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Original Article

Effects of APP 5-mer peptide analogue P165 on the synaptic proteins and insulin signal transduction proteins

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Abstract: Diabetic encephalopathy (DE) is one of risk factors for Alzheimer's disease (AD). Our previous findings indicated that DE animals had impairment of learning and memory and degeneration of hippocampal neurons, which could be improved by neurotrophic peptide. APP 17-mer peptide is a synthesized peptide sequenced from soluble amyloid precursor protein. APP 17-mer peptide has neural protective effect, but is susceptible to enzyme degradation. Soluble APP 5-mer peptide is the active form of APP 17-mer peptide, and composed of arginine, glutamic acid, arginine, methionine and serine. P165, an APP 5-mer peptide analog reconstructed by our lab, is resistant to enzyme degradation, and can be orally used to protect neurons. In the present study, high glucose and $A\beta_{25-35}$ were used to cause injury to human neuroblastoma cell line SH-SY5Y *in vitro*, and streptozotocin was used to induce diabetes in mice *in vivo*. The changes in synaptic proteins and proteins of insulin signal transduction which closely correlate with learning and memory were detected in these cells and the brain of mice. Results showed that P165 could up-regulate the expression of α-synuclein and insulin receptor (IR), down-regulate the expression of insulin receptor substrate-1 (IRS-1), PSD-95, Shank1 and MAPK expression. All these findings suggest that nicorandil might be a potential drug used for the treatment of AD.

Keywords: Alzheimer's disease, APP 5-mer peptide analogue P165, diabetes mellitus, insulin signal transduction, synapse

Introductions

Alzheimer's disease (AD) is an irreversible, progressive and degenerative disease, which attacks the brain, resulting in impaired memory and behaviors. The typical pathological characteristics of AD include senile plaques, neurofibrillary tangles and loss of cholinergic neurons [1]. The pathogenesis of AD has been ascribed to aging, environmental factors, neuroendocrine disorder and other factors.

On one hand, studies show synapses dysfunction occurs before neuron loss, and thus attention has been paid to the synapse-associated proteins, such as postsynaptic protein-95 (PSD-95), Shank1 and α-synuclein [2]. On the other hand, energy homeostasis associated with the regulation of insulin signal transduction is closely related to AD, and thus some investigators proposed that AD might be a form of type 3 diabetes mellitus (DM) [3].

APP 17-mer peptide is a peptide extracted from the soluble amyloid precursor protein. In our previous studies, results show APP 17-mer peptide may exert neuroprotective effect via improving the insulin signal transduction and inhibiting the apoptosis of neurons. APP 5-mer peptide is an active form of APP 17-mer peptide. P165, an APP 5-mer peptide analogue reconstructed in our lab, is resistant to enzyme degradation [4], and can be orally used as a drug to protect neurons. In this study, high glu-

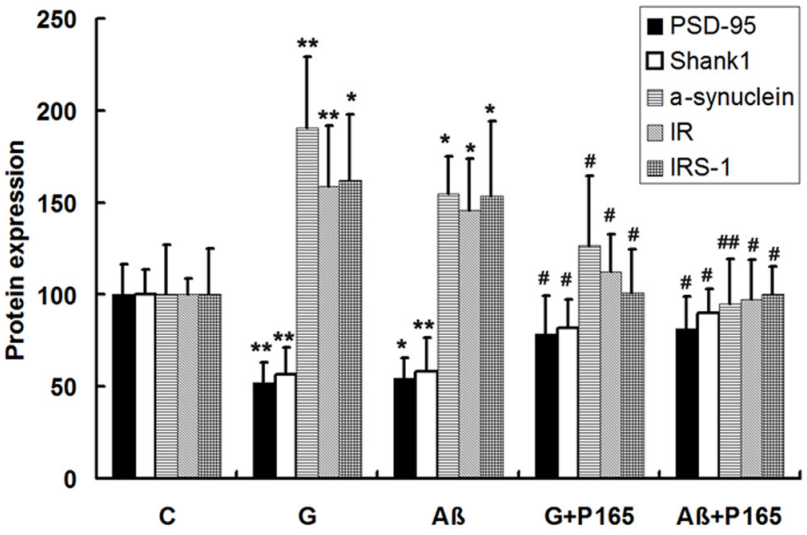

Figure 1. Effects of APP 5-mer peptide analog P165 on the synaptic proteins and insulin signal transduction proteins. **compare with group C, $p < 0.01$, *compared with group C, $p < 0.05$, ##compared with group G and group Aβ respectively, $p < 0.01$, #compared with group G and group Aβ respectively, $p < 0.05$.

cose and $A\beta_{25-35}$ were employed to cause injury to human neuroblastoma cell line SH-SY5Y in vitro, and streptozotocin was used to induce DM in mice in vivo. Then, the changes in synaptic proteins and proteins of insulin signal transduction which closely correlate with learning and memory were evaluated.

Materials and methods

Cell culture and treatments

Human neuroblastoma cells (SH-SY5Y) were obtained from the Karolinska Institute of Sweden. Cells were maintained in Minimum Essential Medium (MEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA), 15 mM HEPES (DNN company, PR China), 100 U/ml penicillin and 100 U/ml streptomycin in an environment with 5% CO_2 at 37°C. The medium was refreshed twice weekly and cells were passaged once weekly.

SH-SY5Y cells were cultured *in vitro*, and divided into normal control group (C group), two model groups (cells in Aβ group and G group were incubated with 10 μmol/L $A\beta_{25-35}$ and 8.33 mmol/L glucose, respectively), and two treatment groups (Aβ+P165 group and G+P165 group) (cells were independently treated with 40 μmol/L P165).

Animals

C57 male mice weighing 28-32 g (n=60) were purchased from the Pharmaceutic al Institute of Aca demy of Medical Science of China and housed in the Animal Center of Xuanwu Hospital affiliated to Capital Medical University in a temperature-controlled environment with a 12:12 h light:dark cycle. Animals were given ad libitum accedd to water and food. All experiments were in accordance to the Beijing regulations and guidelines for the use of animals in research. This study was approved by the Ethics Committee of Xuanwu Hospital. The animals were fasted but given ad libitum access to water before intraperitoneal injection of STZ.

In brief, 1% STZ (Sigma, USA) was freshly prepared with 0.1 mol/L citric acid buffer (pH=4.4) and intraperitoneally injected at 160 mg/kg. In control group, mice were treated with citric acid buffer alone.

After 3 days, venous blood was harvested from the tail and the blood glucose was measured. The blood glucose of greater than 15 mmol/L suggest the successful establishment of DM model. The blood glucose was measured for 5 consecutive weeks. Mice were randomly divided into 5 groups (n=12 per group): control group (C group), DM group, low dose P165 group, medium dose P165 group, and high dose P165 group. P165 was synthesized by PE 431A Peptide Synthesizer (PE, USA) and purified by high performance liquid chromatography with the purity of 99.8%. The dose of P165 was 5 ug/d in low dose P165 group, 10 ug/d in medium dose P165 group and 20 ug/d in high dose P165 group. On the second day after the confirmation of animal model, P165 was intragastrically administered. In control group and DM group, mice were treated with normal saline of the same volume. The mice were treated with P165 once daily for 5 consecutive weeks.

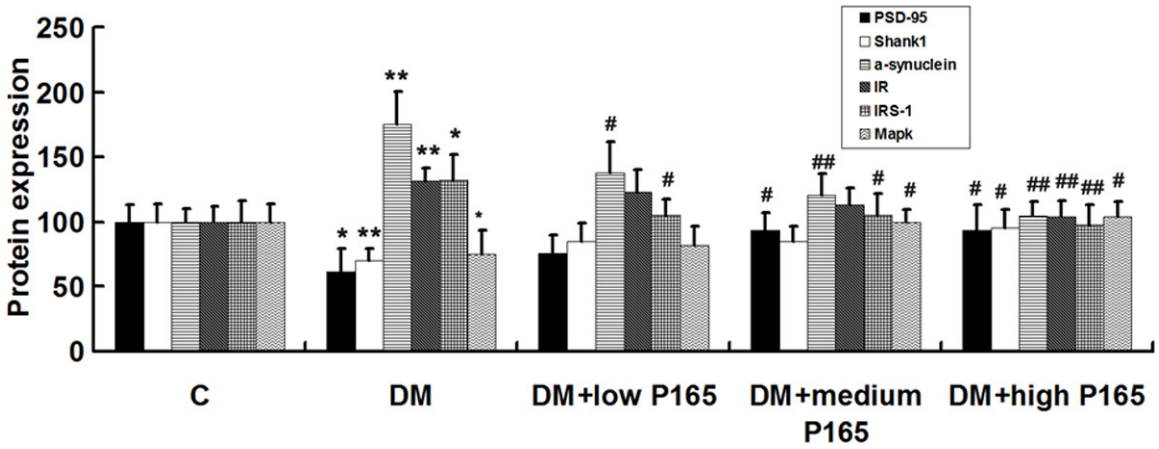

Figure 2. Western blot of synaptic proteins and insulin signal proteins. *compared with group C, $p < 0.05$, **compared with group C, $p < 0.01$, #compared with group DM, $p < 0.05$, ##compared with group DM, $p < 0.01$.

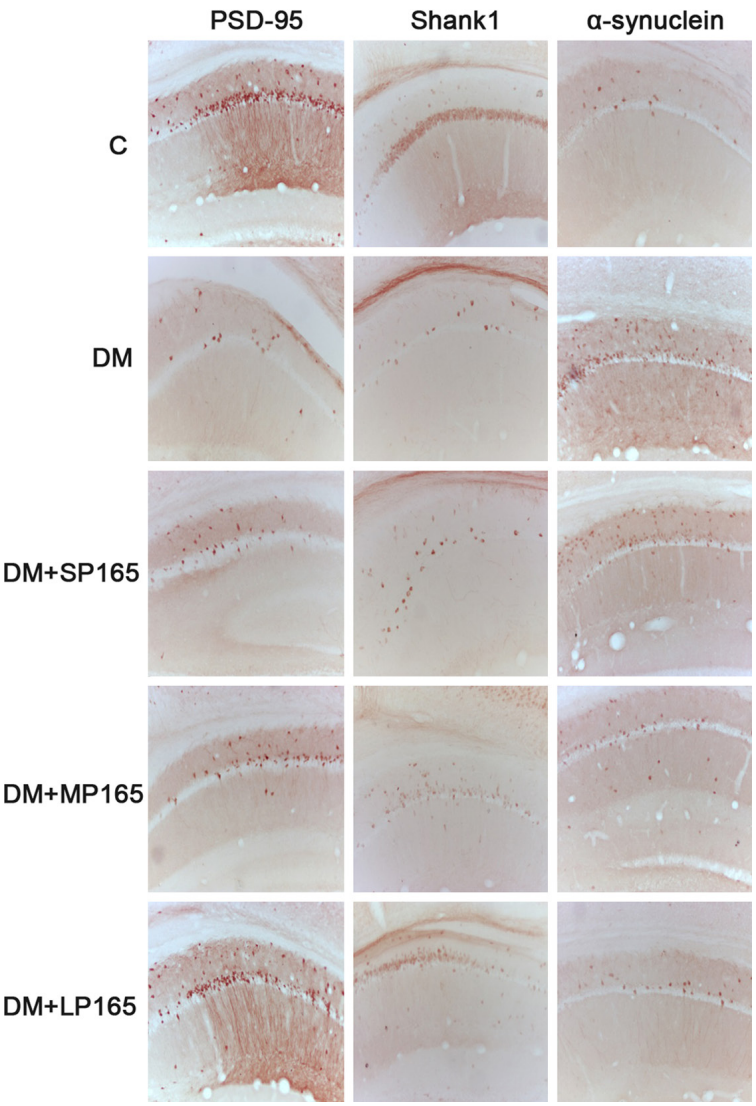

Figure 3. The effect of P165 on synaptic proteins.

The animals were anesthetized with 10% chloral hydrate and transcardially perfused with 250 ml of 0.9% physiological saline and then with 100 ml of 4% paraformaldehyde. The brains were harvested and fixed in paraformaldehyde containing 30% sucrose overnight at 4°C. When the brains were at the bottom of the container, then fixed with later stationary liquid at 4°C. The hippocampus were cut into 35-um sections.

The sections were incubated for 30 min at room temperature, washed thrice in PBS (5 min/time), treated with 3% H_2O_2 for 10 min, and then washed with PBS. Thereafter, the sectionss were treated with 10% goat serum for 30 min, and incubated with primary antibody at 4°C overnight. Then, the sections were washed thrice with PBS (5 min/time), and incubated with biotin-conjugated secondary antibody (1:300, Zhongshan Jinqiao, Beijing, PR China) for 60 min at 37°C. Following washing thrice in PBS (5 min/time), sections were incubated with horseradish peroxide conjugated antibody (1:300, Zhongshan Jinqi-

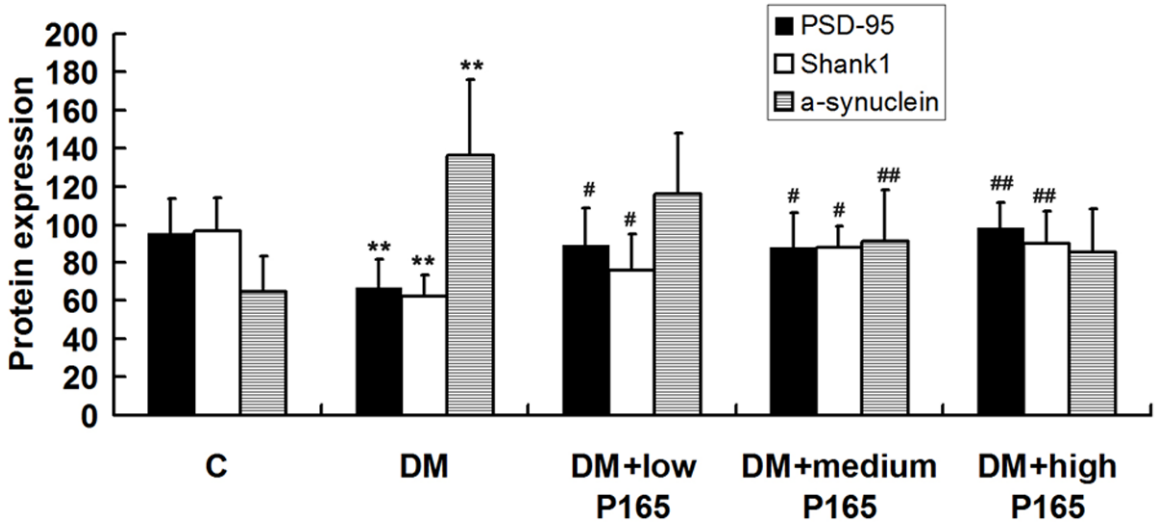

Figure 4. The effect of P165 on synaptic proteins of mice. *compared with group C, $p < 0.05$, **compared with group C, $p < 0.01$, #compared with group DM, $p < 0.05$, ##compared with group DM, $p < 0.01$.

ao, Beijing, PR China) for 60 min at 37°C. After rinsing thrice in PBS (5 min/time), visualization was done with diaminobenzidine (DAB) substrate for 5 min. These sections were mounted, dehydrated, covered, and then observed under a light microscope. The primary antibodies include rabbit anti-PSD-95 (1:2000, Zhongshan Jinqiao, Beijing, PR China), rabbit anti-Shank1 (1:1000, Zhongshan Jinqiao, Beijing, PR China), rabbit anti-α-synuclein (1:1000, Zhongshan Jinqiao, Beijing, PR China), rabbit anti-IR (1:1000, Zhongshan Jinqiao, Beijing, PR China), rabbit anti-IRS-1 (1:1000, Zhongshan Jinqiao, Beijing, PR China), and rabbit anti-MAPK (1:1000, Zhongshan Jinqiao, Beijing, PR China). The positive neurons were counted at a magnification of X10 from the CA1 region of hippocampus, and representative images were captured by using Motic Med 6.0 Image software.

Western-blot assay

SH-SY5Y cells were seeded into a 75-cm^2 flask, and divided into control group, two model groups ($A\beta_{35-45}$ group and G group) (cells were treated with 10 μmol/L $A\beta_{25-35}$ and 8.33 mmol/L glucose, respectively), and two treatment groups (cells were independently treated with 40 μmol/L P165). When cell confluence reached 90%, cells were harvested and protein was extracted for Western blot assay. The protein was quantified with Lowry method and the integrated density of protein bands was detected with NIH Image J.

In addition, hippocampus was collected and placed on ice. Following washing twice in PBS (5 min/time), hippocampus was lysed in lysis buffer (50 mmol/L Tris-HCl [pH7.4], 0.1% Leupeptin, 1 mmol/L PMSF, 0.5 mol/L EDTA, pH8.0) at a ratio of 1:5. Following centrifugation at 15000 r/min for 30 min, proteins were collected and boiled for 3 min in loading buffer. Then, these proteins were load onto gels for SDS-PAGE. These proteins were transferred onto PVDF membrane and then treated with primary antibodies and secondary antibodies. The primary antibodies were rabbit anti-PSD-95 (1:2000, Zhongshan Jinqiao, Beijing, PR China), rabbit anti-Shank1 (1:1000, Zhongshan Jinqiao, Beijing, PR China), rabbit anti-α-synuclein (1:1000, Zhongshan Jin qiao, Beijing, PR China), rabbit anti-IR (1:1000, Zhongshan Jinqiao, Beijing, PR China), rabbit anti-IRS-1 (1:1000, Zhongshan Jinqiao, Beijing, PR China) and rabbit anti-MAPK (1:1000, Zhongshan Jinqiao, Beijing, PR China). The protein bands were scanned and quantified with NIH Image J software.

Statistical analysis

Statistical analysis was performed with SPSS version 11.5 and data were presented as mean ± standard deviation (SD). Comparisons were done with one-way analysis of variance (ANOVA) among groups. A value of $P < 0.05$ was considered statistically significant.

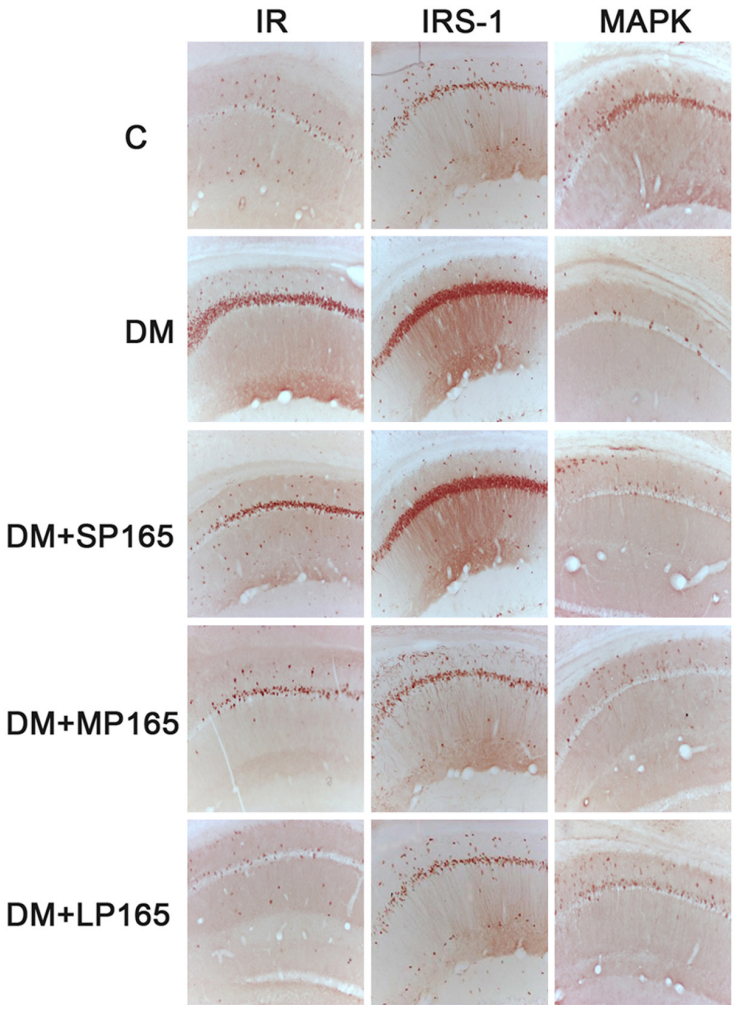

Figure 5. The effect of P165 on insulin signal transduction proteins.

Results

Western blot assay

Impairment of synaptic plasticity underlies the memory dysfunction in AD. Molecules involved in this plasticity (such as PSD-95, Shank1, and α-synuclein) play an important role in the pathogenesis of AD. Western blot assay of SH-SY5Y cells showed that PSD-95 expression decreased in both model groups ($P < 0.01$ and $P < 0.05$, respectively). The Shank1 expression decreased ($P < 0.01$) after the treatment with high glucose or $A\beta_{25-35}$, but the α-synuclein expression increased ($P < 0.01$ and $P < 0.05$, respectively). P165 increased the PSD-95 expression ($P < 0.05$), Shank1 ($P < 0.05$), and decreased the α-synuclein expression in two treatment groups ($P < 0.05$ and $P < 0.01$, respectively). The expression of IR and IRS-1 increased in both model groups ($P < 0.01$ or $P < 0.05$), However, P165 decreased the expression of IR and IRS-1 in two treatment groups ($P < 0.05$) (**Figure 1**).

The expression of PSD-95, Shank1 and MAPK decreased in medium dose group ($P < 0.05$, $P < 0.01$, and $P < 0.05$ respectively), while the expression of α-synuclein, IR and IRS-1 increased ($P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively). Compared with untreated group, P165 increased the expression of PSD-95 after treatment with medium and high dose P165 ($P < 0.05$). The expression of Shank1 increased ($P < 0.05$) after treatment with high dose P165. α-synuclein decreased in the low, medium and high dose P165 groups ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively). IR expression decreased ($P < 0.01$) in high dose P165 group and IRS-1 expression decreased in low, medium and high dose P165 groups ($P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively). The expression of MAPK increased ($P < 0.05$) in medium and high dose P165 groups (**Figure 2**).

Immunohistochemical staining

Effect of P165 on the expression of synaptic proteins in the hippocampus of DM mice: Compared with control group, the number of PSD-95 positive cells in DM group decreased ($P < 0.01$). Compared with DM group, the positive cells increased in low, medium and high dose P165 groups ($P < 0.05$ or $P < 0.01$). Compared with control group, the number of Shank1 positive cells in DM group decreased ($P < 0.01$). Compared with DM group, the positive cells remained unchanged in low dose P165 group, but increased in medium dose group ($P < 0.01$). In high dose P165 group, the Shnak1 positive cells increased significantly ($P < 0.01$). Compared with control group, the α-synuclein positive cells increased in DM group ($P < 0.01$), and compared with DM group, the positive cells remained unchanged in low dose and

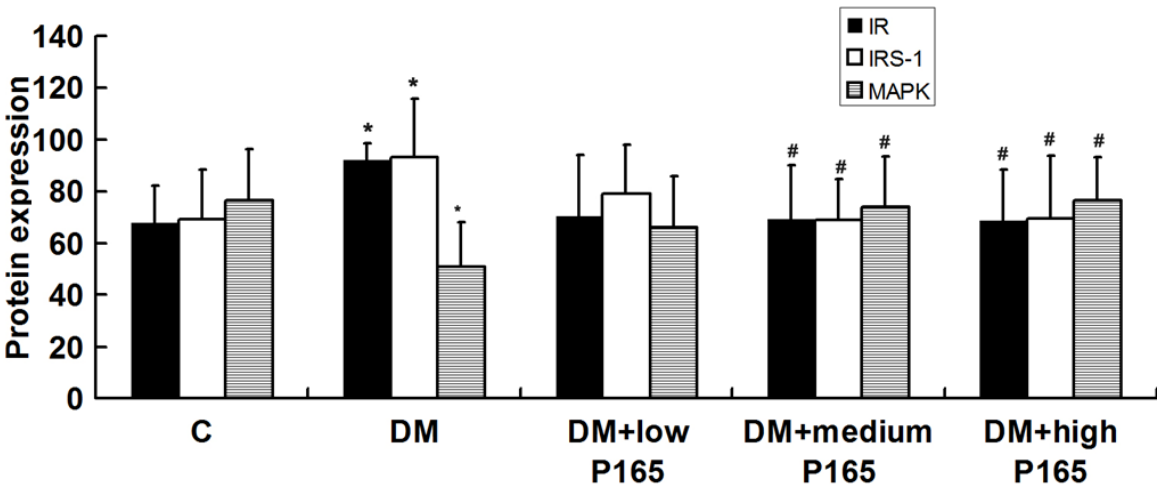

Figure 6. The effect of P165 on insulin signal transduction proteins. *Compared with group C, $p < 0.05$, **compared with group C, $p < 0.01$, #compared with group DM, $p < 0.05$, ##compared with group DM, $p < 0.01$.

medium dose P165 group. In high dose P165 group, the positive cells decreased significantly ($P < 0.01$) (**Figures 3**, **4**).

Effect of P165 on the expression of proteins related to insulin signal transduction in the hippocampus of DM mice: Compared with control group, the IR positive cells in DM group increased ($P < 0.05$). Compared with DM group, the positive cells remained unchanged in low dose P165 group, but decreased in medium and high dose groups ($P < 0.01$). Compared with control group, the IRS-1 positive cells in DM group increased ($P < 0.05$). Compared with DM group, the positive cells remained unchanged in low dose P165 group. Compared with DM group, the positive cells decreased in medium and high dose P165 groups ($P < 0.05$). Compared with control group, the MAPK positive cells in DM group decreased ($P < 0.05$). Compared with DM group, the positive cells remained unchanged in low dose P165 group. Compared with DM group, the positive cells increased in medium ($P < 0.05$) and high dose P165 groups ($P < 0.05$) (**Figures 5**, **6**).

Discussion

AD is a neurodegenerative disease and the most common type of dementia attacking the elderly. Synaptic proteins play an important role in the synaptic plasticity which underlies the higher brain functions such as learning and memory [5]. The change in the insulin signal transduction also plays an important role in the pathogenesis of AD, and glucose metabolic disorder may influence the normal function of synapses. As a result, synaptic proteins and proteins associated with insulin signal transduction were investigated in the present study. SH-SY5Y cells were maintained in a high glucose environment and then presented with characteristics of type 1 DM in mouse model (such as the absolute lack of insulin neurotrophic factor, attenuated neuronal activity, impairment of the synthesis and secretion of neurotrophic factors and injured signal transduction). Thus, through weakening the synaptic transmission and synaptic transmission, P165 may raise the postsynaptic response and enhance the synaptic transmission to improve the learning and memory in animal model. What is more, P165 may activate the insulin signaling pathway and exert neuroprotective effect.

Our Previous studies showed that P165 was resistant to enzyme degeneration, increased the SHSY5Y cell viability, decreased the LDH leakage, and facilitate the growth of neurons *in vitro*. P165 also improved the performance in animal water maze test and the synaptic morphology of hippocampal neurons *in vivo* [4].

The neuron function depends on the synaptic transmission, in which synapse density proteins play a key role. Thus, PSD-95, Shank1, and α-synulein were investigated in the present study [6].

PSD-95 is a specific protein found in the glutamatergic postsynaptic dense zone. PSD-

95 may interacts with other proteins via different domains, which not only aggregates the NMDA receptor and its signal pathways associated proteins, but participates in the formation and maintenance of synaptic connections [7, 8]. Thus, PSD-95 plays a key role in mediating and integrating the NMDA receptor signal transduction.

Investigators co-cultured treated rat hippocampal slices with insulin, and results showed the PSD-95 expression increased in CA1 region, which was inhibited by the PI3K inhibitor LY294002. Insulin increases the PSD-95 expression through activating the receptor tyrosine kinase PI3K pathway, and alters the insulin signaling pathway and synaptic signaling pathway in the same direction [9].

Some researchers confirmed that the PSD-95 expression decreased in the neurodegenerative diseases [10], which was also confirmed in our experiment. *In vitro*, PSD-95 band was thinner, and lighter in color after treatment with high glucose and $A\beta_{25-35}$, but the band became thicker and darker after P165 treatment. *In vivo*, immunohistochemical staining showed PSD-95 positive cells decreased in the hippocampus of DM mice, and Western blot assay showed similar results. In this experiment, because of absence of insulin (insulin can up-regulate PSD-95), the PSD-95 expression decreased in model groups. Previous studies confirmed that P165 up-regulated the expression of PI3K and Akt, suggesting that P165 may up-regulate PSD-95 via the PI3K/Akt pathway.

Shank1 is a scaffolding protein playing an important role in the synaptic signal transduction. It is composed of isolated three postsynaptic membrane linked glutamate receptor through its multidomains [11, 12]. Shank1 knockout may lead to reduction in projection neurons in the hippocampus, while transfecting Shank1 into neurons protects the glutamate receptor function and increase the synaptic connections and the scope and frequency of [13]. Some investigators propose that Shank1 and glutamate receptors have a role in the occurrence and development of neurites and synapses [14]. The Shank1 expression decreased in injury group, but increased after P165 treatment in vitro. In vivo, immunohistochemical staining showed Shank1 positive cells decreased in the hippocampus of DM mice, and P165 returned the Shank1 expression to a nearly normal level, and partly recovered the structure and function of synapses.

The α-synuclein mRNA is highly expressed in the cortex, substantia nigra and limbic system [15]. Ueda isolated non A beta protein from component of amyloid plaques in AD, while the precursor protein of NAC is the α-synuclein [16]. During the development of rat brain, the α-synuclein expression is detectable at the time of gestational age 12-15 d, and increased sharply. α-synuclein participates in the formation of synapses [17]. α-synuclein is also involved in the synaptic plasticity by promoting the release of presynaptic transmitters [18]. α-synuclein and presynaptic vesicles are involved in the synaptic plasticity and the release of neurotransmitters, while its N terminal and C terminal participate in the formation of membrane complex, synthesis, and associated with the presynaptic vesicles to maintain and fusion [19, 20]. In vitro studies showed that NACP can occur aggregation in $A\beta_{25-35}$, and the binding of NACP to Aβ protein promotes this aggregation [21]. In vitro, Aβ can bind to the NACP/α-synuclein NAC fragment, which was also found in our experiment. Our results showed the α-synuclein expression increased in SH-SY5Y cells treated with high glucose and $A\beta_{25-35}$.

The results from in vivo experiments were similar to those from in vitro ones. The increased expression of α-synuclein is an adaptive response to reduced protein expression of PSD-95 and Shank1. In order to promote the release of presynaptic transmitters, it promotes the postsynaptic signal transduction. When this phenomenon occurs in the decompensation phase, the imbalance between synthesis and decomposition of synaptic proteins decreases the synaptic transmission efficacy, which influence the postsynaptic insulin signal transduction. P165 may normalize the expression of proteins to a certain extent, to partially recover the synaptic function.

Insulin signaling pathway related proteins play a very important role in the learning and memory [22]. These proteins can increase the glucose utilization of the specific brain regions (such as the hippocampus), and promote the synthesis of neurotransmitters (such as acetylcholine) [23].

Insulin receptor is composed of two α and two β subunits, and only the C terminal of β subunit contains specific intracellular protein tyrosine kinase activity [24]. Insulin receptor substrate-1 contains 21 potential tyrosine phosphorylation sites and more than 30 potential Ser/Thr phosphorylation sites. IRS-1 can interact with many proteins. As an upstream molecule of the insulin signaling pathway, it is susceptible to the influence by the external environment [25].

When the insulin is significantly insufficient, neurons may fail to compensate this insufficiency. On the contrary, the expression of other neurotrophic factors decreases. Only the supplement of exogenous neural survival factor can improve the functions of neurons [26]. Due to lack of insulin in DM mice, up-regulation of IR expression acts as a compensation for the impaired insulin signal transduction, and supplement of P165 may activate the neuronal signaling, and normalize the protein expression.

The Ras-MAPK pathway is a major signaling pathway of various neurotrophic factors [27]. MAPK is a Ser/Thr kinase and can phosphorylate the transcriptional factors, transduce extracellular signals and induce gene responses [28].

The MAPK expression decreased in injury group, but increased after P165 treatment *in vitro*. *In vivo*, immunohistochemical staining showed MAPK positive cells decreased in the hippocampus of DM mice, and the Western blot assay found similar findings. The increased MAPK may increase the dendrite protein synthesis and improve the synaptic plasticity.

As a kind of small molecular neurotrophic peptide, P165 can improve the expression of synaptic proteins and proteins related to the insulin signal transduction. One of the most important advantages of P165 is its resistance to enzyme degradation. However, the specific site of synapses and the exact mechanism underlying the action of insulin signaling pathway related proteins in postsynaptic membrane warrant further investigations.

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Disclosure of conflict of interest

None.

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