Full paper

Dipotassium N-stearoyltyrosinate ameliorated pathological injuries in triple-transgenic mouse model of Alzheimer's disease

Sha Liu a, Shuang-Qi Tang a, Heng-Jing Cui a, Sha Yin a, Ming Yin b, Hong Zhao b, Ling-Hua Meng a, Ze-Jian Wang b, a, Yang Lu b, a, **

a Institute of Medical Science, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai, China
b School of Pharmacy, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai, China

A R T I C L E   I N F O

Article history:
Received 3 May 2016
Received in revised form 7 August 2016
Accepted 31 August 2016
Available online 12 September 2016

Keywords:
Alzheimer’s disease
APPsw/PS1M146V/TauP301L mouse model
Dipotassium N-stearoyltyrosinate
Animal behavior
Neuroprotection

A B S T R A C T

Recently, anandamide (AEA) analogues have been well recognized for its potent neuroprotective effects in counteracting the deterioration of Alzheimer’s disease (AD) brains through multiple pathological processes. In our previous studies, dipotassium N-stearoyltyrosinate (NSTK), an AEA analogue synthesized by our laboratory was reported to exert significant efficacy through multiple interventions. Within this study, the amyloid precursor protein (APP)sw/PS1M146V/TauP301L, mouse (3×Tg-AD) model was used to explore further the neuroprotective effects of NSTK and its underlying mechanisms. NSTK could increase spontaneous locomotor activity in the open field and low anxiety-like behavior in the elevated plus maze, and improve the spatial memory deficits in the Morris water maze. The biochemical analysis suggested that NSTK could decrease Aβ42 deposition, abnormal tau aggregation, and the expressions of p-APP Thr668, PS1 and p-tau Ser202/Thr205 in the hippocampus of 3×Tg-AD mice. Consistently, NSTK could reduce the level of malondialdehyde, increase the activity of superoxide dismutase and catalase. Up-regulation of Bcl-2, and down-regulation of BAX, caspase-3 and inflammatory cytokines also occurred in the hippocampus of 3×Tg-AD mice after treatment with NSTK. Thus, NSTK could intervene in multiple pathological processes of AD and would be a drug candidate against AD.

© 2016 The Authors. Production and hosting by Elsevier B.V. on behalf of Japanese Pharmacological Society. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Alzheimer’s disease (AD), a neurodegenerative disorder characterized by progressive cognitive dysfunction and behavioral impairment has become a major threat to human health. In AD brain, presenilin-1 (PS1) processes the amyloid precursor protein (APP) to generate amyloid (Aβ) peptides: as a matter of fact, clinical features of AD are manifested morphologically by excessive accumulation of extracellular aggregation of Aβ peptides in the form of amyloid plaques, and intracellular neurofibrillary tangles (NFTs) composed of phosphorylated tau (1). Experimental evidence indicates that Aβ deposition and tau hyperphosphorylation trigger oxidative stress and inflammation, which lead to glial cell proliferation and eventually neuronal loss (2). Although numerous therapeutic agents aiming at the specific pathological process (3–5) have been developed, few of them are efficacious in vivo because of ignoring the integrity of pathogenesis and the relevance of multiple biological processes of AD and their applications in AD are limited. Therefore, it is imperative to find novel agents against AD via multiple pathways of intervention.

In damaged brain tissues, the endocannabinoid system (ECS) which consists of endocannabinoid (anandamide, AEA), its receptors and catabolic enzymes (e.g. fatty acid amide hydrolase, FAAH) regulates multiple pathological pathways in the central nervous system and has emerged as a potential target for the neuroprotection. It has been reported that the inhibitors of FAAH can decrease AEA hydrolysis and elevate AEA levels correspondingly, which are associated with anti-oxidative, anti-apoptotic and anti-inflammatory activities (6–8). Dipotassium N-stearoyltyrosinate (NSTK, Fig. 1), an AEA analog, was developed in our laboratory and is a promising neuroprotective candidate against stroke currently under preclinical studies in China. NSTK could ameliorate cognitive dysfunction induced by chronic cerebral hypoperfusion/global cerebral ischemia in rats/...
gerbils (10,11). We found the mechanisms of NSTK against stroke involve the inhibition of FAAH and the subsequent indirect activation of cannabinoid receptors (12), which finally improves inflammation, oxidative stress and glutamate-induced toxicity and cell viability (9–11). The above researches showed that NSTK has good neuroprotective effects through ECS-mediated multiple pathways.

Since inflammation, oxidative stress and glutamate-induced apoptosis are the common pathological characteristics in stroke as well as in AD (13–15) and NSTK had the potential to improve the pathological processes in cerebral ischemia model, NSTK might be also effective in AD treatment. Indeed, we have demonstrated the effects of NSTK against Aβ-induced toxicity on primary cortical neurons (12). It is interesting to observe the anti-AD effect of NSTK based on the animal model, especially on the triple-transgenic mouse model of AD (3×Tg-AD mice) which over-expresses human APP, PS1 and tau mutations. Since 3×Tg-AD mice not only progressively develop the typical pathological features and show behavioral impairments, but also mimic many aspects of human AD (16), it is of great clinical significance to validate the efficacy of NSTK on the model of AD.

In the present study, 3×Tg-AD mice were used to confirm the efficacy and mechanisms of NSTK in the treatment of AD. We first observed the animal behaviors as well as typical pathological features of the mice to evaluate the efficacy of NSTK against AD. Then, the key factors related to oxidative stress, inflammation and apoptosis in the transgenic mice were investigated to confirm the underlying mechanism of NSTK. In accordance with our previous observations in neuron (12), NSTK obviously improves behaviors and pathological features in 3×Tg-AD mice. Moreover, we found the mechanisms involved not only the improvement of glutamate-induced toxicity, which we have confirmed recently (9), but the decrease of inflammatory response, oxidative stress and the inhibition of apoptosis via ECS indirect activation.

2. Materials and methods

2.1. Chemicals

NSTK with purity over 98% was prepared in our laboratory and its structure was confirmed by 1H NMR and 13C NMR. NSTK and Piracetam purchased from Sigma Chemical Co. (St. Louis, MO, USA) were dissolved in sterilized water and stored at −20°C.

2.2. Animals

The 3×Tg-AD mice were obtained by crossing heterozygous APPswe/PS1dE9 double transgenic mice (Jackson Laboratory, Bar Harbor, ME, USA) with heterozygous P301L tau transgenic mice (Taconic Labs, Germantown, N.Y.). The wild male C57BL/6j mice (Shanghai SLAC Laboratory Animal Co., Ltd, Shanghai, China) and 3×Tg-AD mice were maintained in a controlled environment at 25 ± 1°C with a 12/12 h light-dark cycle. The experimental protocols were performed according to the Guidelines for Animal Experimentation of Shanghai Jiao Tong University.

2.3. Experimental treatment

50 male 3×Tg-AD mice (9-month-old at the start of the study) were randomly divided into five groups (each n = 10): the 3×Tg-AD group, three groups of 3×Tg-AD mice treated with 15, 30 and 45 mg/kg NSTK respectively, and the 3×Tg-AD group treated with piracetam (100 mg/kg) as the positive control. Each mouse was administrated orally in a volume of 0.1 mL per 10 g weight for 2 months. The wild mice were used for the control group (n = 10). Experimental groups and the wild group received an equal volume of NSTK and distilled water, respectively. The animals were behaviorally tested after 2 months of treatment. The behavioral tests were carried out in the following order: open field testing (OFT), elevated plus-maze (EPM), Morris water maze (MWM). The animals were sacrificed and the brains were removed from the skulls after the animal experiment. Half of the brain was used for immunofluorescence, and the rest for western blotting and enzyme-linked immunosorbent assay (ELISA). Since the hippocampus is the major part of learning and memory function, we used the tissue homogenates of the hippocampus for western blotting and ELISA in this study.

2.4. OFT

The OFT was used to assess general locomotor activity. Each mouse was placed in the center of the open field apparatus (50 × 50 × 38 cm) equipped with a video-tracking system and allowed to explore the apparatus for 5 min with the experimenter out of view. The total distance moved in the arena was recorded.

2.5. EPM

The apparatus (50 cm height from floor) is consisted of two open arms (30 × 5 cm) and two enclosed arms (30 × 5 × 15 cm) and is used to measure anxiety-like behaviors of the mouse. Each mouse was placed in the central section facing an open arm and was allowed to explore the maze for 5 min with the experimenter out of view. The time spent in the open arms was recorded using a video camera.

2.6. MWM

The MWM is consisted of a circular pool (1.8 m in diameter) filled with water (24 ± 1°C) and is surrounded with curtains to avoid environmental distraction. It was used to assess the spatial learning and memory. The target quadrant contained an escape platform (9 cm in diameter) which was submerged 1 cm below the surface of water in the center of one quadrant. During the training, each mouse was given 4 trials per day for 5 consecutive days. During each trial, the mouse was placed into the pool facing the wall at one random quadrant and allowed 60 s to locate the platform. Any animal which did not locate the platform was guided and placed on the platform for 30 s. On the 6th day, probe trials were carried out in which the platform was removed and the mice were given 60 s to search for the target quadrant. The escape latency, the time spent in the target quadrant and searching strategy were recorded by the video-tracking system.

2.7. Immunohistochemistry

The 3×Tg-AD mice were anesthetized with pentobarbital, perfused with saline, and then perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. The brains were removed from the skulls, fixed in 4% paraformaldehyde for 24 h and then transferred into PBS containing 30% sucrose. Each brain was
sectioned in the coronal plane at an instrument setting of 10 µm. Free floating sections were washed with PBS three times before being permeabilized with 0.3% Triton X-100 for 10 min, blocked with 3% bovine serum albumin (BSA) diluted in PBS for 1 h and then incubated overnight at 4 °C with the following primary antibodies: rabbit anti-Aβ42 (1:200, Abcam, Cambridge, Cambs, UK) and mouse anti-202/205 phosphorylated tau (AT8, 1:100, Life Technologies, Carlsbad, CA, USA). After several washes in PBS, the slides were incubated for 1 h at room temperature with the secondary antibody: DyLight 594 goat anti-rabbit IgG (1:500, Thermo Scientific, Rockford, IL, USA) in the dark. 4', 6-diamidino-2-phenylindole (DAPI, 1:500, Thermo Scientific) was used to detect cell nuclei. After being washed three times in PBS, the sections were then mounted on charged slides for immunofluorescence detection using an Olympus microscope with DP-70 software. The imaging data were analyzed and quantified using Image pro-plus version 6.0.

2.8. Western blotting analysis

The frozen brains were lysed with an ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) with complete protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche, Indianapolis, IN, USA). Lysates were centrifuged at 12,000 g for 20 min at 4 °C. The supernatants were collected and total protein concentrations were estimated using the Bradford method by means of the protein assay kit (Beyotime Institute of Biotechnology). Total proteins were denatured at 100 °C for 8 min and 4°C overnight at 4 °C with 3% bovine serum albumin (BSA) diluted in PBS for 1 h and then incubated being permeabilized with 0.3% Triton X-100 for 10 min, blocked with 5% BSA in Tris-buffered saline with 1% Tween-20 (TBST) for 2 h and 0.3% Triton X-100 for 10 min, blocked with 5% BSA in Tris-buffered saline with 1% Tween-20 (TBST) for 2 h and then incubated in the secondary antibody: DyLight 594 goat anti-rabbit IgG (1:500, Thermo Scientific, Rockford, IL, USA) in the dark. 4', 6-diamidino-2-phenylindole (DAPI, 1:500, Thermo Scientific) was used to detect cell nuclei. After being washed three times in PBS, the sections were then mounted on charged slides for immunofluorescence detection using an Olympus microscope with DP-70 software. The imaging data were analyzed and quantified using Image pro-plus version 6.0.

2.9. ELISA

Brain hemispheres were homogenized in ice-cold PBS containing 5 M guanidine hydrochloric acid and 1 × protease inhibitor mixture (pH 8.0) (17). The levels of Aβ42 were quantified using ELISA kits according to the manufacturer’s instruction (Invitrogen, Camarillo, CA, USA) and expressed as ng/g protein. The antioxidant status of the brains was assessed by determining the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the concentration of malondialdehyde (MDA). The measurement procedures were performed following the manufacturer’s instruction using commercial kits (Beyotime Institute of Biotechnology).

2.10. Statistical analyses

SPSS statistical software 16.0 for windows was used. All results were evaluated by using one-way ANOVA and Dunnett’s multiple range tests. All values were expressed as mean ± standard error of the mean (S.E.M). Statistical significance was assumed if P < 0.05.

3. Results

3.1. NSTK improved the behaviors of 3 × Tg-AD mice in the OFT, EPM and MWM

The total traveled distance of mice in the OFT was evaluated as spontaneous locomotor activity (Fig. 2A), which of the 3 × Tg-AD mice was less than that of the wilds. NSTK could significantly increase that of the 3 × Tg-AD mice. The anxiety-related behavior (the time spent in the open arms) of mice was evaluated in the EPM (Fig. 2B). The 3 × Tg-AD mice (low anxiety) spent more time in the open arms compared with the wilds. NSTK could significantly improve that of the 3 × Tg-AD mice.

The escape latency in MWM was evaluated per day for 5 consecutive days. The escape latency of the 3 × Tg-AD mice was longer than that of the wilds. NSTK (30 and 45 mg/kg) reversed these effects (Fig. 3A). The swimming time in the target quadrant and the swimming track in the probe trial were evaluated to estimate retention performance at the end of five-days-training period. The swimming time in the target quadrant of the 3 × Tg-AD mice was not longer than that of the wilds. NSTK (30 and 45 mg/kg) reversed the effects (Fig. 3B). The swimming track of the 3 × Tg-AD mice was in an inappropriate way for the platform and NSTK resulted in fewer excursions in the target quadrant (Fig. 3C).

3.2. NSTK ameliorated Aβ and tau pathology

The Aβ42 deposition in the cortex and hippocampus of the 3 × Tg-AD mice was significantly higher than in the wilds, while NSTK (30 and 45 mg/kg) significantly decreased the deposition. The amount of Aβ42 decreased from 27.86 ± 5.075 ng/g in the 3 × Tg-AD mice to 16.3 ± 2.941 ng/g (30 mg/kg) and to 13.9 ± 3.782 ng/g (45 mg/kg) in the NSTK groups, suggesting that NSTK decreased the level of Aβ42 in the brain of the 3 × Tg-AD mice (Fig. 4). As shown in Fig. 4B, the
expressions of p-APP Thr668 and PS1 in the 3×C2Tg-AD mice were significantly increased compared to the wilds, NSTK could significantly reduce that of the 3×C2Tg-AD mice, which were unanimous with the results of Fig. 4C.

The intensity of AT8, which recognizes tau phosphorylated at both Ser202 and Thr205 in the hippocampus was significantly higher in the 3×C2Tg-AD mice than in the wilds (Fig. 5A and C), which was similar with the results of western blotting (Fig. 5B and D). NSTK treatment (45 mg/kg) significantly reversed the phenomena.

3.3. NSTK attenuated oxidative stress

Oxidative stress is an important factor for the progression of AD (14). The antioxidant enzymes play pivotal roles against excess free radicals. The level of MDA is routinely used to reflect the extent of cell membrane damage resulting from attacks by oxygen-radicals.

In the brains of the 3×C2Tg-AD mice, the content of MDA significantly increased and the activities of CAT, SOD and GPx showed a marked decrease compared with those of the wilds, whereas NSTK (30 and 45 mg/kg) only increased the activities of CAT and SOD in the hippocampus of 3×C2Tg-AD mice (Fig. 6) and the 3×C2Tg-AD mice treated with NSTK revealed a non-significant increase in the activities of GPx (data not shown).

3.4. NSTK blunted inflammatory and apoptotic responses

Aβ deposition and tau hyperphosphorylation promote inflammation by stimulating the release of inflammatory cytokines (18). The levels of IL-6, IL-1β and TNF-α in the 3×Tg-AD mice were significantly higher than in the wild mice. Intervention of NSTK (30 and 45 mg/kg) decreased the levels of IL-6, IL-1β and TNF-α in the 3×Tg-AD mice (Fig. 7). The Bcl family and the caspase family are well-known with respect to the apoptosis (19). The expressions of Bcl-2 in the 3×C2Tg-AD mice were dramatically decreased compared to the wilds, whereas that of Bax increased. The expressions of Bcl-2 and Bax in the NSTK groups were reversed (Fig. 8A and C). Furthermore, the expression of the executioner caspase-3 involved in the ultimate of apoptosis was significantly increased in the 3×C2Tg-AD mice compared with the wilds and in the NSTK groups a significant decrease in caspase-3 protein expression was observed (Fig. 8A,D).

4. Discussion

The discovery and application of a candidate intervening in multiple pathological processes is a novel strategy for AD treatment. The inhibitors of FAAH could improve multiple pathological processes (oxidative stress, inflammation, apoptosis, etc.) and showed benign effects (6–8). Preclinical studies of NSTK had demonstrated its biological activities similar to endocannabinoids based on the cellular and animal experiments (9–12). NSTK as an inhibitor of FAAH responsible for AEA inaction is different from cannabinoid receptor agonists. The application of a cannabinoid receptor agonist would result in broad cannabinoid-like effects including therapeutic effects in the damaged tissue and side effects in the normal tissue (20). However, the application of NSTK resulted in cannabinoid-like effects only in the damaged tissue, but had no...
Effect on the normal tissue, since ECS is active only in the damaged tissue and in a resting state in the normal tissue. The facts that NSTK showed curative effects on cerebral ischemia and that the pathological processes in cerebral ischemia are similar to those of AD promoted us to study the feasibility of NSTK in the treatment of AD. In this study we evaluated NSTK’s neuroprotective effects and verified the mechanisms based on an AD animal model, then revealed its value for AD treatment.

Piracetam as a nootropic drug could improve the function of mitochondria, inhibit the pathological processes of oxidative stress, inflammation and apoptosis, and has been widely used in treatment of older patients with dementia or cognitive problems (21-23). Piracetam as a positive drug is also used in the studies (24,25).

The traditional AD animal models (26) show some learning and memory impairments, but do not or only partly mimic the pathological features of AD and therefore are limited in anti-AD drug research. The transgenic mice accurately mimic the regional and specific pathological features of AD and provide a guarantee for the study of AD. To comprehensively evaluate the effects of NSTK on AD, we used the 3×Tg-AD mice in the study. Although the 3-month-old transgenic mice were used in a few reports to evaluate the effects of compounds in treating AD (3), in the present study the 9-month-old transgenic mice were used, which were more closely resemble clinical pathological features of AD (27). To understand the effects of NSTK on transgenic mice, we assessed three indexes related to cognitive and non-cognitive behaviors of transgenic mice: autonomic activity (OFT), anxiety level (EPM) and the ability of learning and memory (MWM). In the NSTK groups, the autonomic activities of transgenic mice have been enhanced and the low anxiety (disinhibitory tendencies) of transgenic mice have been improved, indicating the mice are afraid of the danger (28,29). The improvement of the abnormal behavior might be associated with high emotion induced by brain activation which also improved the dysfunction of learning and memory of mice (30). Aβ deposition and tau hyperphosphorylation could induce animal behavior disorders (3).
NSTK inhibited Aβ deposition and the expressions of p-APP and PS1 which could be resulted from inflammation, oxidative stress and glutamate excitotoxicity (31–33) and reversed by the intervention of multiple pathological pathways (2,34) and inhibited the formation of NFTs and the expression of p-tau, indicating that NSTK alleviated two pathological characteristics from macro and micro perspectives. The above mentioned results comprehensively evaluated neuroprotective effects of NSTK from animal behaviors and pathological features.

Aβ deposition and tau hyperphosphorylation could activate glial cells, then release a number of inflammatory mediators which would further activate other glial cells, damage cell membranes and cause inflammation and oxidative stress (2). Accordingly, NSTK could significantly reduce the release of inflammatory cytokines, decrease the inflammatory cascade reaction, and then improve the disorders of the central nervous system. In the NSTK groups, the product of lipid peroxidation and the activities of antioxidant enzymes were improved, consisted with our previous observations in PC12 cells and on mice in ischemia reperfusion injury (9,10). The treatment of 3×Tg-AD mice with NSTK reduced the level of MDA and increased the activities of CAT and SOD but not that of GPx. The results might be related to the inhibition of active microglia which generate reactive oxygen species (35). Since the microglia-mediated oxidative

Fig. 5. NSTK ameliorated tau pathology in the hippocampus of the 3×Tg-AD mice (n = 6). (A) The aggregation of NFTs (red) in the hippocampus of the 3×Tg-AD mice was significantly higher than in the wilds, while NSTK (30 and 45 mg/kg) significantly decreased the aggregation. Cell nuclei were stained with DAPI (blue). Scale bars represented 100 μm. (B) The expressions of p-tau Ser202/Thr205 in the 3×Tg-AD mice were significantly increased compared to the wilds, NSTK could significantly reduce that of the 3×Tg-AD mice. GAPDH was used as a loading control. (C) Number of NFTs in the hippocampus. (D) Quantification of p-tau Ser202/Thr205 expression. Values are reported as the mean ± S.E.M. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. 3×Tg-AD mice.

Fig. 6. NSTK attenuated oxidative stress in the brains of the 3×Tg-AD mice (n = 6). The activities of CAT (A) and SOD (B) showed a marked decrease and the content of MDA (C) significantly increased compared with the wilds, whereas NSTK (30 and 45 mg/kg) reversed the above phenomena. Values are reported as the mean ± S.E.M. *P < 0.05 and **P < 0.01 vs. 3×Tg-AD mice.
stress is a complicated process, the further studies are required to elucidate the antioxidant mechanisms of NSTK. 

Aβ deposition, NFTs, inflammation, and oxidative stress could activate apoptotic pathways (36–38). NSTK could obviously regulate the level of Bcl-2 and Bax, and reduce the expression of Caspase-3, which were further confirmed our previous immunohistochemical observations that NSTK could improve the morphology of apoptosis in the brain of mice (10). The current observations suggested that NSTK reduced neuronal apoptosis in brain tissue from both regulation and execution of apoptosis from the protein levels.

In damaged brain tissue, ECS is capable of activating distinct signaling pathways in response to different pathogenic events.
through activating cannabinoid receptors and has been demonstrated to modulate the main pathological processes, including neuroinflammation, excitotoxicity and oxidative stress, and then reduce the secondary injuries (6–8). NSTK could regulate ECS by inhibition of FAAH, therefore, it would be a good candidate for the treatment of AD.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (Nos. 81171245, 31201418, 81270432), Projects of Shanghai Municipal Science and Technology Commission (No. 14D11900604).

References