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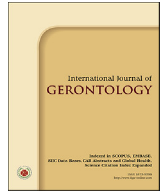

Original Article

Aging effects on circulating adiponectin levels and the expressions of adiponectin and adiponectin receptor 1 in the brains of male rats

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S U M M A R Y

Background: At present, many studies address circulating adiponectin (APN) levels in patients with Alzheimer's disease (AD) and moderate cognitive impairment, but the results of these studies are controversial. We assumed that Alzheimer-like changes in the brain may be accompanied by the changes in APN and its receptor expression in aging process. This study sought to investigate the effects of aging on serum APN levels, APN and AdipoR1 expression in the rat brain.
Methods: Serum APN levels, Tau protein phosphorylation, APN and its receptor expressions in brain tissue were observed in male Sprague-Dawley rats at the age of 12 weeks (young group), 36 weeks (adult group) and 96 weeks (elder group) ($n = 12$).
Results: No significant difference in Tau5 protein expression was detected between those grops ($P > 0.05$). Phosphorylation of Tau at Ser262 and Ser396 was gradually increased with age in the hippocampus ($P < 0.05$), but no significant difference in the cerebral cortex ($P > 0.05$). Serum APN levels gradually reduced with age in rats ($P < 0.05$). Moreover, APN expression gradually diminished in the hippocampus with age ($P < 0.05$). However, no significant difference in APN expression was observed in the cerebral cortex with age ($P > 0.05$). With age, AdipoR1 expression graduallyincreased both in the hippocampus and cerebral cortex ($P < 0.05$).
Conclusion: Aging can simulate Alzheimer's disease-like degeneration of the brain, with reduced circulating APN levels, decreased expression of APN and increased expression of AdipoR1 in the brains of male rats.
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1. Introduction

Clinical manifestations of Alzheimer's disease (AD) are mainly characterized by progressive intellectual deterioration, memory impairment, cognitive impairment, and psychiatric symptoms. The pathogenesis of AD remains unclear, mainly containing genetic factors, neurotransmitter disorders, cytoskeletal changes, hypertension, diabetes, hyperlipemia, high serum homocysteine, central obesity, atrial fibrillation, traumatic brain injury, chronic viral infection, low educational level, smoking, and history of exposure to heavy metals.[1,2] Tau protein phosphorylation can reduce the ability of microtubule assembly and damage nerve cells. Aging can cause the phosphorylation of Tau protein at multiple sites.

Recent studies suggested that the disorders of energy metabolism may be one of pathogenesis of AD, mainly because AD patients often suffer from visceral fat accumulation, insulin resistance and abnormal secretion of leptin and APN. APN, an adipokine secreted by adipose tissue, is involved in the regulation of energy metabolism, enhances insulin sensitivity, glucose uptake and fatty acid oxidation, effectively resists inflammation and atherosclerosis, and protects vascular endothelial cells.[3] The decrease in circulating APN levels is correlated with insulin resistance syndrome and visceral fat deposition, and can be found in some clinical diseases, such as obesity, dyslipidemia, diabetes and depression.[4] APN and its receptors are widely expressed in the brain. AdipoR1 is mainly expressed in the hippocampus. Combining with diverse physiological functions of APN, it is indicated that APN signaling system may exert a crucial effect on cognitive function.

Teixeira et al[3] confirmed that low circulating APN levels were associated with cognitive dysfunction, and the decrease in APN levels could reflect the pathological process of AD. On the

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contrary, Kamogawa et al[5] verified that the increase in plasma APN levels was a protective factor for dementia in males, but did not impact cognitive function in females after the investigation in 517 community members, which was consistent with Une et al's study.[6] Roberts et al[7] suggested that no significant difference in circulating APN levels was visible between mild cognitive impairment (MCI) patients and controls. Interestingly, the results of van Himbergen et al's study[8] are contrary to above results. They believed that the increase in circulating APN levels was an independent risk factor for dementia or AD in females, which was contrary to multiple physiological functions of APN. Another previous study showed that circulating APN levels had obvious sex difference, and the levels were higher in females than in males.[5] Thus, APN levels may be affected by estrogen levels.[9] Therefore, it is presumed that results of van Himbergen et al's study were probably associated with estrogen levels in females. Pakaski et al[10] demonstrated that serum APN levels increased progressively with the time of taking donepezil in AD patients, and also indirectly verified that APN had protective effects on AD patients. Accordingly, current results support the protective effect of on cognitive function.

There is extensive cross-linking on the pathogenesis of AD and type II diabetes, which associates with aging.[11] More and more studies have shown that APN may be the key point of their cross-linking. Age-associated degenerative lesions have been shown to accelerate the pathological process of AD and other neurodegenerative diseases.[12] Taken together, we assumed that Alzheimer-like changes in the brain may be accompanied by the changes in APN and its receptor expression in aging process. This study sought to investigate the effects of aging on serum APN levels, APN and AdipoR1 expression in the rat brain.

2. Materials and methods

2.1. Experimental animals

Unmated male Sprague-Dawley rats at the age of 12 weeks (young group), 36 weeks (adult group) and 96 weeks (elder group) ($n = 12$) were purchased from Dashuo Biotechnology Co., Ltd. (*Chengdu, China; license No. SCXK2008-24*). All rats were housed in the Clean Animal Room, and allowed free access to food and water, housed at 24–26 °C and relative humidity of 70% in 12-h light/dark cycles, with regular ultraviolet disinfection and ventilation. After 3 days of acclimation, rats were sacrificed by excessive intraperitoneal injection of phenobarbital 40 mg/kg body weight. Blood from inferior vena cava was obtained from all rats for detection of serum APN concentration. Brains of six rats from each group were collected for western blot assay. Brains of remaining rats were gained for immunohistochemistry. The protocols were conducted in accordance with the Animal Study Outline of Shanghai Jiao Tong University in China, and approved by the Animal Research Committee of Shanghai Jiao Tong University in China.

2.2. Enzyme linked immunosorbent assay (ELISA) for serum APN concentration

Mice were fasted overnight before sacrifice. Blood was immediately collected from the inferior vena cava after sacrifice. Blood was placed in a pre-cooling tube for 1 h, centrifuged at 1600 *g* for 15 min and 4 °C. Serum was obtained and stored at −80 °C for further use (no more than 2 months). Serum APN concentration was detected with a commercial ELISA kit (*USCN Life Science Inc., Wuhan, Hubei Province, China*). Experiments were performed in triplicate.

2.3. Immunohistochemical staining

After paraformaldehyde perfusion in the heart, brain tissue was obtained and fixed in 4% paraformaldehyde for 24 h, dehydrated, permeabilize, and embedded in paraffin. Above sample was then sliced into 4 μm-thick sections with a RM 2255 hand slicer (*Leica Microsystems, Wetzlar, Germany*). Before staining, sections were dried, dewaxed, dehydrated, rehydrated, and treated with 10 mM citrate buffer (pH 6), boiled in a microwave oven for 3 min, cooled at room temperature for 30 min, blocked with 0.3% H2O2 to inactivate endogenous peroxidase for 10 min, lysed with 0.2% Triton X-100 for 30 min, and blocked with normal goat serum (1:10) for 20 min. Above sections were incubated with mouse monoclonal anti-adiponectin antibody (1:200), mouse polyclonal anti-AdipoR1 antibody (1:200) or rabbit polyclonal anti-GAPDH antibody (1:1000) (diluted in 0.1 M PBS-dissolved 1% bovine serum albumin; *Abcam, Cambridge, USA*) at 4 °C overnight. After three washings in PBS, the specimens were exposed to a PowerVision™ goat anti-mouse IgG antibody-horseradish peroxidase polymer (*Dako, Carpinteria, USA*) for 30 min to detect immunoactivity. Antibody binding was visualized by incubation with DAB (*Boster, Wuhan, China*) for 6 min at room temperature followed by counterstaining with hematoxylin (*Baso, Zhuhai, China*). Subsequently, sections were dehydrated in a graded series of ethanol, cleared in xylene, and coverslipped. Sections that did not incubated with primary antibody served as negative controls. All sections were observed and photographed under the light microscope (*Olympus, Tokyo, Japan*).

2.4. Western blot assay

After sacrifice, the brain was obtained immediately. Cerebral cortex and hippocampus were isolated and stored in liquid nitrogen separately. When proteins were extracted, radioimmune precipitation assay buffer lysate (1:10) and 0.1 mM phenylmethyl sulfonylfluoride (*Beyotime, Nantong, Jiangsu Province, China*) were added, followed by sonication. Samples were lysed on ice for 15 min, and centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant was obtained. Protein concentration was determined with a Bicinchoninic Acid Protein Assay Kit. Total protein was extracted and 5 × loading buffer was added at 4:1. After boiling for 5 min, samples were stored at −80 °C for western blot assay. In strict accordance with the instruction of reagent, western blot assay was conducted. Briefly, 8% sodium dodecylsulfate-polyacrylamide gel was prepared. 40 μg protein of each sample was electrophoresed on above gel, and the separated proteins were electrically transferred to polyethylene membrane (*Beyotime, Nantong, Jiangsu Province, China*). Following three washes with 10 mM Tris Buffered Saline with 1.0% Tween-20 (TBST) for 10 min each, the membrane was blocked with 5% skimmed milk powder (dissolved in 10 mM TBST) for non-specific protein binding. Above membrane was incubated in rabbit monoclonal anti-Tau (phospho S396) antibody dissolved in 1% bovine serum albumin, rabbit polyclonal anti-Tau (phospho S262) antibody, mouse monoclonal anti-Tau 5 antibody, mouse monoclonal anti-adiponectin antibody, mouse polyclonal anti-AdipoR1 antibody (1:1000; *Abcam, Cambridge, USA*), or rabbit polyclonal anti-GAPDH antibody (*1:1000; Bioworld, St. Paul, USA*) at 4 °C overnight. On the next day, the membrane was washed three times with TBST for 10 min each. The polyethylene membrane was subsequently incubated with horseradish peroxidase goat anti-rabbit IgG secondary antibody (*1:1000; Dako, Carpinteria, USA*) at room temperature for 60 min, washed three times with TBST for 10 min each, and visualized with the Super Signal Chemilumnescent Substrate System (*Pierce, Rockford, USA*). Quantitative analysis was performed with Bio-Rad image lab 2.0 software (*Bio-Rad Laboratory, Hercules, USA*).

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