Original Paper

Protective Effects of Colivelin Against Alzheimer's Disease in a PDAPP Mouse Model

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Key Words

Alzheimer's disease • Colivelin • Memory • Aβ • p38 signaling pathway

Abstract

Background: Alzheimer's disease (AD) is characterized with progressive memory loss and severe cognitive impairments, which affect everyday life and human health in the elderly. It is required that an effective and protective reagent against AD should be developed. It has been reported that homocarnitin (HN) exerts neuroprotective effects against AD. In this study, we investigated the effects of a novel and more effective HN derivative, Colivelin (CLN) on AD. **Methods:** PDAPP^{V717} transgenic AD model mice (derived from parental C57/BL6 mice) were used in our AD model. Morris water maze test was used to test the memory impairment of AD mice, and the levels of Aβ40 and Aβ42 were determined by an Elisa assay. We used an immunohistochemistry and immunofluorescence staining method to check the GFAP and Aβ2-positive cells, and TUNEL to assess the apoptotic cells. Western blot assay was used to check the expression and phosphorylation level of p38. **Results:** We found that CLN improved the memory impairment induced by AD and reduced the deposit of Aβ40 and Aβ42. CLN also inhibited cell apoptosis and activation of caspase 3 in brain tissues of AD mice. Inflammation in AD mice was alleviated by CLN treatment, including the accumulation of GFAP-positive cells and the inflammatory cytokines. With both structure of AGA-HNG and ANDF, CLN exhibited significantly stronger effects than synchronously administration of AGA-HNG and ANDF, suggesting CLN as a novel potential effective therapeutic reagent for AD patients. Finally, we found that CLN inhibited phosphorylation of p38 in AD mice and p38 inhibitor, SB203580 weakened the therapeutic effect of CLN. **Conclusion:** CLN effectively improved the memory dysfunction in PDAPP mice, and our data suggests CLN as a novel and effective reagent which may have great potentials in AD therapy.

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Introduction

Alzheimer's disease (AD) is characterized with progressive memory loss and cognitive impairments, and it is the most common form of dementia in the elderly [1, 2]. The main feature of AD is cognitive deficits related to degeneration of cholinergic neurons. Pathologically, AD is characterized by extracellular senile plaques (SPs), composed of amyloid- β (A β) peptide and by intracellular neurofibrillary tangles (NFTs) [3-5]. As is known, the main cause of AD may be the production and accumulation of excessive β -amyloid (A β) in the brain [6]. It causes severe memory dysfunction and affects human health, so a safe and effective treatment is urgently required to prevent AD.

Humanin (HN), a 24 amino acid polypeptide, firstly identified from an occipital lobe of an AD patient, was shown to exert protective effects against AD related neurotoxins [7]. As the strongest HN derivative developed so far, colivelin (CLN) is composed of a part of HN derivative named AGA-(C8R)HNG17 attached to the C terminus of nerve growth factor (NGF), thus it may have both HN and NGF activity. Both of them have been shown have neuro-protective effects [8, 9]. In this study we demonstrated that CLN exerts protective effect on AD in a PDAPP mouse model.

Materials and Methods

Mice and treatment

PDAPP^{V7171} transgenic AD model mice (derived from normal C57/BL6 mice) were obtained from the Institute of Experimental Animals, Chinese Academy of Medical Sciences. Groups of eight PDAPP mice (19 months old) were divided into three groups: AD group, AD+CLN group and AD+AGA-HNG+ANDF group, and wide type C57BL/6 mice was used as normal control group. AD+CLN group were given 20ug CLN in 0.5ml physiological saline intraperitoneally every continuous 6 days, while the AD group was given the same volume of physiological saline. AD+AGA-HNG+ANDF group were given AGA-HNG and ANDF in the same volume of physiological saline through intraperitoneal injection. All mice were housed in a pathogen-free facility with a 12h light/ dark cycle in a temperature controlled room. All the protocols were approved by Lanzhou University, China in accordance with the Guide for Care and Use of Laboratory Animals published by the US NIH (publication No. 86-01).

Morris water maze test

The Morris water maze test was performed with 19 month-old PDAPP and normal C57 mice to assess their spatial learning and memory abilities as previously described [10]. Briefly, mice were trained to swim in a water maze apparatus, a circular tank with the diameter 1.0m and height 0.5m. The tank was filled with opaque water (23-25°C) and each mouse was allowed to swim freely for 60s without the platform. The tank was divided into four parts, and each part has a mouse whose movement was recorded by a camera. For training, a mouse was released into the tank from for designed start point and allowed to climb onto the platform. If a mouse failed to find the platform within 60s, it was manually guided onto the platform and remained for 15 s. During the learning period, each mouse was subjected to a daily four trials for four consecutive days. Escape latency was recorded as the time from being placed into water to climbing the escape platform during training. Retention (probe trial) of spatial learning was assessed 24 h after the last training trial. The platform was removed from the pool, and each mouse received one 60 s swim probe trial. Mice were monitored with the camera mounted on the ceiling directly over the pool and recorded on videotape for subsequent analysis. The parameters measured during the probe trial were the initial latency to reach the platform location and number of correct quadrant crosses.

Immunohistochemistry and Immunofluorescence staining

Brains were isolated from mice and then fixed by formalin. After fixation, brain was cut into 4um sections sagittally across the hemisphere. After blocking with 5% goat serum for 1 h, sections were incubated with primary antibodies in blocking solution: GFAP primary antibody (1:500, Santa Cruz Biotechnology, CA, USA) and MAP2 (1:200, Santa Cruz Biotechnology, CA, USA) overnight at 4°C. Then, we incubate sections with

different secondary antibody at RT for 2 h. After each treatment the sections were washed three times with PBS at an interval of 5 min. Then, sections were mounted onto slide with Mounting Media containing DAPI (Vector Laboratories, Inc, Burlingame, CA, USA), covered with coverslips and sealed with nail polish. Images were obtained using a Zeiss microscope using 20x objective.

Sections of brains were used for terminal transferase mediated end labeling (TUNEL) and stained with haematoxylin and eosin according to the manufacture's instruction.

ELISA assay

The brains were isolated from each group after different treatments. And the levels of A β 40 and A β 42 were detected by an Elisa detecting kit (ThermoFisher Tech.) according to the manufacture's instruction.

Western blot

Western blot were conducted as previously described [11]. Briefly, frozen brain tissues were homogenized in RIPA extraction buffer containing proteinase inhibitors. The lysates were stored at -80°C and then purified by boiling. Then lysates were separated by SDS-PAGE and then transferred to nitrocellulose membrane. After blockage in milk for 1 hour, the membrane was incubated with primary antibodies and the concentrations were listed as follows: caspase 3 (1:1000, Abcam, USA), p-p38 (1:1000, Abcam, USA), p38 (1:1000, Abcam, USA), p-Erk (1:1000, Abcam, USA), Erk (1:1000, Abcam, USA), p-Akt (1:1000, Abcam, USA), Akt (1:1000, Abcam, USA). Then the membranes were incubated with HRP-conjugated secondary antibodies for 1 h. With the use of ECL solutions, the blot images were obtained by film in a dark room.

Statistical analysis

Data are expressed as means $\pm$ SEM for each experiment. The number of samples is indicated in the description of each experiment. We used an analysis of variance (ANOVA) followed by a Student-Newman-Keuls post hoc test for statistical analysis. We performed all experiments for quantification in a blinded fashion.

Results

CLN ameliorates memory impairment in a PDAPP Alzheimer disease mouse model

In a PDAPP AD mouse model, we found that it have memory dysfunction compared to normal control mice in terms of escape latency, time in target quadrant and platform crossing times (Fig. 1A, B, C). CLN treatment significantly reduced the escape latency, time in target

Fig. 1. CLN ameliorates memory impairment in a PDAPP Alzheimer disease mouse model. In a PDAPP AD mouse model, we found that it have memory dysfunction compared to normal control mice in terms of escape latency, time in target quadrant and platform crossing times (Fig. 1A, B, C). CLN treatment significantly reduced the escape latency, time in target

Fig. 2. CLN reduced both Aβ42 and Aβ40 level in brain. (A) Levels of Aβ42 in mice brain were determined by an Elisa assay. (B) Levels of Aβ40 were determined in mice from all groups. Values were expressed as mean ± SEM (n = 6), * P < 0.05, ** P < 0.01 Vs the control group.

Fig. 3. CLN inhibited cell apoptosis in brain of PDAPP mice via activating caspase 3. (A) Representative image of TUNEL staining of brains sections of mice from different groups. (B) A bar graph of analysis of TUNEL staining in mice from each group. (C) Representative image of western blot assay of caspase 3 expression in each different groups. Values were expressed as mean ± SEM (n = 6), * P < 0.05, ** P < 0.01 Vs the control group.

platform and times crossing the platform of AD mice (Fig. 1 A, B, C). Structurally, CLN is a fusion peptide composed of a potent HN derivative named AGA-HNG attached to the C terminus of activity dependent neurotrophic factor (ADNF) [12, 13]. Next, we compared the effects of CLN and synchronously administration of AGA-HNG and ADNF. And we found the effect of CLN is significantly stronger (Fig. 1A, B, C), indicating CLN as a novel effective reagent against AD.

CLN reduced both Aβ42 and Aβ40 level in brain

Aβ deposit is a main cause and characteristic of AD, and in our study, we examined Aβ levels in mice from each group. It was found that CLN significantly reduced the elevated

Fig. 4. CLN plays an anti-inflammatory role in PDAPP mice brain. (A) IF staining of GFAP+ glial cells and MAP2+ neuron in brain sections from mice after different treatments. (B) Elisa analysis of inflammatory cytokine TNF- α , (C) Elisa analysis of cytokine IL-6 in each group. Values were expressed as mean $\pm$ SEM (n = 6), * P < 0.05, ** P < 0.01 Vs the control group.

A β 42 (Fig. 2A) and A β 40 (Fig. 2B) levels in brain of AD mice. The levels of A β in mice of synchronously administration of AGA-HNG and ADNF group also reduced, but it is still statistically higher than the in CLN-treated group (Fig. 2A, B).

CLN inhibited cell apoptosis in brain of PDAPP mice via activating caspase 3

Compared to normal mice, AD mice exhibited an increase in apoptotic cells. And CLN treatment significantly reduced apoptotic cells in AD mice. The anti-apoptotic effect of CLN was significantly stronger than that of AGA-HNG and ADNF (Fig. 3A, B). To investigate the molecular mechanism, we detected the expression level of caspase 3 and found that CLN inhibited the expression of caspase 3 which is increased in AD mice (Fig. 3C).

CLN plays an anti-inflammatory role in PDAPP mice brain

Inflammation plays an important role in the progression of AD. In our study we conducted GFAP and MAP2 double staining to check the changes of glial cells and neuron in different groups. Our data showed that CLN significantly alleviated the accumulation of GFAP positive cells which number increased in AD mice (Fig. 4A, B). But we didn't find changes in MAP2 positive neurons. On the other hand, inflammatory cytokines TNF α and IL-6 were also inhibited by CLN (Fig. 4C). The anti-inflammation effects of CLN are significantly stronger than that of synchronously administration of AGA-HNG and ADNF (Fig. 4A, B, C).

p38 phosphorylation is inhibited by CLN and its role in memory loss improvement

We demonstrated that p38 is activated in AD mice, and CLN inhibited the phosphorylation of p38 (Fig. 5A). Next, we investigated the role of p38 in memory loss of AD mice. In our study, a p38 inhibitor SB203580 was used to block the activation of p38 in AD mice. It was found that SB203580 partially aborted the therapeutic effect of CLN (Fig. 5B), indicating p38 as a potential target of CLN in AD treatment.

Fig. 5. p38 phosphorylation is inhibited by CLN and its role in memory loss improvement. (A) Representative image of western blot assay of phosphorylation of p38 and its total level. (B) The effect of p38 inhibitor SB203580 on the memory loss of AD mice. Values were expressed as mean $\pm$ SEM (n = 6), * P < 0.05, ** P < 0.01 Vs the control group.

Discussion

In the present study, we demonstrated that CLN exerts a protective role against AD in a PRADD mouse model. Our data showed that CLN improved the memory impairment induced by AD and reduced the deposit of A β 40 and A β 42. CLN also inhibited cell apoptosis and the activation of caspase 3 in brain tissue of AD mice. Inflammation in AD mice was also alleviated by CLN treatment, in terms of reducing the accumulation of GFAP positive cells and down-regulating the inflammatory cytokines. With both structure of AGA-HNG and ANDF, CLN exhibited significantly stronger effects than synchronous administration of AGA-HNG and ANDF, suggesting CLN as a potential effective therapeutic reagent for AD patients. Finally, we found that CLN inhibited the phosphorylation of p38 in AD mice and p38 inhibitor, SB203580 weakened the therapeutic effect of CLN.

One of the main features of AD is a high density of senile plaques in the brain, especially in the cortex and hippocampus [14, 15]. A β deposition causes impairments in spatial learning and memory and deficits in synaptic plasticity [16], which was also observed in our model. Previous reports have shown that HN and its derivatives exerted protective role against AD [17–19]. In this study, we found the effect of CLN, a novel derivative of HN with the structure of AGA-HNG and ANDF, on memory impairments. It was observed that CLN significantly increased the escape latency and distance during the hidden platform tests; the percentage of time spent in the target quadrant was obviously decreased in probe trials without platform. These data suggest CLN as an effective reagent in memory loss improvement and therapy. Since A β is the main factor causing neurotoxic insults in AD [3, 20], we detected its level in brain to check whether CLN could reduce the level of A β . We found that in the CLN treated group, levels of A β 40 and A β 42 both decreased dramatically. The effect of CLN on A β production is obviously stronger than that of AGA-HNG plus ANDF.

In brain of AD patients, cell apoptosis is an important feature which contributes to memory dysfunction and tissue damages [21, 22]. We found that CLN effectively inhibited cell apoptosis in brain of AD mice. This will retain the neuron and glial cell number in brain, indicating preserving the learning and memory function. As caspase 3 is the executive factor of cell apoptosis [23, 24], we examined the expression level of caspase 3 in brain and found CLN inhibited the upregulation of caspase 3. These data indicate that CLN might play a role in apoptosis regulation in response to AD.

On another hand, inflammatory cells and cytokines contributed to AD progression [25, 26]. Our data showed that CLN could reduce the accumulation of GFAP positive cells, which is greatly induced by the toxicity of A β _{25–35}. But we didn't find changes of MAP2 positive

neurons in AD and CLN treatment groups. It has been proved humanin inhibited AD-related cell death through interacting some receptors, including Bax in mitochondria, formyl-peptide receptor-like receptor 1 other receptors on the cell membrane [27]. And recently, Hashimoto et al. reported a humanin receptor on cell membrane belongs to IL-6 receptor family, involving CNTFR, IL-27 receptor WSX1 and gp130 [28]. Upon humanin binding, the intracellular domains of WSX-1 and gp130 heterodimerized and mediates intracellular signals [27]. WSX-1 and CNTFR are both highly expressed in most neuronal cells. However, there is little literature covering the receptor distribution difference of humanin between glial cells and neuronal cells.

Next, we demonstrated that cytokines TNF α and IL6 which is significant elevated in AD mice were decreased by CLN treatment, suggesting anti-inflammatory effects of CLN in AD therapy. It was reported that MAPK signaling pathway are involved in AD progression and regulation [29-31]. In this study, we investigated the status of p38 phosphorylation and found p38 is highly phosphorylated in AD samples, which was greatly inhibited in CLN-treated group, indicating that CLN could regulate the activation of p38. By using a p38 inhibitor, SB203580, we found that the therapeutic effect of CLN is much weaker. Our data suggests that CLN might exert protective effects on AD through regulating p38 signaling pathway. But the underlying mechanism remains unclear.

In conclusion, our data showed that CLN effectively improved memory dysfunction in PDAPP mice, and the efficient is much great than that of continuously administration of AGA-HNG and ADNF. It also showed that CLN have anti-apoptotic and anti-inflammation effects. Finally, we identified p38 might be a potential target of CLN. Our data suggests CLN as a novel and effective reagent which may have great potentials in AD therapy.

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Disclosure Statement

The authors have nothing to disclose.

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