyzed by immunoblotting. Ratio of synaptophysin and tubulin was used as relative protein expression of synaptophysin. Data are mean ± SD from independent experiments. B. Levels of HA and synaptophysin in IP group were analyzed by immunoblotting. Data are mean ± SD from independent experiments. *, $P<0.05$ (statistical significance).

Transient transfection of constructs

HEK 293 cells were transfected with HA-ubiquitin, Flag-Siah-1, or Myc-synaptophysin plasmid using Lipofectamine TM 3000, according to manufacturer instructions. Eight hours later, the medium was removed and the DMEM containing 10% FBS, 50 mM glucose, and 50 µM resveratrol was added. Transfected cells were processed for Western blotting with antibody against HA (1:1000, Abcam Co., Cambridge,

lated and the original medium was removed. Fresh medium and Polybrene were added at a final concentration of 5 µg/mL and mixed into the incubator. After incubation at 37°C for 30 minutes, the lentivirus was removed, slowly melting the ice. The virus solution was added according to MOI 75 into the cells and mixed. After culturing at 37°C for 12 hours, the medium containing lentivirus were removed and the fresh medium was added. Transfection efficiency was determined by fluorescence microscopy after incubation at 37°C for 48 hours. Transfected cells were processed for real-time quantitative PCR and Western blotting.
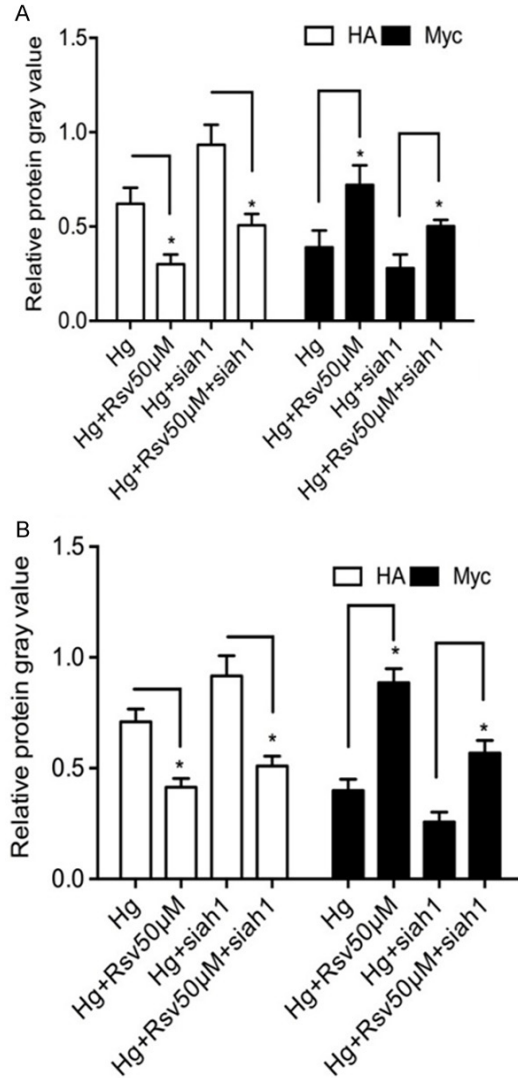
A

RSV µm

NC Hg 10 30 50
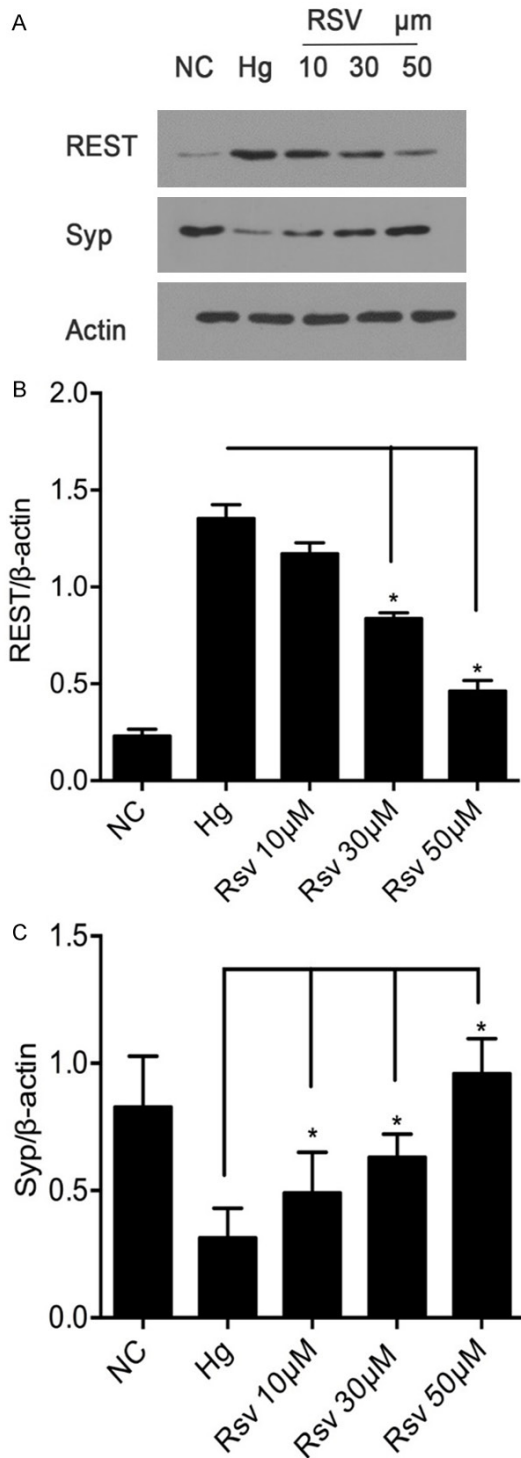
REST

Syp

Actin

B

C

Figure 5. After high glucose exposure, PC12 cells were cultured with different concentrations of resveratrol for 24 hours. A. Levels of REST and synaptophysin were analyzed by immunoblotting. Compared with the NC group, relative expression of Siah-1 in Hg group was significantly increased, while relative expression of synaptophysin was significantly decreased. Compared with the Hg group, relative expression of REST in Rsv group was significantly decreased and relative expression of synaptophysin

was significantly increased after affected by Rsv for 24 hours. B. C. Densitometric quantification of REST and synaptophysin in PC12 cells, which were treated as described above. Data are mean ± SD from independent experiments. *, P<0.05 (statistical significance); NC, normal control; Hg, high glucose (50 mM); Rsv, resveratrol (10 µM, 30 µM, 50 µM).

MA, USA), Flag (1:1000, Abcam Co., Cambridge, MA, USA) or Myc (1:1000, Abcam Co., Cambridge, MA, USA).

Ubiquitination assays

HEK293 cells were transfected with plasmids containing HA-ubiquitin or Myc-synaptophysin in the presence or absence of Flag-Siah-1. Cells were washed twice with PBS and lysed in IP buffer containing a protease inhibitor and phosphatase inhibitor for 1 hour at 4°C. Supernatant was collected after centrifugation at 10,000 rpm for 10 minutes at 4°C. Agarose Protein A + G beads were added and mixed. After washing twice with PBS, centrifugation was performed at 3000 rpm for 5 minutes. The resultant product was divided into two parts, one for the removal of nonspecific binding and the other for binding to the specific antibody. The monoclonal anti-Myc antibody (5 µg, Abcam Co., Cambridge, MA, USA) was added, followed by incubation at 4°C for 12 hours. Agarose Protein A + G (30 µl/tube) was then added, followed by incubation at 4°C for 3 to 6 hours. After washing to remove the nonspecific binding, the bound protein was detected by immunoblotting.

Statistical analysis

All data were analyzed using SPSS version 20.0. One-way ANOVA was employed for comparisons. A value of *P*<0.05 is considered statistically significant.

Results

Cell viability

As shown in **Figure 1**, 24-hour cell viability was as follows: (100.590±0.512)%, (101.140± 1.927)%, (98.201±0.460)%, (99.210±0.515)%, (97.967±1.430)%, (94.509±1.206)%, (76.740± 1.750)%, (43.152±1.509)%, (3.024±1.128)%, (4.201±2.239)% (P<0.05); 48-hour cell viability was as follows: (95.704±4.507)%, (93.401± 2.827)%, (92.150±2.343)%, (90.645±2.20)%,
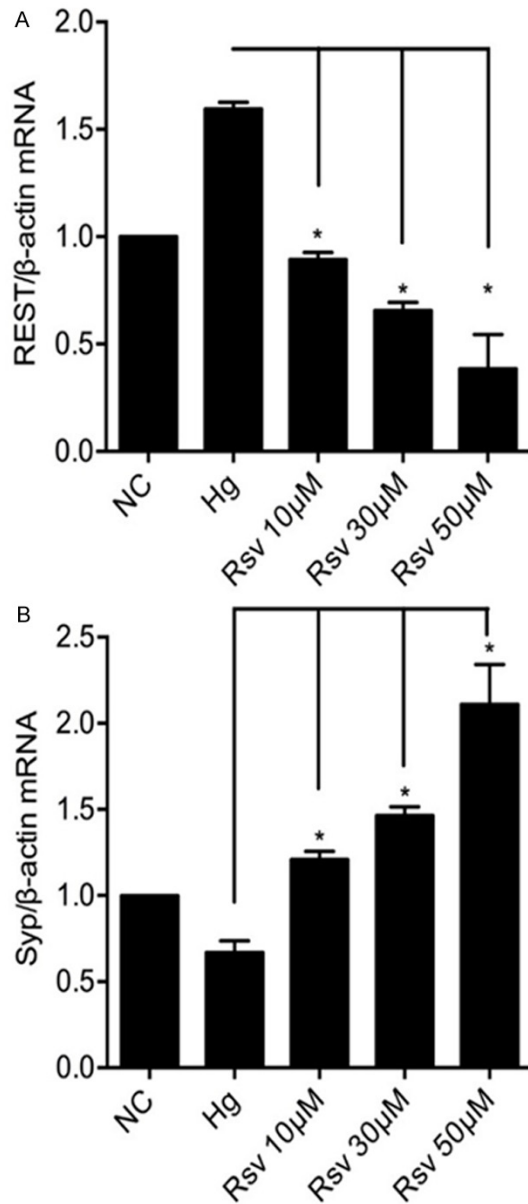
Figure 6. A. The mRNA level of REST in Hg group was 1.594±0.010 times higher than that in control group, while the level of REST in resveratrol group was 0.923±0.11 times (10 µM), 0.656±0.038 times (30 µM), 0.384±0.160 (50 µM) times lower, respectively. The ratio of syp and β-actin was used as relative mRNA expression of syp. There was a significant difference between high glucose treatment group and Rsv treatment group. B. MicroRNA expression of synaptophysin in High glucose group was 0.670±0.067 times lower than that in control group and expression of synaptophysin in resveratrol group was 1.208±0.048 times (10 µM), 1.463±0.052 times (30 µM), 2.110±0.23 times (50 µM) higher, respectively. The ratio of REST and β-actin was used as relative mRNA expression of REST. There was a significant difference between high glucose treatment group and Rsv treatment group. Data are mean ±

SD from independent experiments. *, P<0.05 (statistical significance); NC, normal control; Hg, high glucose (50 mM); Rsv, resveratrol (10 µM, 30 µM, 50 µM).

(92.088±0.710)%, (88.221±1.256)%, (68.289±1.588)%, (45.767±2.970)%, (6.067±4.633)%, (3.100±1.534)%; Resveratrol at 0-60 µM had no influence on cell viability. At >60 µM, however, it significantly reduced the ability of cell proliferation, which was concentration dependent. In the following experiments, resveratrol at 10, 30, and 50 µmol/L was used to treat cells for 24 hours.

Resveratrol upregulates synaptophysin expression with decreased siah-1 expression

After high glucose exposure, PC12 cells were treated with different concentrations of resveratrol for 24 hours. Expression of selective proteins was detected by Western blotting. Siah-1 expression in the high glucose group was markedly higher than the normal control group (0.193±0.006) (P<0.05). There was a significant difference in Siah-1 expression between the high glucose group (0.745±0.047) and Rsv groups (10 µM: 0.441±0.040; 30 µM: 0.392±0.056; 50 µM: 0.401±0.065) (P<0.05). The syp expression in the high glucose group was markedly lower than the normal control group (0.905±0.052) (P<0.05). Significant differences were also observed in syp expression between the high glucose group (0.392±0.026) and Rsv groups (10 µM: 0.667±0.034; 30 µM: 0.986±0.066; 50 µM: 1.089±0.058) (P<0.05) (**Figure 2**).

Resveratrol inhibits the ubiquitination of synaptophysin under high glucose conditions

With Myc-tagged synaptophysin, along with HA-tagged ubiquitin in the absence or presence of exogenous Siah-1, HEK 293 cells cultured were transfected. Immunoprecipitation with anti-Myc antibody was then performed. Target proteins were immunoblotted with anti-HA antibody to detect ubiquitin conjugated synaptophysin. As shown in **Figures 3**, **4**, synaptophysin expression significantly increased in the resveratrol group, but ubiquitination decreased. Additionally, exogenous Siah-1 significantly decreased synaptophysin expression. Increased association of ubiquitin with synaptophysin
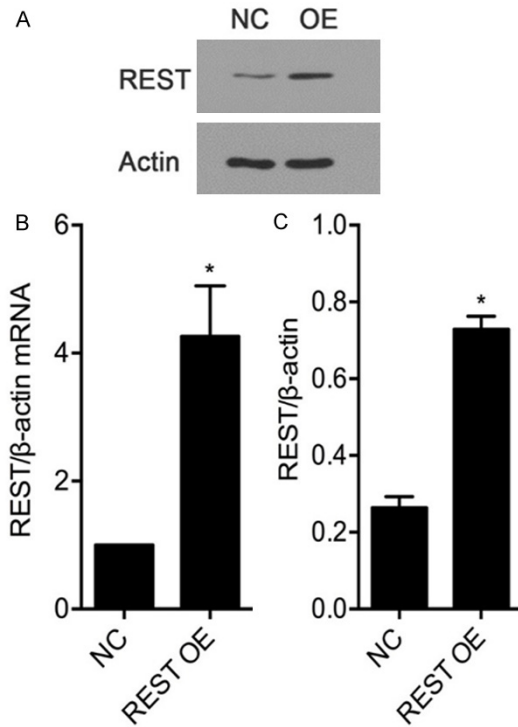
Figure 7. Lentivirus with REST overexpression was used to transfect PC12 cells. A. Levels of REST were analyzed by immunoblotting. B. The ratio of REST and β-actin was used as relative mRNA expression of REST. Results showed that mRNA expression of REST after transfection was significantly higher than that in scramble control group (4.263-fold). C. Western blotting showed that protein expression of REST after transfection was about 2.760 times higher than that in scramble control group, showing significant differences between them. Data are mean ± SD from independent experiments. *, P<0.05 (statistical significance); NC, normal control; OE, overexpression.

was found by overexpression of Siah-1. Synaptophysin or HA-tagged ubiquitin was undetectable in control IP, which using mouse IgG pulled down. Present findings indicate that resveratrol inhibits the ubiquitination of synaptophysin under high glucose conditions by downregulating Siah-1 expression, providing an experimental basis for treatment of diabetic cognitive dysfunction.

Resveratrol upregulates synaptophysin expression with decreased REST expression

There was a significant difference in REST expression between the high glucose group and Rsv group. As shown in **Figure 5**, compared with the normal control group, REST expression in the high glucose group significantly increased

and synaptophysin markedly decreased. Compared with the high glucose group, REST expression in the resveratrol group significantly decreased and synaptophysin markedly increased. The mRNA expression of REST in the high glucose group was 1.594±0.010 times higher than that in the normal control group. In resveratrol groups, it was 0.923±0.11 times (10 μM), 0.656±0.038 times (30 μM) and 0.384±0.160 (50 μM) times lower than that in the high glucose group (**Figure 6A**). Present findings demonstrate that mRNA expression of synaptophysin in the high glucose group was 0.670±0.067 times lower than the control group. In the resveratrol group, it was 1.208±0.048 times (10 μM), 1.463±0.052 times (30 μM) and 2.110±0.23 times (50 μM) higher than in the high glucose group (P<0.05) (**Figure 6B**).

Lentivirus with REST overexpression was constructed

Lentivirus with REST overexpression was used to transfect PC12 cells and mRNA expression of REST was detected. MicroRNA expression of REST, after transfection, was significantly higher than in the scramble control group (4.263-fold) (P<0.05) (**Figure 7B**). Western blotting showed that protein expression of REST, after transfection, was about 2.760 times higher than that in the scramble control group, showing significant differences between the groups (**Figure 7A-C**).

Expression of synaptophysin was downregulated after REST overexpression

After transfection by lentivirus with REST overexpression, PC12 cells were treated with 50 μM resveratrol for 24 hours. Protein and RNA were extracted and expression of REST and synaptophysin was further detected. Expression of synaptophysin in Rsv (50 μM) treated cells after transfection was like that in the high glucose group. This indicates that protein expression of synaptophysin was downregulated after REST overexpression and resveratrol restores expression of synaptophysin by downregulating REST expression (**Figures 8**, **9**).

Discussion

Studies have confirmed that DM is an independent risk factor for dementia [28-33]. The annual rate of global cognitive impairment is up

A

ed cells after transfection was in accord with that in the high glucose group. This indicates that protein expression of REST was upregulated after REST overexpression. B. The ratio of REST and β-actin was used as relative mRNA expression of REST. There was a significant difference between OE group and Rsv treatment group. C. Densitometric quantification of REST in PC12 cells treated with different group. Data are mean ± SD from independent experiments. *, P<0.05 (statistical significance); NC, normal control; Hg, high glucose (50 mM); Rsv, resveratrol (10 µM, 30 µM, 50 µM); OE, overexpression.

to 1.5 times greater in DM patients than in non-diabetic subjects [28]. DM may enhance the risk for dementia and significantly promote the progression of MCI to dementia [34]. Synaptic transmission is an important step in the cognitive process. Synaptophysin is involved in synaptic plasticity, neurotransmitter release, synaptic vesicle reuse, and formation, which are crucial for learning, memory, and other functions. Synaptophysin also serves as a specific marker of synapses [35, 36]. Abnormal synaptophysin expression has been observed in a variety of neurodegenerative diseases [37-39].

Resveratrol is a natural plant antitoxin extracted from a variety of plants, such as grapes, berries, and peanuts. It can regulate cell growth. In addition, resveratrol possesses a variety of biochemical and physiological functions, including anti-inflammatory, anti-apoptotic, anti-oxidative, anti-diabetic, anti-viral, neuroprotective, and cardioprotective properties [40]. In a previous study, results showed that Siah-1 could downregulate Synaptophysin expression under high glucose conditions, which is one of pathological changes in cases of diabetic cognitive dysfunction [10]. Furthermore, REST is a main transcriptional factor in the transcriptional regulation of synaptophysin. There is a functional binding site for REST in the first intron of the human synaptophysin gene [26, 27]. There is evidence showing that resveratrol can protect against neuronal cell death by downregulating REST expression [41].

In the present study, the influence of resveratrol on synaptophysin expression was further investigated and potential mechanisms were explored by focusing on Siah-1 and REST. After high glucose exposure, PC12 cells were treated with different concentrations of resveratrol for 24 hours. Results showed that Siah-1 expression in the high glucose group significantly increased, while synaptophysin expression sig-
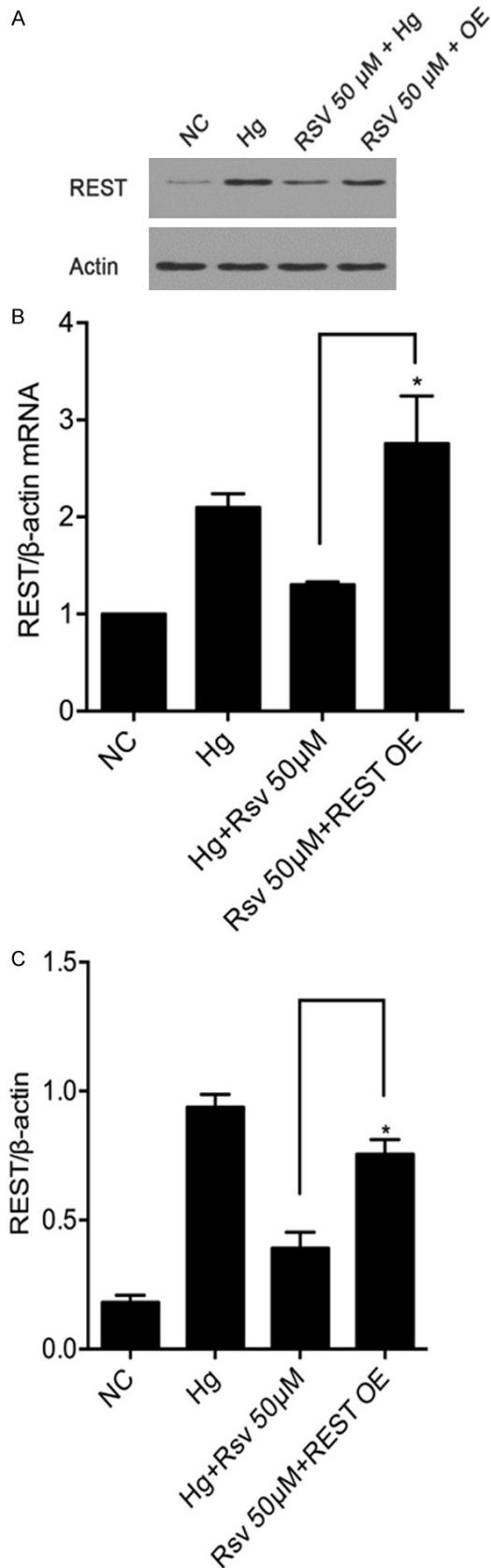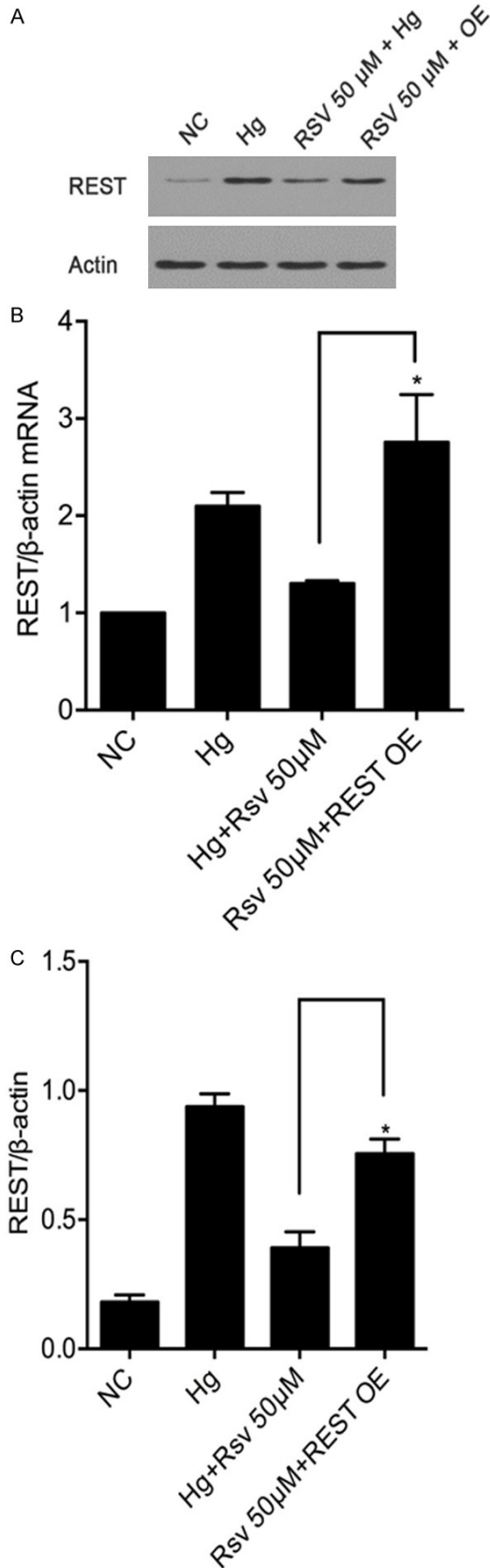
Figure 8. A. Levels of REST were analyzed by immunoblotting. Expression of REST in Rsv (50 µM) treat-

(50 μM) treated cells after transfection was similar to that in high glucose group. This indicates that protein expression of synaptophysin was downregulated after REST overexpression and resveratrol restores expression of synaptophysin by downregulating REST expression. B. The ratio of synaptophysin and β-actin was used as the relative mRNA expression of synaptophysin. There was a significant difference between OE group and Rsv treatment group. C. Densitometric quantification of synaptophysin in PC12 cells treated with different group. Data are mean ± SD from independent experiments. *, P<0.05 (statistical significance); NC, normal control; Hg, high glucose (50 mM); Rsv, resveratrol (10 μM, 30 μM, 50 μM); OE, overexpression.

nificantly decreased, compared with the normal control group. Under high glucose conditions, synaptophysin was ubiquitinated. Ubiquitinated synaptophysin could be detected with anti-HA antibody by immunoblotting in the absence of exogenous Siah protein. Exogenous Siah-1 significantly decreased synaptophysin expression. Additionally, increased association of ubiquitin with synaptophysin was found in cases of Siah-1 overexpression. However, when compared with the high glucose group, Siah-1 expression in the resveratrol group significantly decreased and synaptophysin expression significantly increased after resveratrol treatment for 24 hours. IP showed that protein expression of synaptophysin significantly increased in the resveratrol group and ubiquitination decreased. Studies have shown that E3 ubiquitin ligase Siah1 is a main protease involved in the degradation of synaptophysin through the ubiquitin-proteasome system in the neurons [25]. Present findings indicate that resveratrol can inhibit the ubiquitination of synaptophysin under high glucose conditions, thereby upregulating synaptophysin expression and preventing diabetic cognitive impairment.

Furthermore, REST has been found to be a main transcriptional factor for synaptophysin [26, 27]. In the present study, when compared with the normal control group, REST expression in the high glucose group significantly increased, while synaptophysin expression significantly decreased. However, when compared with the high glucose group, REST expression in the resveratrol group significantly decreased and synaptophysin expression significantly increased. Moreover, synaptophysin expression in PC12 cells with REST overexpression after resveratrol treatment for 24 hours was comparable to that in the high glucose group.

Figure 9. A. Levels of synaptophysin were analyzed by immunoblotting. Expression of synaptophysin in Rsv

This indicates that synaptophysin expression is downregulated after REST overexpression, but resveratrol upregulates synaptophysin expression by downregulating REST expression.

In summary, resveratrol upregulates synaptophysin expression by downregulating E3 ubiquitin ligase Siah-1 and RE1 silencing transcription factor REST. This provides a new method for the prevention and treatment of diabetic cognitive dysfunction.

Acknowledgements

Disclosure of conflict of interest

None.

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