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Research report

Neurotrophic effects of amyloid precursor protein peptide 165 *in vitro*

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ABSTRACT

Diabetic encephalopathy is one of the risk factors for Alzheimer's disease. Our previous findings indicated that animals with diabetic encephalopathy exhibit learning and memory impairment in addition to hippocampal neurodegeneration, both of which are ameliorated with amyloid precursor protein (APP) 17-mer (APP17) peptide treatment. Although APP17 is neuroprotective, it is susceptible to enzymatic degradation. Derived from the active sequence structure of APP17, we have previously structurally transformed and modified several APP5-mer peptides (APP328-332 [RERMS], APP 5). We have developed seven different derivatives of APP5, including several analogs. Results from the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on human neuroblastoma SH-SY5Y cells in the present study showed that P165 was the most neuroprotective APP5 derivative. Furthermore, we tested the effects of APP5 and P165 on the number of cells and the release of lactate dehydrogenase. Western immunoblot analyses were also performed. The digestion rates of P165 and APP5 were determined by the pepsin digestion test. P165 resisted pepsin digestion significantly more than APP5. Therefore, P165 may be optimal for oral administration. Overall, these findings suggest that P165 may be a potential drug for the treatment of diabetic encephalopathy.

1. Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases. AD causes dementia and affects middle- to old-aged individuals. Approximately one in four individuals over the age of 85 suffers from AD (Feng and Wang, 2012). Neurodegenerative diseases are a heterogeneous group of disorders characterized by impaired neuronal structure and function, and are generally accompanied by neuronal loss (Peruffo and Cozzi, 2014). Diabetic encephalopathy (DE) is one of the risk factors for AD. Our previous findings indicated that animals suffering from DE are learning and memory-impaired and exhibit hippocampal neurodegeneration, both of which improve with amyloid precursor protein 17-mer peptide (APP17) treatment. APP17 is neuroprotective (Yamamoto et al., 1994; Make et al., 1994; Sheng et al., 2001; Wang et al., 2004a) despite its susceptibility to enzymatic degradation (Feng et al., 2014). Our previous findings in streptozotocin-induced diabetes mellitus rats have shown that APP17 is neuroprotective by modulating insulin signaling, inhibiting hyperphosphorylation of Tau protein, and reducing apoptosis (Wang et al., 2003; Zhao et al., 2005). Because of its susceptibility to enzymatic degradation, APP17 was required to be administered via injection to optimize its effects. However, under clinical conditions, the development of orally administered APP17 may provide more patient compliance. The discovery of an active sequence within APP17 has led to another APP17-related peptide, APP5 (Wang et al., 2011). We have synthesized several analogs of APP 5, and using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to measure the metabolic rate, we have identified the most potent candidate.

The aim of the present study was to investigate the neuroprotective effects of APP analogs *in vitro* for novel anti-dementia drugs to identify those that exhibit the most potent neurotrophic effects that are also resistant to pepsin digestion.

2. Materials and methods

2.1. SH-SY5Y cell culture

SH-SY5Y cells were obtained from Professor Bengt Winblad of The Karolinska Institute, Sweden. Cells were grown in Dulbecco's Modified Eagle's medium: nutrient mixture F-12 media (Gibco

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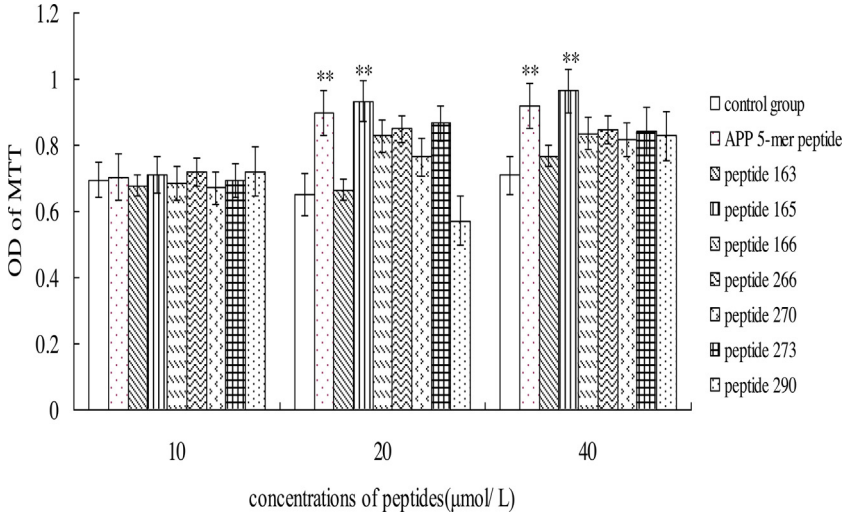

Fig. 1. Effect of amyloid precursor protein 5 (APP5) and its analogs on cell metabolic rate using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The optical density of MTT exposed to SH-SY5Y cells ($n = 8$) is shown. $^{**}P < 0.01$ *vs.* control group.

BRL, New York, USA) containing 10% heat-inactivated fetal calf serum (Hyclone, Los Angeles, CA, USA), penicillin (100 IU/ml), and streptomycin (100 μg/ml) in T_{75} tissue culture flasks under 95% air, 5% CO_2 at 37 °C.

2.2. Peptide synthesis

APP5 and its seven derivatives (P165, P163, P166, P266, P270, P273, and P290) were synthesized according to the solid-phase methodology using a peptide synthesizer machine (Takato and Eisuke, 1998; Wang et al., 2004b). Peptide purity was confirmed by analytical reversed-phase high-performance liquid chromatography (HPLC) and amino acid analysis.

2.3. MTT assay

SH-SY5Y cells were plated in 96-well plates (Costar) at a density of 2×10^3 cells/200 μl. After 72 h, the cells were exposed to the control or one of the peptides at 10, 20, or 40 μM for 72 h. The cells were then exposed to MTT (0.5 mg/ml) to measure the metabolic rate (Wang et al., 2004b). P165 was selected from this assay for further analysis.

2.4. Cell survival

SH-SY5Y cells were plated in 24-well plates at 1.0×10^7/l for 24 h. The cells were then exposed to Trypan blue followed by the control, APP, or 20 μM P165 for 24, 96, or 144 h. Cells were then quantified using a light microscope (Wang et al., 2004b).

2.5. Lactate dehydrogenase (LDH) release rate

SH-SY5Y cells were seeded into a 48-well plate at 5×10^3 cell/ml for 48 h. The cells were then exposed to the control, APP 5, or P165 ($n = 6$ wells per group) for 24 h. The supernatant from each well was collected and LDH activity was measured (at 450 nm) according to the manufacturer's instructions (Beijing Chemical Reagent Company, Beijing, China) (Wang et al., 2011).

2.6. Western immunoblot

Western immunoblotting was performed to determine if P165 affects the cell signal transduction pathway proteins, B-cell lymphoma 2 (Bcl-2) and phosphorylated-cAMP response element-binding protein (p-CREB), which are both involved in apoptosis. SH-SY5Y cells were seeded in 75-ml cell culture bottles. When the cells were 80% confluent, they were exposed to the control, APP-5, or P165 for 24 h. The cells were then collected, and cellular proteins were extracted and protein concentrations measured using the Lowry method. Beta (β)-actin served as the internal control. The bands were quantified and analyzed using NIH Image J software (Wang et al., 2011).

2.7. Pepsin digestion test

P165 and APP 5 were treated with pepsin (2 mg or 5 mg) and HCl (0.01 mol/l, 50 μl) at 25 °C for 4.5 h to give a final concentration of 40 g/l or 100 g/l. Controls consisted of P165 or APP 5 with only HCl (0.01 mol/l, 100 μl) at 25 °C for 4.5 h. The decomposition ratios of the two peptides were then collected and the peptides analyzed by HPLC. These data were used for the statistical analysis of the sample rate (Ma, 2001).

2.8. Statistical analysis

All data are expressed as the mean ± SD and were analyzed by one-way analysis of variance. The *F*-test value for equal variance was used for statistical analysis. $P < 0.05$ or $P < 0.01$ was considered statistically significant. Statistical analysis was performed using SPSS 11.0 software.

3. Results

3.1. MTT metabolic rate

Results from the MTT analysis showed that all eight peptides significantly enhanced the metabolic rate when compared with the control group (Fig. 1). Furthermore, compared with the control group, SH-SY5Y cells grew significantly ($P < 0.01$) faster when treated with 20 μM or 40 μM P165 (Fig. 1). These results therefore confirmed that 20 μM P165 was optimal for further analysis. The OD of MTT in 10 μM dose of control group, APP 5-mer peptide, P163, 165, 166, 266, 270, 273, 290 were 0.697 ± 0.014, 0.703 ± 0.007, 0.676 ± 0.009, 0.711 ± 0.010, 0.685 ± 0.012, 0.721 ± 0.006, 0.673 ± 0.010, 0.697 ± 0.008, 0.722 ± 0.013. The OD of MTT in 20 μM dose of control group and eight differ-