A New Vanadium Complex Improves the Spatial Learning and Memory by Activation of Caveolin–MAPK–CREB Pathway in Diabetic Mice

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Abstract

Diabetic encephalopathy, characterized by impaired spatial cognitive functions and the decline of learning and memory ability, involves direct neuronal damage caused by intracellular glucose. Vanadium, a required trace element of human body, is reported that it can reduce the blood glucose values of glycemia animals and has an effect on the treatment of diabetes complications. To investigate the role of vanadium in the pathogenesis of diabetic cognitive function impairment, Kunming mice were divided into control group, diabetes group and vanadium-treatment group. Diabetic mice were induced by intraperitoneal injection of 200 mg/kg of alloxan and in vanadium-treatment group diabetic mice were treated by intragastric infusion for three weeks with 5 mg/kg of VO(HB(3,5-Me_pz))(SCN)(SCNH)2, a new vanadium complex with 3,5-dimethyl-pyrazolyl ligand and relative lower toxicity. The three groups were trained by Morris water maze and then the expression of proteins related to learning and memory in the hippocampus of mice were examined by Western blot. The results showed that (1) the latency to find platform was longer (p<0.05) and the percent time in target quadrant was lower (p<0.05) in diabetes group compared with that in control group. The learning and memory score of vanadium-treatment group was obviously higher than that of diabetes group (p<0.05) and equivalent with the control group; (2) the phosphorylation level of p42/p44MAPK protein was remarkably decreased in diabetes group and then increased after vanadium treatment. Furthermore, Cavolin-1 expression was remarkably reduced and CREB2 expression was higher in diabetes group while after vanadium treatment caveolin-1 expression was significantly increased. These results suggest that vanadium can improve the learning and memory ability of diabetic mice and its mechanism may be involved in the activation of Caveolin–MAPK–CREB pathway in the neuron.

Keywords: Diabetes; Vanadium; Caveolin-1; Learning and memory

Abbreviations: CREB: cAMP responsive element binding protein; Cav-1: Caveolin-1; IR: Insulin receptor; mGluR1α: Metabotropic glutamate receptor 1α; MTT: 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide

Introduction

Diabetes mellitus, a common metabolic disease with a rising global prevalence, is associated with long-term complications of peripheral nervous system and the central nervous system [1-6]. Numerous studies indicate that diabetes mellitus might be accompanied with a certain damage of brain function involved in impairment of attention, information processing speed and spatial learning and memory [7-11]. The pathogenesis of the impaired brain function relating to diabetes has been studied involved in dysregulated innate immunity [12], vascular and metabolic mechanism [13], hippocampal neuronal apoptosis [14] and other factors. However to date there is still no convincing empirical evidence on the pathogenesis of this disorder and the drug candidates for improving cognitive deficits caused by diabetes are still few.

The transcription factor cAMP responsive element binding protein (CREB) is a nuclear protein that modulates the transcription of genes with cAMP responsive elements in their promoters. Genetic and pharmacological studies in mice and rats have demonstrated that CREB is essential for the induction of many forms of long-term synaptic plasticity, including spatial and social learning [15,16]. CREB1 is a transcriptional activator necessary for induction of long-term synaptic facilitation, while CREB2 functions as a transcriptional repressor that poses inhibitory constraints on the induction and formation of long-term memory. The repression by CREB2 could be relieved, possibly via phosphorylation of CREB2 by mitogen-activated protein kinase (MAPK) [17]. CREB1 and CREB2 interact via interlocked positive and negative loops [18]. Since CREB is critical for converting short- to long-term memory, the proteins in CREB pathway have been demonstrated as important targets for memory-modifying drugs [19].

Insulin/insulin receptor (IR) plays diverse roles in brain functions via activating specific signal transduction cascades, including spatial learning and memory formation [20,21]. As a receptor tyrosine kinase, IR in the hippocampus has been shown responding to learning experiences by alterations in its gene expression and activation of downstream molecules such as Shc/Erk1/2 and IRS-1/AKT in the stage of memory formation [20,21]. Caveolae, a subset of membrane microdomains, and its resident protein Cavolin-1 (Cav-1) are reported playing a major role in insulin signaling. IR is highly concentrated in caveolae and it has been reported that there is a critical important interaction between Cav-1 and IR in executing successful insulin signaling in adipocytes [22]. Recently, Cav-1 has been demonstrated to be essential for estrogen receptor a activation of metabolic...
glutamate receptor 1 α (mGluR1α), leading to MAPK-dependent CREB phosphorylation in neuron [23]. In addition, our previous results have shown that Cav-1 was involved in the discrimination learning [24].

Vanadium, a required trace element of human body, has been proved to have insulin-like function [25]. Since the insulin-like effects of vanadium salts in isolated rat adipocytes were reported, the insulin-like actions of vanadium complex have been examined in a large variety of insulin-responsive cells and tissues [26]. Vanadium complex not only improved the hyperglycaemia but also were involved in the treatment of diabetic complications such as obesity and hypertension [26–28]. Recently, more and more studies focused on the role of vanadium complex in brain function. It was reported that vanadium complex protected the streptozotocin-induced oxidative damage [29] and ameliorated the altered antioxidant status and membrane linked functions in diabetic rat brains [30]. Recently, vanadium-enriched chickpea was reported to ameliorate some hyperglycemic symptoms of the diabetic rats and reduce diabetes relating spatial learning and memory impairment [31]. Study also indicated that vanadium complex could be viewed as potential therapeutic agent to enhance ischemia-induced neurogenesis through PI3K/AKT and Erk activation [32]. However, potential short and long-term vanadium toxicity has slowed the acceptance for therapeutic use [33].

In this study, aimed at looking for a new vanadium complex with relative lower toxicity, we synthesized a oxovanadium-hydrotopolyazolylborate complex VO(3.5Me[Pz])2 (SCN)(SCN)2 [34].

With all above background, we hypothesize that the new vanadium complex may ameliorate the learning and memory of diabetic mice and its mechanism may be involved in Cav-1-PI3K/AKT or Erk-CREB activation. In present study, to investigate the effect of the new vanadium on diabetes relating spatial learning and memory impairment, the vanadium complex were administrated to diabetic mice, which were induced by alloxan. The expression of proteins related to learning and memory in hippocampus of mice was examined by Western blot following Morris water maze.

Materials and Methods

Animals

All animal experiments followed the guidelines of the International Council for Laboratory Animal Science (ICLAS). Efforts were made to minimize animal suffering and only the number of animals necessary to produce reliable scientific data was used. 3-5 months male Kunming mice (weighing 20±2 g, specific pathogen free) were purchased from Dalian Medical University (Dalian, China). The mice were housed five per cage at 23±2°C (12-h light/dark cycle) with ad libitum access to food and water. Mice were sacrificed by decapitation immediately after behavioral test. Hippocampus tissues were isolated and stored at -80°C until processed for further processing.

Reagents

Vanadium complexes were synthesized by the method described in previous study (34). Alloxan was purchased from Biosharp (USA). Polyclonal anti-rabbit Caveolin-1 (N-22) were from Santa Cruz Biotechnology (USA). Monoclonal anti-mouse β-actin and polyclonal anti-rabbit CREB2 were from Boster Biotechnology (China). Monoclonal anti-rabbit p44/42 MAPK, polyclonal anti-mouse phospho-p44/42 MAPK, monoclonal anti-rabbit AKT and polyclonal anti-rabbit phospho-AKT were from Cell Signaling Technology (USA).

Horseradish peroxidase-labeled goat anti-rabbit and horseradish peroxidase-labeled goat anti-mouse secondary antibodies were from Boster Biotechnology (China). Other biochemical reagents were from Sigma (USA) and Promega (USA).

Cell culture

3T3-L1 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM glucose, 100 µg/mL penicillin, 100 µg/mL streptomycin and 10 % fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5 % CO2. After 70–80 % confluence, cells were subcultured using 0.25 % trypsin in Ca2+-Mg2+ free phosphate buffered saline (PBS).

MTT reduction assay

Cell viability was measured with blue formazan that had been metabolized from colorless 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial dehydrogenases, which are only active in live cells.

Cells were pre-incubated in 96 multi-well plates at a density of 5×104 cells/well. When cells reached 70 % confluence, the monolayers were washed twice with DMEM. Cells were incubated in the medium with vanadium complexes of 10 μM for 24 h. Then the culture medium was replaced by a solution of MTT (5 mg/mL) in serum-free growth medium. After 4 h incubated at 37°C in a 5 % CO2 atmosphere, the supernatant was poured off, and the reaction was stopped with DMSO. The extracted was quantified at 570 nm with a microplate reader (Multiskan Ascent, Thermo Labsystems, USA), and the percentage viability was calculated.

Diabetes model induction and administration of vanadium complex

A single dose of 200 mg/kg alloxan freshly prepared in 20 mL/kg 0.9 % saline buffer was injected intraperitoneally to induce diabetes. The control mice received the same volume of 0.9 % saline buffer. Diabetes was confirmed after 3 days of alloxan injection for four times. After 48 h following alloxan injections, the blood samples were collected through tail vein and blood glucose levels were estimated by Glucoval Compact Meter and GlucoVal Strips (Biochemical Systems International s. r. l., Italy). On day 7 following alloxan injections, the mice with fasting blood glucose levels 540 mg/dL or higher were considered diabetic.

Diabetic mice were randomly divided into diabetes group (Diabetes, n=8) and vanadium treatment group (Vanadium, n=7). Vanadium group were treated with vanadium complex (5 mg/kg, p.o.) for consecutive three weeks. Vanadium complex was dissolved in 0.9 % saline buffer before administration in a constant volume of 15.6 mL/kg body weight. Control group (Control, n=10) and diabetes group received 15.6 mL/kg 0.9 % saline buffer. Glucose level was estimated weekly. Body weight was measured daily.

Open-field test

The mice were evaluated in big square box (100 cm×100 cm×50 cm) for a period of 5 min. The bottom of testing box was divided into 25 grids. The testing box was wiped clean with ethanol after each test. Following each mouse adapting to the box environment for 1 min, spontaneous locomotor activity was evaluated. We chose the number of crossing grids and the rearing times within 5 min as indicators [24].

Morris water maze test

After open-field test, animals were tested in a spatial version of...
Morris water maze test [35]. The apparatus consisted of a circular water tank (100 cm in diameter and 50 cm high). A platform (9 cm in diameter and 29 cm high), invisible to the mice, was set inside the tank which was filled with water maintained at approximately 20±1°C at a height of 30 cm. The tank was located in a large room where there were several different colored cues external to the maze; these were visible from the pool and could be used by the mice for spatial orientation. The position of the cues remained unchanged throughout the study.

The water maze task was carried out for 5 consecutive days. The mice received four daily training trials, with each trial having a ceiling time of 60 s and a trial interval of approximately 20 min. For each trial, each mouse was put into the water at one of four starting positions, the sequence of which being selected randomly. During test trials, mice were placed into the tank at the same starting point, with their heads facing the wall. The mouse had to swim until it climbed onto the platform and remained there for 10 s. The escape platform was kept in the same position relative to the distal cues. If the mouse failed to reach the escape platform within the maximally allowed time of 60 s, it was gently placed on the platform and allowed to remain there for the same amount of time.

On the next day a probe trial was performed where in the extent of memory consolidation was assessed. The time spent in target quadrant indicates the degree of memory consolidation that has taken place after learning. In probe trial, the mice were placed into the pool as in the training trial, except that the hidden platform was removed from the pool.

**Western blot**

Hippocampus tissues were homogenized with an lysis buffer (0.15 M NaCl, 1 % Triton X-100, 1 % Deoxycholic acid sodium salt, 0.1 % SDS, 10 mM Tris-HCl pH 7.4, 1 % phenylmethylsulfonfluoride) and centrifuged (10,000 rpm) for 10 min at 4°C. The supernatant was stored at -20°C for Western blot analysis.

The protein content of the samples was measured using the Coomassie technique. 100 μg of total protein were separated by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 2 h and transferred onto PVDF membrane (Hybond, USA) at room temperature (220 V for 40 min). Equal protein loading was verified by Coomassie Blue Staining. Membranes were incubated with polyclonal anti-rabbit Caveolin-1 (N-22) antibody (1:1000), polyclonal anti-rabbit CREB2 antibody (1:800), monoclonal anti-rabbit p44/42 MAPK antibody (1:1000), polyclonal anti-mouse phospho-p44/42 MAPK antibody (1:800), monoclonal anti-rabbit AKT antibody (1:1000), polyclonal anti-rabbit phospho-AKT antibody (1:800) or monoclonal anti-mouse β-actin antibody (1:1000) diluted in 0.1 % phosphate buffered saline (PBS) containing 0.1 % Tween 20 (PBST) overnight at 4°C. After the incubation, membranes were washed 3 times (10 min/time) in 0.1 % PBST and incubated in secondary antibody, horseradish peroxidase-labeled goat anti-rabbit (1:5000) or goat anti-mouse (1:5000) and rocked at 37°C for 1 h. Another 3-time wash in PBST, membranes were detected via enhanced chemiluminescence kit (Amersham Corp, USA) and developed to Kodak XAR films from 1 to 3 min.

Quantitative measurement of protein level was performed using the Image J program (NIH). And the value of protein levels was designed as 1 in the control group. The results were expressed as mean proportion of the control group values. Experimental design was shown in (Figure 1).

**Statistical analysis**

One-way ANOVA followed by Tukey test for multiple pairwise examinations was used for comparison. Changes were identified as significant if p was less than 0.05. p<0.01 was taken to indicate marked difference. Mean values were reported together with the standard difference of the mean (S.D.).

**Results**

**Lower-toxicity of the new vanadium complex with 3,5-dimethyl-pyrazolyl ligand**

To evaluate the toxicity of the new oxovanadium complex with 3,5-dimethyl-pyrazolyl ligand compared with other oxovanadium complexes, MTT reduction assay was conducted on 3T3-L1 cells. As shown in (Figure 2), incubation with 10 μM VO(ph-acac) (HB(pz)) or VO(O₂) (HB(pz))(pz) for 24 h resulted in a cell survival rate of 95.5 % or 82.6 % compared with VO(acac). However, incubated with VO(HB(3,5-Me,pz))(3,5-Me,pzH)(SCN)(SCNH), the cell livability was increased by 9 % compared with VO(acac).

The new vanadium complex ameliorate hyperglycaemia symptoms of diabetic mice

Body weight and blood glucose level after three weeks' vanadium treatment were measured to estimate the effect of vanadium on the
Blood glucose level of control, diabetic and vanadium-treatment mice were measured on a weekly basis and the body weight of each group were measured daily. The final measured results at the end of three weeks vanadium treatment are shown. Data are presented as mean±S.D. *

| Group       | Body weight(g) | Blood glucose (mg/dL) |
|-------------|----------------|-----------------------|
| Control(n=10) | 32.4±0.9       | 142.2±12.6            |
| Diabetes(n=8)  | 24.7±0.7**  | 540.0±0**             |
| Vanadium(n=7)  | 33.7±2.4      | 325.8±7.0             |

Blood glucose level of control, diabetic and vanadium-treatment mice were measured on a weekly basis and the body weight of each group were measured daily. The final measured results at the end of three weeks vanadium treatment are shown. Data are presented as mean±S.D. **p<0.01 vs. Control, p<0.05 vs. Diabetics.

Table 1: Effect of vanadium on body weight and blood glucose levels in the three groups.

Number of rearing and number of crossing were observed to evaluate spontaneous locomotor activity of each group mice. Data are presented as mean±S.D.

| Groups       | Number of rearing | Number of crossing |
|--------------|-------------------|-------------------|
| Control(n=10) | 6.00±0.05         | 26.8±4.32         |
| Diabetes(n=8)  | 7.25±1.05         | 24.8±2.92         |
| Vanadium(n=7)  | 4.71±0.87         | 22.29±1.44        |

Number of rearing and number of crossing were observed to evaluate spontaneous locomotor activity of each group mice. Data are presented as mean±S.D.

Figure 3: Effects of vanadium on the latency to find platform in diabetic mice. After spontaneous locomotor activity testing, average latency to locate the platform of each group mice in five days' acquisition trial were examined by Morris water maze. Data are presented as mean±S.D.

Figure 4: Vanadium complex prolong the time spent in target quadrant. Percent of time spent in target quadrant of each group mice in five days' acquisition trial were calculated to examine the learning ability of mice. Data are presented as the mean±S.D. P<0.05 vs. control group, P<0.05 vs. diabetes.

Figure 5: Percent of time spent in each quadrant in probe trials. After five days' acquisition trial, probe trial was performed to determine how well each group mice had learned and consolidated the platform location. In probe trial, platform, which is original in SE quadrant, was removed. Percent of time spent in each quadrant (NE, NW, SW and SE) were calculated, in which the percent of time spent in original target quadrant indicates the degree of memory consolidation that has taken place after learning.

Figure 6: Protein expressions of AKT and phospho-AKT in the hippocampus. Following Morris water maze protein expressions of AKT and phospho-AKT in hippocampus of each group mice were analyzed by Western blot. Densitometry values represent the ratio of MAPK/Actin, phospho-AKT/Actin and phospho-AKT/Actin (normalized to 1 in Control). Data are presented as the mean±S.D. There is no significant difference between each group.

Figure 7: MAPK pathway associated protein expressions in the hippocampus of each group. Following Morris water maze protein expressions of MAPK, phospho-MAPK, Cav-1 and CREB2 in hippocampus of each group mice were analyzed by Western blot. Densitometry values represent the ratio of MAPK/Actin, phospho-MAPK/Actin, Cav-1/Actin and CREB2/Actin (normalized to 1 in Control). Data are presented as the mean±S.D. P<0.01 vs. Control, P<0.01 vs. Diabetes, p<0.05 vs. Diabetes.

Improved performance of diabetic mice in Morris water maze by vanadium complex

Before testing cognitive function of the three groups, spontaneous locomotor activity was tested and the results showed no difference in the three groups’ mice (Table 2).

The learning and memory ability was assessed by Morris water maze. The mean escape latency of all the trained mice decreased over the course of the five days learning trials. There is no difference between diabetes and control group on the first and second days. But the mean escape latency of diabetic mice was longer than that of control mice in the third and fifth days. Vanadium treatment shortened the mean latency in diabetic mice (Figure 3). Diabetic mice showed a lower ability to find the platform and learn its location. This poorer performance was improved by the vanadium treatment. Although there is no marked difference in the escape latency, the percent time in target quadrant of the three groups suggests the same performance. The percent time in target quadrant did not differ between any of the groups on first and second days. But from the third day onwards there was significant symptoms of diabetes. There was a marked decline in the body weight and a dramatic increase in blood glucose level in diabetic mice compared with control mice (p<0.01). Treatment of diabetic mice with the new vanadium complex effectively increase the body weight to normal level and lowered the high blood glucose level (p<0.05) (Table 1).
Discussion

In the probe trial of the Morris water maze study, which measures how well the mice had learned and consolidated the platform location during the five days' training, differences were shown in the three groups (Figure 5). Control mice spent almost half of the time in the target quadrant while diabetic mice spent equal time in each quadrant. Subtle increase of percent time spent in target quadrant was shown in vanadium treatment mice compared diabetic mice, but it is obvious that the orientation of platform in vanadium treatment mice was not exact. The results showed that diabetic mice were accompanied with impaired spatial memory retention and vanadium had subtle improvement on the impaired spatial memory retention.

Changes of protein expressions by the new vanadium complex in diabetic hippocampus

To investigate the mechanism of improvement of vanadium on the impaired learning and memory ability in diabetes, here we examined the expression of proteins involved in insulin signaling by Western blot. The expressions of AKT and phospho-AKT did not significantly differ in each group (Figure 6), while the phosphorylation level of p42/p44 MAPK protein was remarkably decreased in diabetes group and then increased after vanadium treatment (Figure 7). The results suggest that MAPK signal pathway might be involved in the effect of vanadium on the learning and memory in diabetic hippocampus. In addition, the expression of upstream protein Cav-1 and downstream transcription factor CREB2 were following examined. Cav-1 expression was remarkably reduced and while after vanadium treatment Cav-1 expression was significantly increased. The expression of CREB2, which inhibits long-term memory, was higher in diabetic and vanadium treatment mice and vanadium failed to change the CREB2 expression in diabetes (Figure 7).

Evidence has shown that the insulin and insulin receptor (IR) play a role in cognitive function. IR signaling may play a modulating role to aid functions of the mainstream neurotransmitter receptors during memory processing. Learning-specific increases in levels of downstream molecules such as IRS-1 and Akt were detected in the synaptic membrane accompanied by decreases in Akt phosphorylation. Translocation of Shc protein to the synaptic membrane and activation of Erk1/2 were also observed after long-term memory formation [21]. In our results, p42/p44 MAPK phosphorylation was decreased in diabetic mice which were deficient in learning ability and spatial memory retention while after vanadium treatment it was remarkably increased. The expressions of Akt and phospho-AKT did not differ in each group. The results suggest that MAPK signal pathway may involve in the improvement of vanadium on the learning and memory ability in diabetic hippocampus.

In conclusion, this new vanadium complex treatment improved the learning and memory ability in these diabetic mice. The mechanism may be involved in the activation of Caveolin-MAPK-CREB pathway in the neuron. Moreover, due to its relative lower toxicity and high hypoglycemic effect efficiency, organic vanadium may find clinical application in the treatment of diabetes mellitus.
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