Ameliorative effects of angiotensin-IV on the expression of hippocampus synaptophysin and the cognitive function of diabetic rats

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Cognitive impairment has been a common complication of diabetic. Recent evidences have pointed out angiotensin-IV (Ang-IV) can facilitate memory acquisition and recovery, whether it also can improve cognitive functions of diabetic rats with cognition disorder and the possible mechanisms if it really can are yet unknown. In this study, 45 Sprague Dawley (SD) male rats were divided into three groups randomly: control group, diabetic model group and diabetic model with Ang-IV treatment group. Their cognitive functions were evaluated through Morris water maze task; the synapses ultrastructure and relative mRNA concentrations and protein expression levels of synaptophysin in hippocampus were analysed and detected by transmission electron microscope, reverse transcription polymerase chain reaction (RT-PCR), immunohistochemistry and western blotting, respectively. Results show that, for Ang-IV treatment group, the cognitive functions were obviously improved, and the ultrastructure of synapses in hippocampus became more normalized, and the relative mRNA concentrations and protein levels of synaptophysin in hippocampus were also significantly increased, compared with DM. It is concluded that Ang-IV plays an important role in improving cognitive function of diabetic rats with cognition disorder, and the possible mechanisms are up-regulating the expression of synaptophysin and normalizing the ultrastructure of synapses in hippocampus.

Key words: Angiotensin-IV, synaptophysin, diabetes, cognition, hippocampus, rats.

INTRODUCTION

Diabetes mellitus is a common metabolic disorder characterized by long-term hyperglycemia, and there are a number of studies suggesting a connection between cognitive function disorder and diabetes mellitus. Its morbidity is rising year by year, and has become a significant public health problem (Nita and Nicholas, 2010). A growing body of evidence suggests that hyperglycemia can damage the central nervous system (CNS), and these diabetes-induced CNS complications were known as diabetic encephalopathy (DE) (Pasquiera et al., 2006; Biessels et al., 2008). The predominant manifestations of diabetic encephalopathy, which was closely associated with brain aging process and Alzheimer disease (AD), involve deficits in cognitive function and failure of learning and memory (Arvanitakis et al., 2004; Profenno et al., 2010; Jolivalt et al., 2010).

Renin-angiotensin system (RAS) is systemic distribution. The brain RAS, besides being known for regulating blood pressure, sodium and water balance, also plays an...
important role in sensory information, learning, memory and emotional activities. Recently, many researches have pointed out angiotensin-IV (Ang-IV) can facilitate memory acquisition and recovery. It is a six peptide fragment of Ang-α, whose binding site localized in structures associated with memory function, such as hippocampus, neocortex and cerebellum and so on. As its corresponding ligands, AT4 receptor system, an insulin-regulated membrane aminopeptidase (IRAP), is acknowledged recently with the effect of memory enhancement (Wright et al., 1993; Wright and Harding, 2004, 2008, Wright and Harding, 2011; Albiston et al., 2003). Synaptophysin is one of the most abundant polypeptide components of synaptic vesicles. It is a part of neuronal cytoskeleton proteins, and the expression of which is decreased in hyperglycemia. It is believed that synaptophysin can modulate the synaptic vesicle cycle, and have a close relationship with synaptic plasticity (Janz et al., 1999; Schmitt et al., 2009). The mechanism of cognitive dysfunction induced by diabetes mellitus is not clear so far. RAS plays an important role in normal cognitive processing, and perhaps it has a hand in dysfunctional cognition related to diabetes mellitus (DM). Accordingly, the aim of this study was to investigate the effect of Ang-IV on the expression of hippocampus synaptophysin and the behavior transformation of diabetic rats, and explore the probable mechanism of Ang-IV impacting on cognitive function.

MATERIALS AND METHODS

Animals

60 adult male normal Sprague dawley (SD) rats (300 to 320 g, approximately 10 weeks old) offered by the Experimental Animal Center of Chongqing Medical University, were used for this study. They were group-housed with regular feeding of food and water, with a regular 12 h light-dark cycle, and living under standard room condition (temperature: 25 to 28°C, humidity: 60 to 65%), with a diurnal cycle. Experiments were performed according to internationally followed ethical standards, and approved by the Ethics Committee of Chongqing Medical University.

Experimental induction of diabetes

60 rats were divided into three groups randomly: normal rats group (control group), diabetic rats with no medicine treatment group (DM group); diabetic rats with Ang-IV treatment group (DM group + Ang-IV); and each group contained 20 rats. As described by Bhutada et al. (2010), the diabetic rat was successfully induced. Briefly, for DM group and DM group + Ang-IV, after abrosia for 12 h, streptozotocin (STZ, Alexis Company, USA) were given, which were dissolved in 0.1 mmol/L citric acid-sodium citrate buffer, pH 4.2, at the dose of 60 mg/kg through routine intraperitoneal injection (i.p.), while ‘control group’ were injected the same dose buffer solution also by i.p. at the same time. 72 h later after that, only those animals with fasting blood glucose levels over 16.67 mmol/L (LifeScan glucometer: B7MD-BY, Johnson and Johnson Compant, USA) were considered as diabetic and will be used for the further study.

At about 12 weeks, the diabetic rat models were induced successfully. Finally, 15 rats in each group were brought into this study. After that, the Morris water maze test followed, and then every rat in DM group + Ang-IV were injected with Ang-IV (Phoenix pharmaceuti
cal Company, LTD, USA), with 5 uL (1 nmol/uL), while each rat in DM group and control group was injected the same volume of physiological saline solution (PSS) instead. The whole periods lasted for one week (Park et al., 2010). At last, one rat in DM group died due to the poor tolerance ability, and was removed from the study.

Morris water maze task

Morris water maze test was used to assess cognitive function. The circular pool (diameter: 150 cm; height: 50 cm; depth of water: 22 cm) was made of gray plastic and divided equally into four quadrants. The platform (10 cm in diameter and 20 cm high) was randomly placed in one of the four maze quadrants labeled with A–B–C–D, and was submerged 1.0 cm below the water surface. The platform remained in the same quadrant during the whole experiment (we chose quadrant A as the place where the platform localized in present study). The rats were required to find the platform using only distal spatial extra-maze cues that was available in the testing room. Theses cues should remain the same throughout the test. All the rats were trained continuously for four days, with each trial having a top limit time of 90 s and a trial interval of approximately 30 s. After climbing onto the platform, the rats were made to stay there for 30 s ahead of the next trial.

On condition that the rat failed to reach the escape platform within the allotted time of 90 s, it was softly placed on the platform and was made to stay there for the same amount of time and the time of reaching the platform (escaping latency in time) was measured. On day five, we made the rat swim until it climbed onto the platform submerged underneath the water, and then analyzed the incubation period, and investigated its mentality. The next day, we removed the platform, observed the rats’ swimming trajectory, counted the times of crossing the primary platform, and tested memory abilities. After corresponding intervention for a week in different groups, the Morris water maze test was performed again. After completion of the last trial, rats were gently dried with a towel, kept warm for an hour and returned to their home cages.

Surgery

Anesthetized with chloralhydrate (10 mLkg), the rat’s head was fixed on the brain stereotaxic device (Stoelting, USA) for the lateral ventricle positioning. Setting the anterior fontanellae as the reference, lateral ventricle was located with 1.0 mm back, 1.5 mm right, and 3.5 mm depth of the anterior fontanellae (Park et al., 2008), and a hole was drilled on the skull by miniature electric drill. Then, a stainless steel cannula with 0.9 mm external diameter and 0.5 mm inner diameter was inserted into the lateral ventricle, vertically following the hole. When it is inserted with approximately 3.5 mm depth, cerebrospinal fluid would flow out if the inner tube was pulled out slowly, which could be considered as success of the surgery. Then, the cannula was fixed with dental cement, and when the dental cement cooled, the wound was stitched up. Next, penicillin was used to prevent infection, and then rats were group fed uncea-singly. Four days after the surgery, the lateral ventricle micro-injection was carried out. Before the injection, the surface of the cannula need be sterilized; then the core tube was pulled out, and 5 uL Ang-IV mixed with physiological saline into 1 nmol/uL was immitted into the lateral ventricle by micro syringe in 3 min. The whole course was required in a constant speed, one minute later, the syringe was extracted. At last, the core tube was inserted back.
Ang-IV solution was injected one time a day per rat in DM group + Ang-IV, while the rats in ‘control group’ and ‘DM group’ were given isopyknic physiological saline solution instead for a week.

**Ultramicrostructure observation**

After the ethology tests were finished, the rats were anesthetized with 10% chloral hydrate (350 mg/kg, i.p.), the brain was quickly removed, and the whole hippocampus was carefully dissected. Tissues were snap-frozen in liquid nitrogen and stored at -80°C. The left hippocampus was used for reverse transcription PCR (RT-PCR) and ultramicrostructure observation; the right hippocampus was used for western blotting and immunohistochemistry. According to the material and methods requirements of transmission electron microscope (TEM), briefly, three pieces of hippocampus tissues with 1 mm diameter of each tissue were obtained; then, 4% paraformaldehyde fixation for 2 h and 1% osmium tetroxide fixation for 1 h; and then gradient dehydration with ethyl alcohol and acetone were carried out. Then, they were saturated, embedded, aggregated with epoxy resin 618°. Next, the 70 nm ultrathin sections of them were made by microtome. At last, electron stains of hippocampus tissues with uranyl acetate and folic acid were carried out, and the synapse ultrastructure were observed by TEM (Hitachi-7500, Japan).

**Immunohistochemistry**

Hippocampus tissues were embedded in paraffin, and were cut serially into coronary slice, and then the expressions of synaptophysin were detected according to the kit’s instructions (Wuhan Boster Company Limited, China). The specific processes were as follows: the slices were dehydrated by conventional paraffin, hydrated by alcohol and endogenous enzymes were inactivated with 3% hydrogen peroxide; and then normal goat serum was used to block the non-specific antibodies after the microwave hot fix. Primary antibody (mice anti-rats synaptophysin) was used with dilution rate 1:100 4°C overnight. Then, incubated with second antibody: biotinylated goat anti-mice IgG with 37°C, 30 min, afterwards incubated with Strept Avidin-Biotin Complex (SABC) at 37°C for 30 min. Next step was colour development with benzidine tetrahydrochloride (DAB) at room temperature. The specific bands were scanned and measured by image analysis software (HPIAS 2000, Tongji Qianping Company, China).

**Results were expressed as mean ± standard deviation (Mean ± SD) and were analysed by SPSS17.0, and homogeneity of variance among groups were tested. Then the data obtained were analyzed by one-way analysis of variance (AVOVA), and followed by student’s t test. Statistical significance was considered at P < 0.05.**

**RESULTS**

**Test result of Morris water maze**

This is represented in Table 1.

**Unvisible platform trials**

Compared with control group, the DM group showed escaping latency prolonged, and the difference was significant (p < 0.01). The latency of DM group + Ang-IV was shortened, obviously compared with DM group (p < 0.05), while prolonged slightly compared with control group, and it was not statistically significant (p > 0.05) (Table 1).
Table 1. The result of spatial exploring trials and unvisible platform trials of Morris water maze in each group (Mean ± s).

<table>
<thead>
<tr>
<th>Group</th>
<th>Rats number</th>
<th>Times of GTP@</th>
<th>Escaping latency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>15</td>
<td>2.62±0.22</td>
<td>42.7±5.93</td>
</tr>
<tr>
<td>DM group</td>
<td>14</td>
<td>1.48±0.27*#</td>
<td>74.98±6.03*#</td>
</tr>
<tr>
<td>DM group+Ang IV</td>
<td>15</td>
<td>2.31±0.28</td>
<td>52.44±7.54</td>
</tr>
</tbody>
</table>

*Compared with control group: *P < 0.01; *comparing with DM group+ Ang-IV: #P < 0.05; @GTP: getting through the platform.

Spatial exploring trials

Compared with control group, the times of DM group getting through the original platform were declining visibly (P < 0.01). These parameters were increasing in DM group+ Ang-IV comparing with DM group (P < 0.05), while there was no marked statistical difference between DM group + Ang-IV and control group (p > 0.05) (Table 1).

Results of transmission electron microscope

In control group the structures of presynaptic membrane, postsynaptic membrane, and synaptic cleft in the hippocampus were easy to distinguish through electronic microscope; the postsynaptic membrane was a tad thicker than the presynaptic membrane; synaptic vesicles and cell organelles were copious in the neurons, while in DM group, the synapses showed pervasive changes compared with control group: the axoplasm of presynaptic membrane and the axoplasm of postsynaptic membrane were blending together; the structures were ambiguous, and part of synapses could not tell the differences between presynaptic membrane and postsynaptic membrane; the synaptic cleft became faint or even disappeared; the number of synaptic esicles were significantly reduced, furthermore the mitochondrial cristae became short and the mitochondria swelled in the neurons. However, in DM group + Ang-IV, the ultra structures were gradually normalized, and there were un conspicuous differentiations compared with the control group (Figure 1).

Immunohistochemical assay

Compared with control group, the mean optical density of positive synaptophysin neurons in hippocampus were dropped dramatically in DM group (P < 0.01). After intervention, the expression of synaptophysin increased obviously in DM group + Ang-IV compared with DM group (P < 0.05), and there was no evident difference comparing with control group (p > 0.05) (Table 2, Figure 2).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

The water maze test indicated that cognitive impairment occurred with diabetes mellitus and the immune histochemical assay had demonstrated the synaptophysin expression were different among the three groups. Thus the RT-PCR we used to estimate whether altered expression of synaptophysin mRNA was involved. Moreover, relative concentrations (RC) of them were calculated.
Table 2. The protein express of synaptophysin in rat hippocampus of each group (Mean ± s).

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat number</th>
<th>The mean optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>15</td>
<td>0.223±0.016</td>
</tr>
<tr>
<td>DM group</td>
<td>14*#</td>
<td>0.151±0.021*</td>
</tr>
<tr>
<td>DM group+Ang-IV</td>
<td>15</td>
<td>0.206±0.019</td>
</tr>
</tbody>
</table>

*Compared with control group: *P < 0.01; # compared with DM group+Ang-IV: #P < 0.05.

Figure 2. Expression of synaptophysin in hippocampus of each group (staining methods of SABC in immunohistochemical method, ×400). A. control group: the sites where the arrow point can reflect the mean optical density of positive synaptophysin neurons in hippocampus. B. DM group. C. DM group + Ang-IV.

DISCUSSION

Large cohort studies have clearly demonstrated that the hippocampus plays an important part in processing of stored information (Ingelsson et al., 2004). In the four subregions of the hippocampus, CA1 subregion has a close association with learning and memory functions (Hoge and Kesner, 2007). Synapsis is a specialized structure which plays an important role in the information transmission between neurons; synaptophysin is a membrane protein closely related to the structure and function of the synapse. Therefore synaptophysin is a specific marker in the aspect of detecting the density and distribution of synapses. In addition, synaptophysin, as a functional presynaptic membrane protein in the hippocampus neurons, participates in multiple physiological activities associated with the learning and memory (Boncristiano et al., 2005). In this study, the synapse ultrastructure in CA1 region of diabetic rats showed pervasive changes: the axoplasm of presynaptic and postsynaptic membranes were blending together, thus the whole structures became indistinct; the synaptic cleft became faint or even disappeared; and the number of synaptic versicle was significantly reduced. These changes of structures caused the impairment of the synapses physiological function.

Morris water maze is one of the most frequently-used water mazes for forcing experimental animals (rats, mice) swim and by making the animals learn to find the unvisible platform in the water. It further estimated their learning and memory ability in spatial topesthesia and sense of direction (space orientation) by analyzing the time it took them finding the original platform and the trajectory they chose. In this experiment, the result of Morris water maze suggested that: diabetic rats had obvious cognitive dysfunction. Compared with control group, the escaping latency of DM group was prolonged distinctly (p < 0.05), which showed the learning ability of diabetic rats had

were performed by statistical analysis (Figure 3).

Western blot assay

Western blot was carried out for the accurate quantitative comparison of the synaptophysin. The relative optical density values were calculated as synaptophysin/β-actin and the corresponding comparison of statistics among them were also implemented (Figure 4).

through synaptophysin/β-actin. The RC values of three groups were showed as mean ± standard deviation, and
already decreased; and the times of crossing the target area reduced, which also proved that the memory ability of diabetic rats were impaired to a certain degree. Combining with the results of the TEM, it was inferred that destruction of synaptic structure could lead to cognitive function impairment of diabetic rats.

Ang-IV, as a recently discovered metabolin of ANG-α, has an impact on learning and memory ability. Some studies indicated that the lateral ventricle injection of Ang-IV could promote memory retention and reminiscence. In addition, the lateral ventricle injection of Ang-IV with pharmacological doses could ameliorate the damaged spatial learning ability in the aged rat dementia model (Olson et al., 2004). In our study, Morris water maze test displayed that the performance abilities of DM group + Ang-IV were observably improved compared with DM group; the escaping latency of DM group + Ang-IV were significantly shortened; and times of crossing the target area by them were also increased. These results aforementioned confirm that Ang-IV can obviously improve the cognitive functions of diabetic rats.

Synaptophysin is a kind of glycoprotein located in presynaptic vesicles, and it belongs to distinctive markers of synaptic terminal, participating in the membrane fusion between synapse vesicles and presynaptic membrane, as well as in the neurotransmitters releasing (Sze et al., 2000). The quantity and the density of synaptophysin distribution can reflect the quantity and the density of synaptic structure indirectly (Calhoun et al., 1996). The synaptophysin combining with Ca^{2+} causes neurotransmitter releasing, information transferring and processing. The changes of synaptophysin level indirectly reflect the changes of rats' learning and memory ability.

In present study, whether the immunohistochemistry detection or the RT-PCR of mRNA or the western bolt assay, they all showed that the synaptophysin expression levels were significantly reduced in DM group, compared with control group ($p < 0.01$); while the expression levels of synaptophysin were up-regulated significantly after the Ang-IV treatment ($p < 0.05$), and had no observable difference with that of control group ($p > 0.05$). Therefore, it could be inferred that the effect of Ang-IV on improving the learning and memory ability was associated with the up-regulation of synaptophysin expression. Whether angiotensin-IV stimulates synaptophysin expression improves cognitive function of diabetic rats through AT4 receptor (AT4R), we will do the further contrast study with angiotensin receptor blocker to testify.

**Conclusion**

Ang-IV treatment can ameliorate cognitive dysfunction, and the possible mechanisms are up-regulating the expression of synaptophysin and normalizing the ultra structure of synapses in hippocampus.

**ACKNOWLEDGEMENTS**

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**Figure 3.** RT-PCR analysis of the mRNA levels of synaptophysin. Left picture was the RT-PCR image (A). Lane M, 2 kb plus DNA ladder; lane 1, control group; lane 2, DM group+Ang-IV; lane 3, DM group. Upper ribbons were the synaptophysin segments; while nether bars were PCR products from the cDNA of β-actin. Right histogram showed the relative concentrations of three groups (B). The mRNA levels of DM group were observably reduced compared with control group ($#P < 0.01$) and DM group + Ang-IV ($&P < 0.05$); while the difference between control group and DM group + Ang-IV was not significant ($^*P > 0.05$).


Abbreviations

Ang-IV, Angiotensin-IV; CNS, central nervous system; DE, diabetic encephalopathy; AD, alzheimer disease; RAS, renin–angiotensin system; IRAp, insulin-regulated aminopeptidase; DM, diabetes mellitus; STZ, streptozotocin-induced diabetic rats; Co., company; SD, sprague dawley; i.p., intraperitoneal injection; PSS, physiological saline solution; TEM, transmission electron microscope; Icv, Intracerebroventricularly; SABC, strept avidin-biotin complex; BUAA, Beijing university of aeronautics and astronautics; AOD, the average optical density; GABA, acide gamma-aminobutyrique; BDZ, benzodiazepines; TGM, transgenic mouse; RT-PCR, reverse transcription-polymerase chain reaction; SYP, synaptophysin.

REFERENCES