Novel peptide VIP-TAT with higher affinity for PAC1 inhibited scopolamine induced amnesia

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A novel peptide VIP-TAT with a cell penetrating peptide TAT at the C-terminus of VIP was constructed and prepared using intein mediated purification with an affinity chinin-binding tag (IMPACT) system to enhance the brain uptake efficiency for the medical application in central nervous system. It was found by labeling VIP-TAT and VIP with fluorescein isothiocyanate (FITC) that the extension with TAT increased the brain uptake efficiency of VIP-TAT significantly. Then short-term and long-term treatment with scopolamine (Scop) was used to evaluate the effect of VIP-TAT or VIP on Scop induced amnesia. Both short-term and long-term administration of VIP-TAT inhibited the latent time reduction in step-through test induced by Scop significantly, but long-term administration of VIP aggravated the Scop induced amnesia. Long-term i.p. injection of VIP-TAT was shown to have positive effect by inhibiting the oxidative damage, apoptosis and the cholinergic system activity reduction that induced by Scop, while VIP exerted negative effect in brain opposite to that in periphery system. The in vitro data showed that VIP-TAT had not only protective but also proliferative effect on Neuro2a cells which was inhibited by PAC1 antagonist PACAP(6-38). Competition binding assay and CAMP assay confirmed that VIP-TAT had higher affinity and activation for PAC1 than VIP. So it was concluded that the significantly stronger protective effect of VIP-TAT against Scop induced amnesia than VIP was due to (1) the enhanced brain uptake efficiency of VIP-TAT and (2) the increased affinity and activation of VIP-TAT for receptor PAC1.

Introduction

Vasoactive intestinal peptide (VIP) is a 28 amino acids peptide first reported in 1970 [29], which is now known as a neurotransmitter, neuro-modulator, neurotrophic and neuro-protective factor widespread in the peripheral and central nervous systems (CNS) [6,11]. VIP has 68% amino acid homology with a C-terminal truncated form of neuropeptide PACAP27 (pituitary adenylate cyclase activating polypeptide), which is also a member of to VIP/secretin/growth hormone releasing hormone/glucacon super family [26]. VIP and PACAP share two receptors VPAC1 and VPAC2, while VIP has affinity about 1000 folds weaker than PACAP for PACAP preferring receptor PAC1 [15].

VIP and its receptors are found in several regions of the brain responsible for learning and memory such as cerebral cortex, hippocampus, amygdala, and suprachiasmatic nucleus [1,16,25], which suggests a crucial role of this peptide in cognitive function [11]. Investigation on the gene-deficient animals [4] and transgenic model [13] has also confirmed that VIP is involved in learning and memory process. It has been reported that a single intraperitoneal (i.p.), subcutaneous (s.c.) or intracerebroventricular (i.c.v.) injection of VIP ameliorates the scopolamine (Scop) induced amnesia [34]. But there are also some contradictory data, such as injection of VIP into the lateral cerebral ventricle at nonphysiological high doses and chronic intracerebroventricular infusion of VIP was reported to produce amnesia [31,32]. Yet the positive reports of VIP such as VIP lipophilic analogues against β-amyloid peptide induced Alzheimer disease [12] and the neurotrophic and neuron-protective effect of VIP in CNS [27] push forward to the development and the application of VIP and its analogues as the therapy for neurological diseases and disorders.

VIP is known to cross the blood–brain barrier (BBB) by transmembrane diffusion, a non-saturable mechanism [30]. But the rapid degradation of VIP in vivo hampers its application in CNS. In our previous reports, an 11-amino acid cell penetrating peptide (TAT peptide) derived from the human immunodeficiency virus type 1 (HIV) Tat protein had been used to improve the efficiency

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of PACAP to traverse biological barriers [36,38]. So in this research we constructed a novel recombinant peptide VIP-TAT which was hypothesized to have enhanced ability of traversing BBB into brain compared with VIP.

After the efficiencies of VIP-TAT and VIP traversing into brain via i.p. injection were assayed, the effects of VIP-TAT and VIP on the food intake in mice were compared to confirm the traversing efficiency and the biological function in vivo of the recombinant peptide VIP-TAT. And then two types of amnesia model induced by short-term (one dose) administration and long-term administration of Scop in mice combined with step-through test were used to detect the effect of short-term (one-time) and long-term (14 days) treatment with VIP-TAT against the Scop induced amnesia. It was shown that VIP-TAT had more significant and more stable inhibitory effect against Scop induced reduction in the latent time than VIP. Long-term administration with VIP-TAT ameliorated the oxidation and apoptosis, improved the cholinergic system reactivity, while long-term administration with VIP exerted negative effect in brain.

In order to explain the significant difference between VIP-TAT and VIP in Scop induced amnesia, some in vitro assays including the detection of the peptides against Scop-induced apoptosis in Neuro2a neuroblastoma cells, competition binding assay and CAMP assay in Chinese hamster ovary (CHO) cells expressing PAC1 stably (named PAC1-CHO) were performed. And it was indicated that VIP-TAT had more affinity and activation for PACAP preferring receptor PAC1 than VIP.

Materials and methods

Preparation of recombinant VIP-TAT

Recombinant peptide VIP-TAT, which is composed of the 27 amino acids VIP with the 11-aa TAT peptide (YGRKKRRQRRR) at the C-terminus of VIP (shown in Fig. 1A), was produced using the IMPACT (intein mediated purification with an affinity chitin-binding tag) system (New England Biolabs, USA) in the same way as PACAP-TAT reported before [36,38]. In brief, the recombinant pKX-VIP-TAT plasmid (shown in Fig. 1B) was transformed to Escherichia coli strain ER2566, which allowed VIP-TAT to be expressed with intein and the chitin-binding domain (CBD) as a fusion protein in the presence of IPTG (isopropyl-β-D-thiogalactoside). The fusion protein, after binding to chitin column, was cleaved in the presence of 100 mM β-mercaptoethanol, releasing target peptide VIP-TAT. The target peptide firstly submitted to dialysis to remove β-mercaptoethanol was then further purified by the molecular sieve chromatography using Sephadex G-50 (GE Healthcare Life Sciences, USA). The recombinant peptide VIP-TAT has a residue Met at the N-terminus and five amino acid residues (LEGSS) at the C-terminus from the vector pKXB (New England Biolabs, USA) after the cleavage with β-mercaptoethanol. Western blotting using monoclonal antibody against VIP (Santa Cruz, USA) was used to characterize the preparation of VIP-TAT. High performance liquid chromatography (HPLC) with C18 reversed phase column using acetonitrile/water/methanol mobile phase (1.0 mL/min) and DAD detection at 210 nm was used to characterize the purity of VIP-TAT. Meanwhile VIP was chemically synthesized by Qiangyao Biotech (Shanghai, China).

Peptides labeled with FITC

Both the recombinant VIP-TAT and VIP were labeled with fluorescein isothiocyanate (FITC) using a FITC Protein Labeling Kit from ChangRui (Guangzhou, China) following the manual. In brief, the lyophilized purified peptide powder diluted in PBS was firstly activated with reaction buffer. FITC dissolved in DMSO (10 mg/mL) was added to the activated protein solution to a final ration of 1 mg FITC: 5 mg protein, and the reaction was carried on at room temperature for 1.5–2 h protected from light. The FITC-labeled proteins were further purified from the free FITC by gel filtration columns and were subjected to the protein concentration determination using K4000 Bradford Protein Quantification Kit (Innovative com., Guangzhou, China). Fluorescence measurement was performed with a multi-wavelengths scanner Victor 3 (PerkinElmer, USA). Excitation and emission wavelengths were 495 and 520 nm respectively with spectral bandwidth of 5 nm. And the label efficiency was calculated following the formula: Label Efficiency (LE) = Fluorescence value (FV)/Peptide mass number (PM), representing the fluorescence intensity (AU) of per mole peptide (mol).

Efficiency assay of traversing into brain by fluorimetry

Male NIH mice were purchased from the Medical and Experimental Animal Center (Guangdong, China) and used at 7–8 weeks of age. Animals were housed in an air-conditioned room at 22–25°C with a 12 h light: 12 h dark cycle. Drinking water and standard mouse diet were freely available. All animal procedures were done in accordance with the applicable institution and governmental regulations concerning the ethical use of animals under the ethical permission for in vivo animal experiments (No. SCXK-2012-0097). Mice were randomly assigned to one of three experimental groups (9 rats per group) and submitted to the i.p. injection of VIP-TAT–FITC (100 nmol/kg) or VIP–FITC (100 nmol/kg) or saline. After bloodletting from the retinal vein plexus, the mice were sacrificed by cervical dislocation and the brain tissues were separated and weighed, washed by PBS for three times, and subjected to ultra-sonication in PBS. The supernatant was collected after centrifugation and the fluorescence intensity in the supernatant was determined using a 1420 multilabel counter Victor 3 (PerkinElmer, USA). Excitation and emission wavelengths were 495 and 520 nm respectively with spectral bandwidth of 5 nm. The valid fluorescence intensity (FI) for each sample treated with FITC labeled peptide was corrected by subtracting the fluorescence value of the sample treated with saline, which was used as a background. The brain uptake efficiency after i.p. injection was expressed as the percentage of the FITC labeled peptide mass number in the brain tissue to the total FITC labeled peptide mass number used for i.p. injection. The brain uptake efficiency (BUE) was calculated using the formula: BUE = tFl/LE/PM (tFl presents the total fluorescence intensity in brain tissue (arbitration unit; AU); LE presents the label efficiency of each peptide which has been determined above; PM presents the peptide mass number totally used during the i.p. injection.

Food intake assay

In order to detect the in vivo function of VIP-TAT and VIP on the food intake via i.p. injection, male NIH mice were starved for 24 h with only water supply. The mice were randomly assigned to one of three experimental groups (9 rats per group) and submitted to the i.p. injection of VIP-TAT (100 nmol/kg) or VIP (100 nmol/kg) or saline (used as blank control). Food was offered to the mice 15 min after the i.p. injection and the food intake was recorded every 1 h for 4 h from the time of the feeding.

Step-through test

Step-through test was conducted using a passive avoidance reflex apparatus JD-500 with 6 detection channels from Jide Expre. Instru. Pla. (Shanghai, China). The test was conducted in three consecutive days. On the first day, male NIH mice aged from 7 to 8 weeks were accommodated to the step-through test instrument
one by one by placing them into the bright chambers and ensuring that they had made positive passive avoidance into the dark chambers without any electric shock. The next day, the mice were i.p. injected with or without Scop (3 mg/kg) 30 min before they were replaced into the bright chambers to receive learning training by suffering 60 V electric shock when they made passive avoidance into dark chambers. The learning training time lasted for 3 min to ensure that the mice received electric shock at least for 3 times and stayed in the bright chambers. On the third day, 24 h after the learning training, the mice were placed into the bright chambers for the third time. And the time counters fixed on the tunnels between the bright chambers and the dark chambers recorded the latent time (within 5 min) for the mice from being put into the bright chambers to passing into the dark chambers. The longer latent time indicated that the mice had better memory of the electric shock received in the dark chambers. Saline (i.p. injection) instead of Scop was used as normal control.

Scop induced amnesia and peptide administration

Male NIH mice aged from 7 to 8 weeks were randomly assigned to one of the experimental groups (9 rats per group) submitted to short-term (10–1000 nmol/kg for one time) and long-term (100 nmol/kg/day for 14 days) administration of VIP-TAT or VIP as indicated in Table 1.

In short-term administration, during the step-through test, before receiving learning training on the second day, mice were submitted to the i.p. injection of VIP-TAT (10–1000 nmol/kg) or VIP (10–1000 nmol/kg) or saline just before the i.p. injection of Scop (3 mg/kg) to detect the inhibitory effect of the peptide against the one-dose Scop induced amnesia. During the step-through test, i.p. injection of saline instead of Scop was used as normal control.

In long-term administration, mice were randomly assigned to one of four groups. Two groups were treated with i.p. injection of VIP-TAT (100 nmol/kg/day) + Scop (3 mg/kg/day) and VIP (100 nmol/kg/day) + Scop (3 mg/kg/day) respectively. One group treated with i.p. injection of saline + Scop (3 mg/kg/day) was used as negative control, while one group just treated with i.p. injection of saline without Scop was considered as normal control. After the treatment for 14 days, on the 15th day, the body weight was recorded and the step-through test was conducted. Scop or peptide was not used during the step-through test anymore, because the amnesia had been induced by the long-term administration of Scop. After the step-through test the mice were sacrificed, and blood samples were then collected and sent to the Inspection Center in the Affiliated First Hospital of Jinan University for the measurement of hemoglobin (HGB) level and serum superoxide dismutase (SOD) level. Furthermore, the brain tissues of the mice treated with VIP-TAT, VIP or saline for 14 days were collected and subjected to the determination of the oxidative, anti-oxidative

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indexes and apoptotic index including the level of malondialdehyde (MDA), SOD, the concentration of neurotransmitter regulator acetylcholinesterase (AChE) and the caspase 3 activity.

**Determination of MDA, SOD and AChE levels in brain tissues**

The mice treated with VIP-TAT, VIP or saline combined with or without Scop for 14 days following the procedure described above were sacrificed by cervical dislocation and the brain tissues were separated and weighted, washed by PBS for three times, and subjected to ultrasonication in PBS with a concentration of 200 mg tissue per milliliter. The supernatant was collected after centrifugation and the levels of MDA, SOD and AChE in the supernatant were all determined. The levels of MDA and SOD in the supernatant were both assayed based on colorimetry using detection kits from Jiancheng Biotech. Com. (Nanjing, China). The detection of MDA is based on the chromogenic reaction between MDA and thiorbarbituric acid (TBA), while the assay of SOD depends on its inhibitory effect on the superoxide anion. The AChE level in the brain tissue was determined using Mice AChE Elisa Kits from Yanyu Biotech. Com. (Shanghai, China). All the detections were made following the procedures in the manuals of the corresponding kits, and all the results were normalized by the protein concentrations in the samples. The protein concentration was determined by BCA (bicinchoninic acid) method using BCA protein concentration detection kit (Jiancheng, Nanjing, China).

**Cell viability assay**

To investigate the protective effect of VIP-TAT and VIP against Scop induced apoptosis, Neuro2a neuroblastoma cells were plated in 6-well plates and allowed to grow in DMEM with 5% Fetal Bovine Serum (FBS) to a fusion rate about 80%. VIP-TAT or VIP (0.01–10 nmol/L) with or without PACAP6-38 (10 nmol/L) was added to the cellsstarved without FBS overnight for 3 h incubation before Scop (0.06 mg/mL) was added. After the incubation for 12 h, the viability of the cells was determined using the colorimetric MTT assay (methyleneblue tetrazolium bromide, Sigma, USA). The cell viabilities were expressed as the percentage of the MTT value of the blank control (treatment without any peptides and without scopolamine). All experiments were run with at least four parallel samples and were repeated three times.

**Caspase 3 activity assay**

For in vitro assay, apoptosis related index caspase 3 activity was determined in the cells treated with VIP-TAT or VIP (1 nmol/L) with Scop (0.06 mg/mL). Neuro2a neuroblastoma cells were seeded in 6-well plates and allowed to grow in DMEM with 5% FBS to a fusion rate about 80%. VIP-TAT (1 nmol/L) or VIP (1 nmol/L) was added to the cellsstarved without FBS overnight for 3 h incubation before Scop (0.06 mg/mL) was added. After the incubation with peptides and Scop for 6 h, cells were harvested and subjected to the assay of caspase 3 activity based on the catalytic activity of caspase 3 on Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroanilide) to produce colored pNA (p-nitroaniline) using the caspase 3 activity detection kit from Biyuntian, Biotech. Com (Shanghai, China). Assays were achieved following the procedures in the manuals of the kits and the data were normalized by the protein concentrations in the samples measured by BCA method. All the results were plotted as the folds of blank control (treatment without any peptides and without scopolamine). All experiments were run with at least four parallel samples and were repeated three times.

For in vivo assay, the mice treated with VIP-TAT, VIP or saline combined with or without Scop for 14 days following the procedures described above were sacrificed by cervical dislocation and the brain tissues were separated and weighted, washed by PBS for three times, and subjected to ultrasonication in PBS with a concentration of 200 mg tissue per milliliter. The supernatant was collected after centrifugation and the caspase 3 activity in the supernatant was assayed following the procedures in the manual of the kit described above and the data were normalized by the protein concentrations in the samples measured by BCA method. All the results were plotted as the folds of control (mice treated with saline). All experiments were run with at least four parallel samples and were repeated three times.

**Membrane preparation and competition binding assay**

Membrane preparation and competition binding assay were performed following the protocol described before [39]. Membranes were prepared from CHO cells expressing PAC1 stably named PAC1-CHO. PAC1-CHO cells were harvested with a rubber policeman and pelleted by low speed centrifugation; the supernatant was discarded and the cells lysed in 1 mM NaHCO₃ solution and immediately frozen in liquid nitrogen. After thawing, the lysate was first centrifuged at 4 °C for 10 min at 400 × g and the supernatant further centrifuged at 20,000 × g for 10 min. The pellet resuspended in 1 mM NaHCO₃ was used immediately as a crude membrane preparation. To measure the receptor binding of VIP-TAT and VIP, 10 mg membrane was incubated with 0.1 nmol/[125I]PACAP38 (1767–34 Ci/mmol) (PerkinElmer, USA) in the presence of increasing concentration of peptide, in a total volume of 100 mL of 20 mM HEPES (pH7.4), 150 mM NaCl, 0.5% BSA, 2 mM MgCl₂, and 0.1 mg/mL bacitracin. After incubating at 37 °C for 20 min, bound ligand was collected on GF/C filters pretreated with 0.1% polyethylenimine. The filters were washed with cold 25 mM NaPO₄ containing 1% BSA and counted in a gamma counter. Non-specific binding was defined as the residual binding in the presence of 1 mM PACAP38 and was always below 20% of the total binding. And the data were plotted as the percentage of the maximum binding by [125I]PACAP38. All experiments were run with at least four parallel samples and were repeated three times.

**cAMP accumulation assay**

The PAC1-CHO cells cultured in DMEM nutrient at 37 °C were scraped off the surface with rubber policeman, washed with PBS for two times and the density of the cells was adjusted to 2 × 10⁵/mL. VIP-TAT or VIP was added to 500 mL cells suspension, and the working concentrations of the peptide were changed from 10⁻¹⁰ to 10⁻⁶ mol/L. The reactions were incubated at 37 °C for 5 min and then were incubated at room temperature for 20 min after 2 volume 0.2 mol/L HCl was added. The mixture was dissociated by pipetting up and down until suspension was homogeneous. The precipitate was removed by centrifugation at 1000 × g for 10 min, and the supernatant was collected to the clean test tube and submitted to the assay for the cAMP quantities using the enzyme immunoassay kit for cAMP (Biyuntian, Shanghai, China) following the operating instruction. All experiments were run with at least four parallel samples and were repeated three times.

**Statistical analysis**

The data were compared by ANOVA followed by the Student–Newman–Keuls posthoc test. Groups of data from both statistical tests were considered to be significantly different when P < 0.05. All results were expressed as means ± SEM (standard error of the mean).
**Results**

**The preparation of VIP-TAT**

The recombinant VIP-TAT was produced by the IMPACT system using chitin affinity chromatography combined with molecular sieve chromatography. SDS-PAGE and western blotting (Fig. 1C) showed that VIP-TAT was prepared with expectant immunogenicity.

**The traversing efficiency of VIP-TAT**

The FITC labeling combined with fluorometry showed that i.p. injection of VIP-TAT (100 nmol/kg) resulted in VIP-TAT traversing blood–air barrier into the brain with the brain uptake efficiency of 1.81 ± 0.34%, which was about 2 folds of the brain uptake efficiency of VIP (0.78 ± 0.22%). (B) Both i.p. injection of VIP-TAT (100 nmol/kg) and VIP (100 nmol/kg) significantly inhibited the food intake (P < 0.01, VIP-TAT and VIP vs. saline), while the inhibitory effect of VIP-TAT was significantly stronger than that of VIP (ΔP < 0.01, VIP-TAT vs. VIP).

**Effect of VIP-TAT on latent time in Scop induced amnesia**

As shown in Table 1, two types of amnesia model induced by short-term and long-term treatment of Scop were used combined with short-term and long-term i.p. injection of VIP-TAT or VIP to evaluate the short-term and long-term effect of i.p. injection of VIP-TAT or VIP on the amnesia induced by Scop. In the short-term test (Fig. 3A), Scop i.p. injected before the learning training impaired the memory storage and reduced the latent time significantly (P < 0.01, saline + Scop vs. normal). Administration of VIP-TAT (10–1000 nmol/kg) for one time just before the i.p. injection of Scop significantly inhibited the reduction of the latent time induced by Scop in dose dependent manner (P < 0.01, VIP-TAT + Scop vs. saline + Scop), and the effect of VIP-TAT was significantly higher than that of VIP in the same concentration (P < 0.01, VIP-TAT + Scop vs. VIP + Scop). Administration of VIP for one time via i.p. injection showed inhibitory effect against Scop induce reduction of latent time, but the effect of VIP was significantly weaker and less stable than that of VIP-TAT, because VIP in the concentration of 1000 nmol/kg displayed somehow negative effect compared with that in lower concentration.

When long-term i.p. injection of Scop was used to produce another type of amnesia, it was found that (Fig. 3B) even without any interference of Scop in the learning train during the step-through, the mice treated with Scop for 14 days showed significant cognitive impairment with significant reduction in latent time (P < 0.01, saline/Scop vs. Normal). Administration of VIP-TAT before i.p. injection of Scop every day for 14 days significantly protected the mice against the Scop-induced amnesia by prolonging the latent time significantly (P < 0.01, VIP-TAT/Scop vs. saline/Scop). On the contrary i.p. injection of VIP before i.p. injection of Scop every day for 14 days aggravated the Scop induced amnesia by further decreasing the latent time (P < 0.05, VIP/Scop vs. saline/Scop). It was showed the long-term effect of VIP-TAT on the Scop induced amnesia was positive and opposite to the long-term effect of VIP, which seemed to be negative, indicating that VIP-TAT may have different activity mechanism from VIP.

**Effect of long-term treatment with VIP-TAT in Scop induced amnesia**

In order to explain the significantly difference between the long-term effect of VIP-TAT and that of VIP in Scop induced amnesia, some physiological and biochemical indexes were assayed. It was shown that long-term i.p. injection of Scop did not affect the body weight, while long-term i.p. injection of VIP-TAT or VIP induced reduction in the body weigh (Fig. 4A).

In the brain tissues (Fig. 4B–E), it was shown that long-term i.p. injection of VIP-TAT significantly reversed the Scop induced oxidation by promoting the SOD concentration and decreasing MDA level,ameliorated the Scop induced apoptosis by inhibiting caspase-3 activity (Fig. 4B–E, P < 0.01, VIP-TAT/Scop vs. saline/Scop). Furthermore, long-term i.p. injection of VIP-TAT not only inhibited the Scop induced increase in AchE level (Fig. 4E, P < 0.01, VIP-TAT/Scop vs. saline/Scop), but also decreased the AchE level so effectively to make the AchE level significantly lower than that in normal mice (Fig. 4E, P < 0.01, VIP-TAT/Scop vs. normal). It was found that long-term i.p. injection of VIP did not inhibit the oxidation and reduction in the cholinergic system activity that induced by Scop, and even raised the MDA level in the brain tissues (Fig. 4C, P < 0.05, VIP/Scop vs. saline/Scop).

As for the indexes in the periphery system (Fig. 4F and G), opposite with the effects of VIP in brain, the effects of VIP were positive. Both long-term i.p. injection of VIP and VIP-TAT ameliorated the injury induced by Scop by increasing the serum SOD level and blood HGB levels (Fig. 4F and G, P < 0.01, VIP/Scop vs. VIP-TAT/Scop vs. Control).
saline/Scop). But the effect of VIP-TAT in raising peripheric SOD level and blood HGB level was more stronger than that of VIP (Fig. 4F and G, P < 0.01, or VIP-TAT/Scop vs. VIP/Scop).

**Effect of VIP-TAT on Scop induced apoptosis in Neuro2a cells**

In order to explain the stronger inhibitory effect of VIP-TAT against Scop than VIP in more detail, Neuro2a cells were used to assay the protective effect of VIP-TAT against the Scop (0.06 mg/mL) induced apoptosis. The MTT results (Fig. 5A) found that 0.06 mg/mL Scop induced significant cell viability reduction about 20%, because only about 80% cell remaining viability after the incubation of cells with Scop. Both pre-treatment with VIP-TAT and VIP (0.01–10 nmol/L) inhibited the reduction of cell viability induced by Scop, but the viability of cells treated with VIP-TAT (0.01–1 nmol/L) was significantly higher than that treated with VIP in the same working concentration (Fig. 5A, P < 0.01, VIP-TAT vs. VIP). Furthermore VIP-TAT (0.1–10 nmol/L) showed significant proliferative effect by increasing the cell viability over 100%, significantly higher than the cell viability of blank control (without peptide and Scop), while VIP did not exhibited such significant proliferative effect. The significant difference between VIP-TAT and VIP indicated that they may have different receptor activation profiles. Considering that VIP has high homology with PACAP27 and the secondary structure detection showed that TAT (YGRKQRRRQRRR) has similar helix structure with PACAP28–38 (GKRYQRRKVRKNK), we
Fig. 4. The long-term effects of Scop combined with VIP-TAT or VIP on body weight (A), SOD level in brain (B), MDA concentration in brain (C), AchE activity in brain (D), Caspase 3 activity in brain (E), serum SOD level (F) and blood HGB concentration (G). (A) Long-term treatment of VIP-TAT (100 nmol/kg/day × 14 days) or VIP (100 nmol/kg/day × 14 days) significantly decreased the body weight (*P < 0.01, VIP-TAT/Scop and VIP/Scop vs. saline/Scop), while long-term i.p. injection of Scop (3 mg/kg/day × 14 days) did not affect body weight. (B–E) In brain tissues, long-term i.p. injection of Scop (3 mg/kg/day × 14 days) resulted in significant decrease of SOD level and significant increase in MDA level, caspase 3 activity and AchE activity (#P < 0.01, saline/Scop vs. Normal). Long-term administration of Scop combined with VIP-TAT significantly inhibited the above mentioned oxidative and apoptotic lesion induced by Scop (*P < 0.01, VIP-TAT/Scop vs. saline/Scop), and promoted the cholinergic nervous activity by decreasing AchE levels even significantly lower than that in normal control (#P < 0.01, VIP-TAT/Scop vs. Normal). Long-term treatment with VIP did not inhibited the Scop induced oxidation, apoptosis and reduction in cholinergic nervous activity (#P < 0.01, VIP/Scop vs. Normal), and even aggravated the Scop induced oxidation by increasing MDA levels in brain higher than that in mice treated with Scop (ΦP < 0.01, VIP/Scop vs. saline/Scop). It was shown that in brain, the long-term effects of VIP-TAT was significantly positive, while the long-term effects of VIP was significantly negative (ΔP < 0.01, VIP-TAT/Scop vs. VIP/Scop). F–G. In peripheral system, long-term treatment of Scop decreased the serum SOD level and blood HGB level (#P < 0.01, saline/Scop vs. Normal). Both long-term treatment with VIP and VIP-TAT were positive by inhibiting the negative effects of Scop (*P < 0.01, VIP-TAT/Scop and VIP/Scop vs. saline/Scop). And the effects of VIP-TAT were significantly stronger than that of VIP (ΔP < 0.01, VIP-TAT/Scop vs. VIP/Scop).
hypothesized that VIP-TAT had higher affinity for PACAP preferring receptor PAC1 than VIP. PAC1 antagonist PACAP6-38 was used to verify this hypothesis. It was found that the proliferative effect of VIP-TAT (1 nmol/mL) was significantly inhibited by PACAP6-38 (10 nmol/mL) (Fig. 3B, *P < 0.01, VIP-TAT/PACAP6-38 + Scop vs. VIP-TAT + Scop), while PACAP6-38 (10 nmol/mL) did not interfere the effect of VIP significantly.

The results of caspase 3 activity assay showed that Scop induced increase in caspase 3 activity (Fig. 5C, *P < 0.01, Scop vs. Control). Pretreatment with VIP-TAT (1 nmol/mL) and VIP (1 nmol/mL) significantly inhibited the increase in caspase 3 activity induced by Scop, and the effect of VIP-TAT (1 nmol/mL) in reducing caspase 3 activity was significantly stronger than that of VIP (Fig. 5C, *P < 0.01, VIP-TAT + Scop vs. VIP-TAT + Scop). But the effect of VIP-TAT in reducing caspase 3 activity was inhibited by the addition of PACAP6-38 significantly (Fig. 5B, *P < 0.01, VIP-TAT/PACAP6-38 + Scop vs. VIP-TAT + Scop), which indicated that part of VIP-TAT activity was mediated by PAC1.

Affinity and activation of VIP-TAT for PAC1

Competition binding of [125]PACAP38 on membranes purified from CHO cells expressing PAC1 (PAC1-CHO) and cAMP assay were used to confirm the affinity and activation of VIP-TAT for PAC1. It was shown that VIP-TAT competitively displaced [125]PACAP38 from PAC1 with a half-maximal inhibitory concentration (IC50) of 53 ± 7 nM, whereas VIP had an IC50 over 1 μM (Fig. 6A). And VIP-TAT had significant stronger activation for PAC1 by promoting cAMP levels in PAC1-CHO cells than VIP in the working concentration of 10⁻¹⁰–10⁻⁸ mol/L (Fig. 6B).

Discussion

Scop is widely considered as a cognitive damage inducer by inducing oxidative stress [19,22] and impairing cholinergic system activity by increasing the AChE activity [14]. In this research, short-term administration and long-term administration of Scop were both used to induce amnesia, which may be different from each other, because it has been showed that an effective amnestic dose of scopolamine negatively modulates long-term memory expression but not memory storage in the crab [3]. Long-term treatment of Scop used in this research was shown to disrupt the memory in a larger scope than short-term treatment of Scop, because Scop was not used during the step-through test but exerted significant negative effect on the latent time, which indicating that long-term i.p. injection of Scop had destroyed the nervous system activity.

In this research, cell penetrating peptide TAT was originally designed to help VIP improve the brain uptake efficiency of VIP, which will help to expand the medical application scope of peptide and simplify the administration way. For example, TAT helped functional protein and large molecular increase the brain uptake efficiency via intranasal inhalation [21,35]. It was also shown in this research that VIP-TAT did have higher brain uptake efficiency after i.p. injection than VIP, which suggested that VIP-TAT might be developed as naristilae for its medical usage in brain diseases and disorders.

But the enhanced traversing ability could not explain all the significant different effects of VIP-TAT from VIP on the Scop induced amnesia and Scop induced apoptosis. In short-term administration, VIP-TAT (10–1000 nmol/kg) inhibited Scop induced amnesia in a dose dependent manner, while VIP in higher concentration (1000 nmol/kg) exerted weaker effect than VIP in lower concentration on Scop induced amnesia. In long-term Scop induced
amnesia, opposite to VIP-TAT, long-term i.p. injection of VIP displayed negative aggravating effects by shortening the latent time and increasing MDA level in brain tissue. Just like the contrary of VIP on amnesia shown here, some similar contradictory results have been reported, e.g. in some cases amnesic effects were described after intrahippocampal administration of VIP [18,31,32]. But the long-term effects of VIP in periphery system still remained positive, i.e., long-term treatment with VIP increased the SOD and HGB levels in blood, which was in consistent with the previous related reports [7,9]. These results indicated that the functional mechanisms of VIP and VIP-TAT on Scop induced amnesia were different from each other. The in vitro data that VIP-TAT (0.1–1 nmol/L) had the extra proliferative effect on Neuro2a cells confirmed that VIP-TAT had different receptor activation profiles from VIP.

When we analyze the structures of VIP and VIP-TAT, it was found that TAT sequence had helix structure similar with the helix structure of PACAP(28–38). On the other side, PACAP(11–38) with helix structure had been proven to be a novel cell penetrating peptide [5], supporting that TAT had similar helix structure and similar function with PACAP(28–38). Furthermore Goure et al. had proven that the presence of the PACAP(28–38) extension not only increased at least 100-fold the VIP- or VIP fragment affinities for the selective PACAP receptor (PAC1), but also stabilized the structure of N-terminally truncated VIP [10]. So VIP-TAT was hinted to have higher affinity for PAC1 than VIP. In this research PAC1 antagonist PACAP(6-38) significantly inhibited VIP-TAT effects of promoting the proliferation of Neuro2a cells and of reducing caspase 3 activity against Scop, without apparent interference on the effects of VIP, help us confirm firstly that VIP-TAT has higher affinity and activation for PAC1 than VIP. Competition binding assay for PAC1 and cAMP assay further confirmed the affinity and activation bias of VIP-TAT for PAC1.

PACAP with about 1000 folds higher affinity for its preferring receptor PAC1 [15] had been reported to promote the proliferation of Neuro2a cells through PAC1, while VIP dose not show proliferative effect in Neuro2a cells [24]. These previous reports help to explain why VIP-TAT with higher affinity for PAC1 than VIP promoted the proliferation of Neuro2a. Furthermore, there have been some positive reports such as PACAP38 improved the learning and memory processes in a passive avoidance paradigm through PACAP38 preferring receptor [33] and PAC1 was involved in the positive lateralized effect of VIP on learning and memory [17]. Our data combined with these related reports indicated that PAC1 in some degree attributed to the significant protective effect of VIP-TAT against Scop induced amnesia in this research.

When we looked into three PACAP/VIP receptors PAC1, VPAC1 and VPAC2, the expression patterns of these receptors are different [2,16]. The largest number of VPAC1 receptors can be found in the hippocampus and cerebral cortex, while the highest concentration of VPAC2 mRNA can be detected in the thalamus and suprachiasmatic nucleus and the lowest levels in the hippocampus, dorsal root ganglia, spinal cord and brainstem. Brain regions containing high amounts of PAC1 mRNA are the thalamus, hypothalamus, hippocampus (dentate gyrus), cerebellum and the olfactory bulb. Furthermore, in aged brain, VPAC1 expression reduced significantly [20], PAC1 expression increased significantly, while VPAC1 remain unchanged [23]. These different expression patterns indicate that the functions mediated by three receptors in learning and memory are absolutely different. The results VIP-TAT with higher affinity for PAC1 displayed more stable and more positive anti-amnesia effects than VIP, in our opinion, resulted from not only the enhanced brain uptake efficiency but also the changes of receptor activation profiles of VIP-TAT.

Furthermore, the finding that i.p. injection of VIP-TAT for one time inhibited the food intake more effectively than VIP but did not reduce the body weight more effectively than VIP after long-term treatment, in our opinion, also resulted from the difference of VIP-TAT and VIP in not only the traversing efficiency but also the receptors affinity and activation bias. It has been reported that VIP and its VPAC receptors in suprachiasmatic nucleus regulate the energy homeostasis by increasing energy expenditure and reducing food intake [40]. Meanwhile, activation of PAC1 in hypothalamic ventromedial nuclei has also been shown to have anorexigenic effect [28]. In other hand the activation of PAC1 in brain or in peripheral system also results into the other hormones release such as insulin [37] involved into the body composition control and energy homeostasis. These complex effects led to that VIP-TAT, which had bias for PAC1, displayed stronger anorexigenic effect than VIP in short-time treatment, but showed no stronger effect in reducing body weight than VIP after long-term administration.

In conclusion, in this research, a novel recombinant peptide VIP-TAT with a cell penetrating peptide TAT linked at the C-terminus of VIP was produced and was shown to have significantly stronger protective effects against Scop induced amnesia than VIP, which were considered owing to (1) the enhanced brain uptake efficiency of VIP-TAT and (2) the increased affinity and activation of VIP-TAT for PACAP preferring receptor PAC1.
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