Effect of Folate and Vitamin B12 on Tau Phosphorylation in Aged Rat Brain

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Abstract: Alzheimer's disease (AD) is the cause of one of the most common types of dementia. In AD brain, abnormal hyperphosphorylated tau composed the major protein of neurofibrillary tangles (NFTs), one of the two neuropathological hallmarks of AD. To prevent and relieve the tau protein abnormal hyperphosphorylation in AD patients' brain is thought to be the key point of therapy. Recent study suggested that there is some relationship between folates, vitamin B12 and AD. In our study we aim to investigate the possible mechanism of AD especially the correlation between folates, vitamin B12 and tau phosphorylation. We examined tau protein phosphorylation state in rats' hippocampus of different age stages: two and forty months old by phosphorylation dependent and independent tau antibodies. We found that tau phosphorylation in aged rats' brain showed significant high level than these two months old. And we also found that folates plus vitamin B12 can decrease the level of tau phosphorylation in aged rats' brain. It suggests folates and vitamin B12 may play an important role in preventing the neurodegenerative change by influencing tau phosphorylation in brain. [Life Science Journal. 2006;3(3):35 - 40] (ISSN: 1097 - 8135).

Keywords: Alzheimer's disease; aged; folate; vitamin B12

Abbreviations: AD: Alzheimer's disease; BCA: bicinchoninic acid; CSF: cerebrospinal fluid; DAB: diaminobenizidine; NFTs: neurofibrillary tangles; PHF: paired helical filaments; SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

1 Introduction

Dementia is a syndrome characterized by an acquired global impairment of memory and other cognitive functions sufficient to interfere with normal life[1]. Alzheimer's disease (AD) is the cause of one of the most common types of dementia. The World Health Organization has estimated that 25 - 29 million people in the world suffer from dementia. Approximately 6% - 8% of all older people over the age of 65 years have AD[2], and the prevalence increases steeply with age[3]. AD is characterized by the presence of two histopathological hallmarks called senile plaques and neurofibrillary tangles, which are involved in the process leading to progressive neuronal degeneration and death. It is reported that neurofibrillary tangles (NFTs) are structures present in the neuronal body and consist of paired helical filaments (PHF), mainly composed of highly phosphorylated tau protein[4,5]. Tau is a microtubule-associated protein expressed mostly, but not exclusively, in the nervous system, and its normal physiological function is to bind and stabilize microtubules[6]. In AD brain, tau is found aberrantly hyperphosphorylated[7]. Abnormal phosphorylation of tau in brain seemed to be an important pathogenesis of AD.

The possible involvement of nutritional factors in the aetiology (causes) or pathogenesis (mechanisms of brain damage) of dementia has been widely considered. In particular, dietary deficiency of folates has been postulated as contributing to the aetiology AD. Folates are vitamins essential to the development of the central nervous system. And researcher also found that vitamin B12 deficiency not only produces anaemia but also causes irreversible damage to the central and peripheral nervous systems. In this study we aim to investigate the mechanism of the relationship between AD and folates and vitamin B12. We focus on the tau protein and to study whether folates plus vitamin B12 have effect on its phosphorylation status.

2 Materials and Methods

2.1 Animal

Male Wistar rats were from Experimental Animal Central of Henan Medical College. All animals were observed daily for clinical signs of disease. All

animal experiments were performed according to *Policies on the Use of Animals and Humans in Neuroscience Research*, revised and approved by the Society for Neuroscience in 1995. The subjects were allocated into two groups: two months old rats and forty months old rats. Group of forty months old rats were treated with folates (40 mg/kg diet) by the gut and vitamin B12 (20 μg/kg) by intraperitoneal injection every day for one month.

2.2 Antibodies and reagents

Antibodies to tau are listed in Table 1. Rabbit polyclonal antibody R134d against total tau, monoclonal antibodies PHF-1 against PHF-tau phosphorylated at Ser396/404, and Tau-1 against PHF-tau unphosphorylated at Ser199/202 were gifts from Dr. Chengxin Gong (New York State Institute for Basic Research, Staten Island, NY, USA). Bicinchoninic acid (BCA) protein detection kit, goat anti-rabbit peroxidase-conjugated secondary antibody, chemiluminescent substrate kit and phosphocellulose units were obtained from Pierce Chemical Company (Rockford, IL, USA). Detection kit (Histostain-SP) for immunohistochemistry. Goat anti-mouse and goat anti-rabbit alkaline phosphatase-conjugated secondary antibodies, diaminobenizidine (DAB) and other chemicals were purchased from Maixin Biotechnology Company (Fu Zhou, China). Folates were from Peking University Pharmaceutical Co. Ltd. and vitamin B12 was from Yangzhou Zhong Bao Pharmaceutical Co. Ltd.

Table 1. Tau antibodies employed and their properties

Antibody	Dilution	Type[a]	Specificity	Phosphorylation sites[b]
Tau-1	1:30000	Mono-	unP	Ser-198/Ser-199/Ser-202
PHF-1	1:500	Mono-	P	Ser-396/Ser-404
R134d	1:2000	Poly-	P + unP	

[a] Poly-, polyclonal; mono-, monoclonal; unP, unphosphorylated epitope; P, phosphorylated epitope

[b] Numbered according to the largest isoform of human brain tau

2.3 Preparation of rat brain extracts

The rat is deeply anesthetized with sodium pentobarbital (75 mg/kg, *i. p.*) and then decapitated. Immediately remove the brain and separate the brain sagittally into hemisphere and put into ice-chilled PBS. The left hemisphere was fixed for immunohistochemistry. And the right hippocampus was homogenated and supernatant was for Western blot.

2.4 Western blot

Western blots of hippocampus homogenate were to determine the phosphorylation state of tau of different aged rats. The homogenizer contained cold homogenizing buffer containing 50 mM Tris-HCl, pH 7.0, 10 mM β-mercaptoethanol, 1.0 mM EDTA, 0.1 mM phenylmethylsufonyl fluoride, and 2.0 μg/ml each of aprotinin, leupeptin, and pepstatin A. Then they were homogenized in the same buffer at a ratio of 9.0 ml of buffer/1.0 g tissue with phosphatase inhibitor mixture containing 20 mM β-glycerophosphate, 1.0 mM Na_3VO_4, and 50 mM NaF, pH 7.0. The homogenates were spin at 15,000 rpm for 3 min at 4 ℃ for biochemical analysis. The phosphorylation of tau in the above samples was analyzed by Western blots using 10% SDS-PAGE. The separated protein bands were transferred into nitrocellulose membrane and probed with specific anti-tau antibodies. Then all blots were probed with peroxidaseconjugated secondary antibody and developed with chemiluminescent substrate kit. The protein bands were quantitatively analyzed, and the amount of protein was expressed as relative level of total optical density.

2.5 Immunohistochemistry

The hippocampus of the left hemisphere were fixed by 10% neutrality formaldehyde, 90% 0.01 M PBS solution at room temperature for 6 h, paraffin embedded, and cut into 5 μm-thick sections. Dry the slides (processed by acetone and APES) with tissue sections in an 80 °C oven for 30 min. Sections were blocked with 0.3% H_2O_2 in absolute methanol for 20 min and non-specific sites were blocked with instant calf serum for 60 min at 37 ℃. Then incubate sections overnight at room temperature with primary antibodies as described. The slides were developed by biotinylated secondary antibodies (1:200) and Avidin-peroxidase conjugate (1:200)/diaminobenzidine tetrachloride (0.05%) system.

2.6 Statistical analysis

Data were expressed as $\bar{x} \pm SD$ and analyzed using SPSS 11.0 statistical software (SPSS Inc, Chicago, Illinois, USA). The One-Way ANOVA procedure followed by LSD's *post hoc* tests was used to determine the different means among groups ($P<0.05$).

3 Results

3.1 Tau phosphorylation at ser396/404 site

To study whether folates and vitamin B12 affect tau protein phosphorylation and their possible

relationship, the status of tau phosphorylation of different ages in rats' hippocampus were analyzed. The status of tau of different age rat were carried out by Western blot using three well characterized phosphorylation-dependent and site-specific tau antibodies as listed in Table 1. Compared with two months old rats group, a remarkable increase of phosphorylated tau for forty months old rats was detected by PHF-1 which recognize phosphorylated tau at ser396/404 site. And tau-1 recognizes unphosphorylated tau at ser199/ser202 sites. Tau phosphorylation in forty months old rats group have the high expression (Figure 1 A and B). The level of total tau was indicated by blot developed with R134d, a phosphorylation-independant antibody. Data showned that there was no remarkable difference between two months old group and forty months old group (Figure 1C). So the phosphorylation of tau protein in rat's hippocampus is notably increased with the aging process. And based this result we do the next step.

3.2 Folates plus vitamin B12 can decrease hyperphosphorylation of tau in aged rat brain

After rats were treated with folates and vitamin B12, the status of tau protein phosphorylation were detected on different age stage groups in rats' brain. Gained the extract of rats' hippocampus was for immunoblot analysis. We found that bands of the drug treated group were sharply decreased compared to the group which was not treated with drugs (Figure 2A). It indicates a large decrease in phosphorylation of tau at Ser396/404 sites. This result was corroborated by the greatly diminished staining of tau bands by phosphorylation-independent anti-tau antibody tau-1, which has an optimal immunoreactivity when tau is dephosphorylated at ser199/ser202 (Figure 2B). It demonstrated that folates and vitamin B12 have the effect on decreasing the tau phosphorylation in aged rat brain. We also found that the level of total tau still had no remarkable difference between groups(Figure 2C).

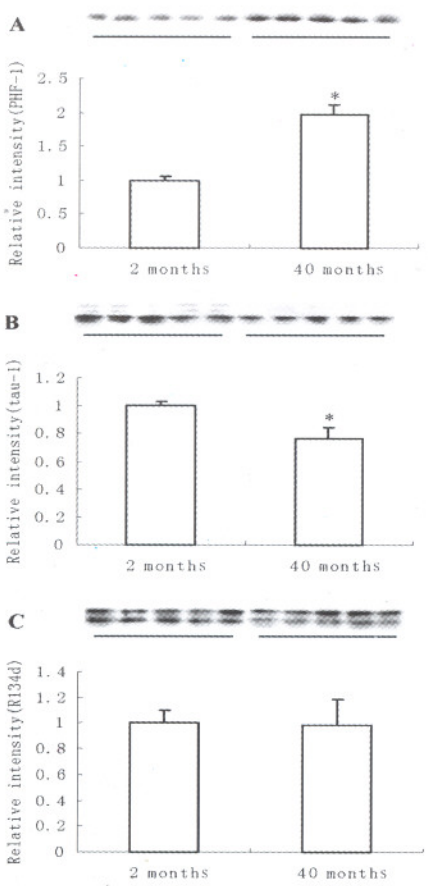

Figure 1. Level of tau phosphorylation in different age rat brain by Western blot.
15 μg of protein per lane was employed with antibody PHF-1, tau-1 and R134d. * $P < 0.05$, *vs*. 2 months old rat.

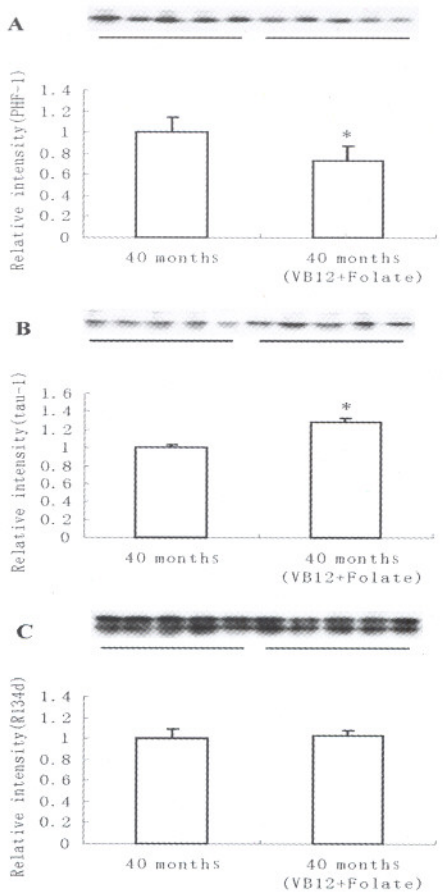

Figure 2. Effect of folate plus vitamin B12 on tau phosphorylation in 40 months aged rat brain by Western blots.
15 μg of protein per lane was employed with antibody PHF-1, tau-1 and R134d. * $P < 0.05$, *vs*. aged rat that was not treated with folate and vitamin B12.

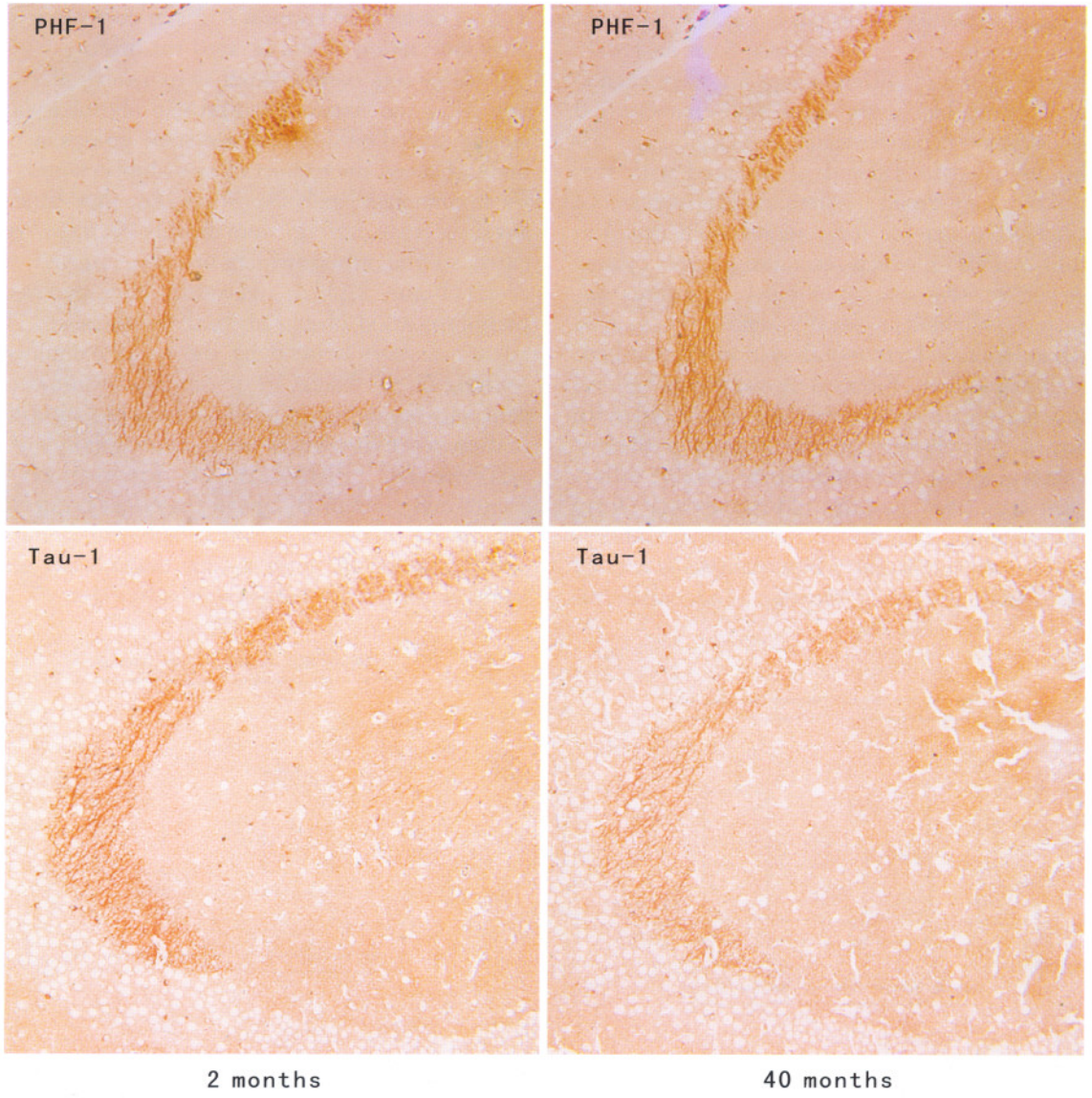

Figure 3. Level of tau phosphorylation in different age rat brain by immunohistochemistry
Paraffin embedded (5 μm-thick) sections were immunostained with PHF-1 and tau-1.

To learn the change of phosphorylation state of tau and its topographical distribution correlate with folates and vitamin B12 treatment in the brain tissue, we immunostained the sections cut from the left hemisphere with phosphorylation dependent and phosphorylation independent anti-tau antibodies. From these pictures we found that the immunohistochemistry results were consistent with Western blots results. The level of tau phosphorylation in 40 months aged rat brain is higher than that of 2 months old rat (Figure 3). After treated with folates and vitamin B12 for one month we can see that at ser396/404(PHF-1) the rat brain sections were stained gradually weak. Oppositely, they were stained gradually increased when detected by antibodies tau-1 (Figure 4). Immunohistochemistry results also demonstrated that folates and vitamin B12 could decrease the level of tau phosphorlation in rats' hippocampus.

4 Discussion

This experiment studied the possible associations between vitamin B12/folate and tau protein phosphorylation in aged rat brain. In the study we detected the phosphorylation state of the aged and young adult rats. We found that the level of tau phosphorylation in aged rat brain was higher than that of young adult rat. The result suggests that the phosphorylation of protein tau changes dynamically according to the physiological state associated

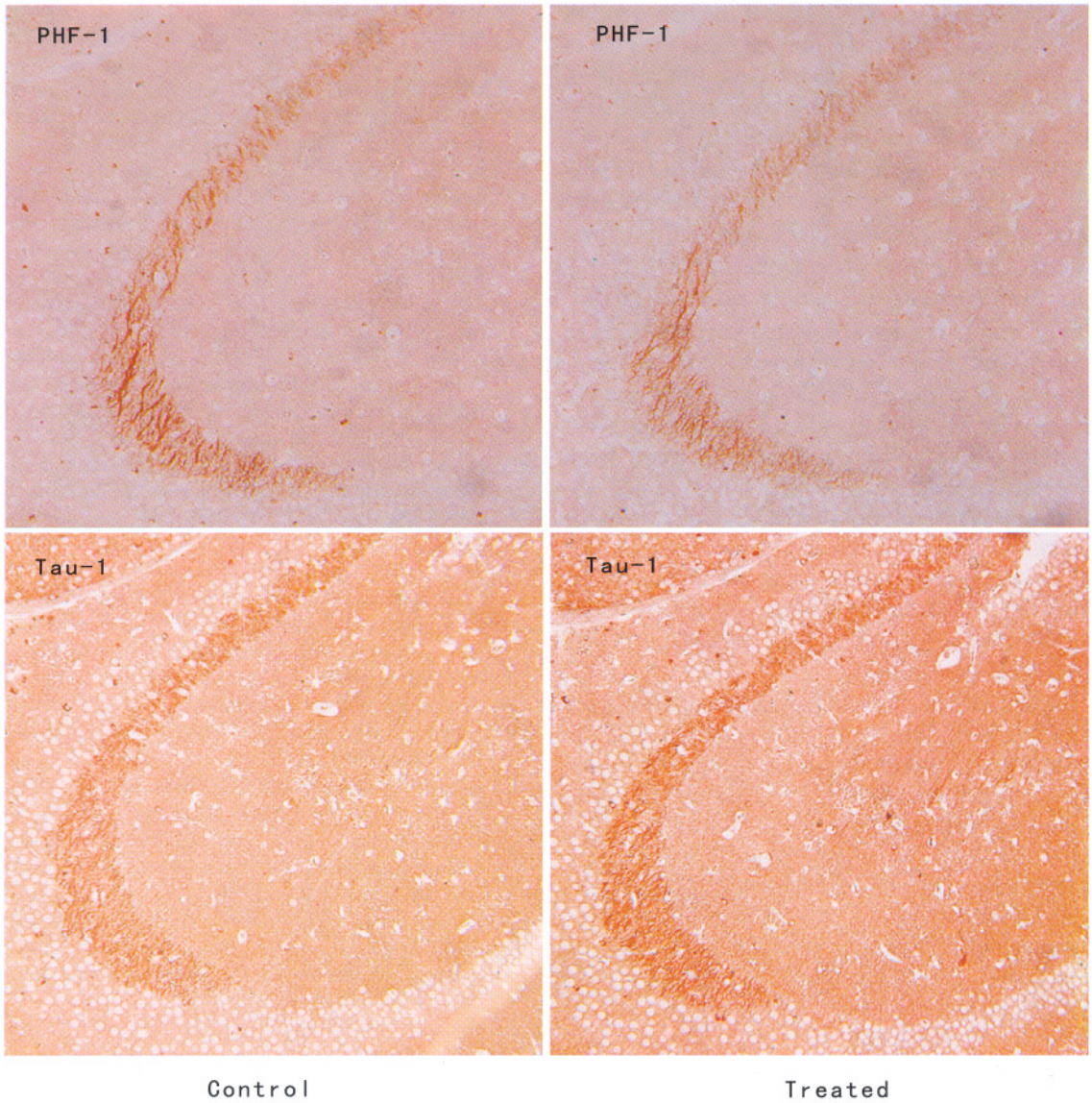

Figure 4. Effect of folate plus vitamin B12 on tau phosphorylation in 40 months aged rat brain by immunohistochemistry. Paraffin embedded (5 μm-thick) sections were immunostained with PHF-1 and tau-1.

with the aging process. In fact, recent studies had reported that high incidence rate of AD in aged population. And abnormal hyperphosphorylation of tau protein is closely related to AD. Biochemical and anatomy also had proven these foundings. But the mechanism of the tau phosphorylation in aged people which probably cause AD is still not clear. Recently, researcher found that older people with low levels of folate are twice as likely to develop AD as are those with normal levels[8]. In some observational studies, researchers found that low serum folate levels have been associated with AD and with all types of dementia[9, 10]. Red blood cell folate and CSF folate levels are lower in patients with AD than in controls[11]. It is known that low folate levels can be the result of inadequate dietary intake, diminished absorption from the gastrointestinal tract or increased utilization. In older people folate metabolism disturbance usually happened probably because body regular function becomes declined. So supplement folate could be reasonable for these older people.

In our study we treated the aged rat with folate and vitamin B12 for one month the level of hyperphosphorylation of tau in rat brain extract decreased. It suggested that folate and vitamin B12

could improve abnormal hyperphosphorylation of tau, which composed NFT, one of the hallmarks of AD. This result probably due to supplemental of folate and vitamin B12 decreaseing the levels of blood homocysteine. It has been reported that blood levels of homocysteine was elevated in patients who were lack of folate and vitamin B12[12]. High homocysteine levels are associated with decreased cognitive function and dementia[13]. Individuals with AD have been found to have higher plasma homocysteine levels than agematched controls[14], and it has been reported that elevation of plasma homocysteine levels precedes clinical manifestations of AD[15]. The underlying mechanisms of homocysteine as a risk factor for Alzheimer's dementia are still uncertain, but there are many ways in which homocysteine could damage neurons, including through endothelial dysfunction, cerebral microangiopathy and increased oxidative stress. In rats, homocysteine induces apoptosis in hippocampal neurons, and *in vivo* it increases excitotoxicity and oxidative damage[16]. The detail mechanism still need further study.

In summary, this study demonstrated that folate and vitamin B12 could relieve the level of tau protein hyperphosphorylation in aged rat brain. And this founding could be a reference for clinicians and medicine researchers.

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