Original Paper

Honokiol Attenuates Oligomeric Amyloid β_{1-42} -Induced Alzheimer's Disease in Mice Through Attenuating Mitochondrial Apoptosis and Inhibiting the Nuclear Factor Kappa-B Signaling Pathway

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Key Words

Alzheimer's disease • Honokiol • Apoptosis • Mitochondria • NF- κ B

Abstract

Background: Increasing evidence indicates that amyloid β oligomer ($A\beta$ O) is toxic to neurons in Alzheimer's disease (AD) brain. The aim of the present study is to evaluate the effects of honokiol on $A\beta$ O-induced learning and memory dysfunction in mice. **Methods:** AD mice model was established by $A\beta$ O intrahippocampal injection. The cognitive function was evaluated using Morris water maze (MWM). Nissl staining was used to examine the hippocampal neuron damage. Primary hippocampal neurons were exposed to $A\beta$ O. The apoptosis in the hippocampal tissues and primary neurons was assessed using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-neuronal nuclei (NeuN) and Hoechst staining, respectively. The mitochondrial membrane potential and radical oxygen species were detected using standard methods. Western blotting assay was used to check the expression levels of apoptotic and nuclear factor kappa-B (NF- κ B) signaling-associated proteins and electrophoretic mobility shift assay was used to detect NF- κ B-DNA binding. **Results:** Honokiol increased the time spend in the target zone of the AD mice in the MWM. In addition, honokiol dose-dependently attenuated $A\beta$ O-induced hippocampal neuronal apoptosis, reactive oxygen species production and loss of mitochondrial membrane potential. Furthermore, $A\beta$ O-induced NF- κ B activation was inhibited by honokiol, as well as the upregulated amyloid precursor protein and β -secretase. **Conclusion:** Honokiol attenuates $A\beta$ O-induced learning and memory dysfunction in mice and it may be a potential candidate in AD therapy.

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Introduction

Dementia is a kind of central nervous system (CNS) disease characterized by progressive memory loss and cognitive decline. Almost 50 million people worldwide are living with dementia, and approximately 60-70% was resulted from Alzheimer's disease (AD) (<https://blog.alz.co.uk/2016/12/18/alzheimer-community-between-disappointment-and-hope-regarding-drug-development-at-ctad-conference-2016/> accessed in March 2017). A major pathological hallmark of AD is extracellular senile plaques in brain, which is formed by accumulation of amyloid β -protein ($A\beta$)₁₋₄₂ [1]. $A\beta$ ₁₋₄₂ is derived from the large amyloid precursor protein (APP) precursor in a process called APP processing. In general, β -site APP-cleaving enzyme 1 (BACE1) and α -secretase mediates amyloidogenic and non-amyloidogenic pathway, respectively. In the amyloidogenic pathway, APP is cleaved by BACE1 and produces $A\beta$. The $A\beta$ is released into the extracellular space and self-aggregates to form oligomers that toxic to neuronal cells. In the non-amyloidogenic pathway, APP was cleaved by α -secretase within the $A\beta$ sequence to produce a soluble secretory amyloid precursor protein (sAPP α) [2, 3]. Thus, factors that triggered overwhelming amyloidogenic pathway and inhibited non-amyloidogenic pathway will result in overproduction of $A\beta$. Several forms of $A\beta$ ₁₋₄₂, such as soluble $A\beta$ oligomers ($A\beta$ O), $A\beta$ fibril and amyloid plaques, are well studied in the pathological mechanisms of AD. Nowadays, soluble $A\beta$ O, rather than $A\beta$ fibrils or amyloid plaques, has been believed to be neurotoxic [4, 5]. Studies have demonstrated that $A\beta$ O targeted to synaptic terminals [6] and its accumulation was associated with the spatial learning deficits in AD transgenic mice without amyloid plaque formation [7, 8]. Therefore, direct targeting $A\beta$ O or attenuation of $A\beta$ O-induced neurotoxicity is a common approach of AD treatment.

Honokiol (3',5-di-2-propenyl-1, 1'-biphenyl-2, 4'-diol) is isolated from the barks of *Magnolia officinalis*, and has multiple pharmacological activities. Previously, studies has shown that honokiol exhibited activities against anxiety [9, 10], depression [11, 12] and cerebral ischemia/reperfusion-induced memory deficiency [13]. In addition, honokiol has been found to improve learning and memory through preserving cholinergic neurons in the brain of senescence-accelerated prone mouse strain 8 (SAMP8) mice [14] and inhibiting the activity of acetylcholinesterase scopolamine-treated mice [15]. Importantly, Wang and colleagues have demonstrated that honokiol has the ability to cross the blood-brain barrier (BBB) [16], which enabled it to be a candidate for AD treatment. However, no study has reported the effects of honokiol in $A\beta$ -accumulation-based cognition deficiency. Only an *in vitro* studies has reported that honokiol could attenuate aggregated form of $A\beta$ ₁₋₄₂ induced oxidative stress, mitochondrial dysfunction and apoptosis in PC-12 cells [17]. The present study aims to evaluate the effects of honokiol on $A\beta$ O-induced cognitive dysfunction and investigate the possible mechanisms.

Materials and Methods

Animals

Male wild-type C57BL/6 mice (19-22 g, 6 weeks old) were purchased from the Experimental Animal Center of Dalian Medical University (Dalian, China). The mice were housed in standard animal house under a 12-h light/dark cycle at 22-23 °C with 60 ± 10 % humidity and were provided with food and water ad libitum. All of the methods and experimental procedures were approved by the Institutional Ethics Committee of Dalian Medical University.

$A\beta$ ₁₋₄₂ oligomer preparation

Oligomers were generated as described elsewhere [18]. $A\beta$ ₁₋₄₂ (GL Biochem Ltd., Shanghai, China) was dissolved in cold HFIP at a concentration of 1 mg/mL for 2 - 4 h at room temperature. HFIP was removed by evaporation under N₂, and the thin transparent film of peptides at the internal surface of the tube was stored at 80 °C. For oligomer preparation, HFIP-treated $A\beta$ ₁₋₄₂ peptide was dissolved in dimethylsulfoxide (DMSO) at 5 mM and diluted to 500 μ M in artificial cerebrospinal fluid (aCSF) or cell culture medium and aged by incubation at 4 °C for 24 h.

Experimental designs

The animals were randomly divided into 5 groups (n=6): control (con), A β_{1-42} oligomer treatment (A β), honokiol (0.7, 7, and 70 μ g/kg, GL Biochem Ltd., Shanghai, China) treatment (H-L, H-M, H-H, respectively) groups. The mice were anesthetized with 10 % chloral hydrate and fixed on a stereotaxic instrument. Then oligomeric A β_{1-42} (2 μ g/ μ L, 2 μ L) was injected into both lateral hippocampi in the model and honokiol groups via a 5- μ L micro-injector daily for 7 days. The mice in the control group received aCSF injection. The injection site was posterior from the bregma (AP) 2 mm, mediolateral from the midline (MR) 1.6 mm, and dorsoventral from the skull (DV) 1.5 mm. After 7 days injection, mice in the honokiol group were administered intraperitoneally with indicated dosage of honokiol daily for 14 days, while mice in the sham and model groups were administered with equal volume of 1% DMSO. The mice were sacrificed after the MWM test and the hippocampal tissues were collected for the following examinations.

Morris water maze (MWM) test

The ability of spatial learning and memory of the mice was assessed using a MWM test. The test was performed on the 14th day after honokiol administration. The test was performed in a circular water tank (120 cm in diameter) containing water (23 $\pm$ 1 $^{\circ}$ C) at a depth of 24 cm and divided into four quadrants. A hidden escape platform (9 cm in diameter) was placed in the center of one quadrant 1 cm under the surface of the water. Each mouse received four trials per day for five consecutive days. The mice were initially placed in the water facing the wall of the tank in the three quadrants which did not have the platform. The escape paths and latency were recorded by a camera. If the mouse failed to find the platform within 60 s, its escape latency was recorded as 60 s. The probe test was carried out at day 5 by removing the platform and the mice were placed at the starting point at the quadrant opposite to the platform quadrant and allowed to swim freely for 60 s. The time that the mice spent in the target quadrant and the number of times the mouse crossed over the platform location was recorded.

Cell culture and drug treatment

Primary cultures of hippocampal neurons were generated from fetal brains (embryonic day 18; E18) obtained from female Sprague-Dawley rats (the Experimental Animal Center of Dalian Medical University, Dalian, China). The brains were removed and hippocampi were dissected in dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA) containing 0.125% trypsin solution (Beyotime, Haimen, China) for 10 min at 37 $^{\circ}$ C. Fetal bovine serum (10%, Gibco, Carlsbad, CA) was then added to terminate the digestion. The dispersed tissues were centrifuged at 300 g for 5 min and were resuspended in DMEM (Invitrogen) containing 2 % B27 supplement (Gibco) and 0.5mM L-glutamine (Gibco). For the hoechst staining, neurons were plated in glass cover slips. For the enzyme linked immunosorbent assay (ELISA), real-time PCR, and Western blotting analysis, neurons were plated onto six-well plates coated with poly-D-lysine. The neurons were maintained in a humidified atmosphere of 95% air and 5% CO $_2$ at 37 $^{\circ}$ C. The culture medium was half-replaced every 3 days. Neurons cultured for 8 days were treated with indicated concentrations of honokiol (10, 50 or 100 μ M) for 24 h and followed by incubated with 10 μ M A β 0 for 12 h.

ELISA

The hippocampal tissues were homogenized in cooled PBS and subjected to repeated freezing and thawing in liquid nitrogen. The homogenate was centrifuged at 10,000 g for 10 min at 4 $^{\circ}$ C. The supernatant was used for ELISA. The culture medium were collected and centrifuged at 150 g for 20 min. The supernatant was used for ELISA. The levels of pro-inflammatory cytokines tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β and IL-6 in the hippocampal tissues and culture medium were determined using commercial ELISA kits according to the manufacture's protocols (Wuhan Boster Biological Technology, Ltd., Wuhan, China).

Neuronal nuclei (NeuN)-terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining

The hippocampal tissues were fixed in 4 % paraformaldehyde (PFA) for 24 h and embedded in paraffin. The paraffin blocks were cut into 5- μ m thick sections. The sections were deparaffinized in xylene, hydrated using a series of ethanol washes and incubated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 8 min. Then, the hippocampal sections were stained using an In Situ Cell Death Detection Kit (Wanleibio, Shenyang, China) and then counterstained with haematoxylin. The sections were observed under an optic microscope (DP73; Olympus).

Hoechst staining

The neurons grown on poly-L-lysine-coated glass cover slips were staining using a Hoechst 33258 Staining Kit (Beyotime). Briefly, the neurons were fixed in the fixing solution in the kit for 20 min at room temperature. After a wash stage with PBS, the neurons were then staining with the Hoechst-33258 for 5 min at room temperature. Finally, the cells were mounted with anti-fade fluorescence mounting medium and observed under a fluorescence microscope (IX53, Olympus, Tokyo, Japan).

Measurement of intracellular reactive oxygen species (ROS) and mitochondrial membrane potential (MMP)

Intracellular ROS was assessed using commercial ROS assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) by DCF-DA method and MMP was assessed using commercial MMP assay kit (Beyotime) by JC-1 method according to the manufactures' protocols.

Western blotting analysis

The hippocampal tissues and neurons were homogenized in cooled RIPA buffer and centrifuged at 12,000 g for 10 min at 4 °C. Protein concentration was determined using a BCA protein assay kit (Beyotime). Tissue homogenates (40 µg protein) from each mouse were boiled in loading buffer and electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5 % nonfat milk and incubated with primary antibodies overnight at 4 °C. After incubation at 37°C for 45 min with HRP (horseradish peroxidase)-conjugated secondary antibodies (1:5000; Beyotime), the membranes were developed using the enhanced chemiluminescence reagent (Beyotime) and visualized with autoradiography film. The intensity of each band was quantified with Gel-Pro-Analyzer software (Media Cybernetics, Bethesda, MD, USA). The primary antibodies used in the present study were listed in table 1.

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted from the hippocampal tissues and neurons using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime), followed by determination of protein concentrations using the BCA protein assay kit (Beyotime). The levels of NF-κB activation in each group hippocampal tissues and neurons were determined by EMSA using the nonradioactive NF-κB EMSA kit (Viagene biotech Inc., Changzhou, China) following the manufacturer's instructions.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis of differences between groups was carried out by one-way ANOVA followed by the LSD test. P values < 0.05 were considered significant.

Results

Honokiol improves spatial learning and memory in Aβ oligomer-treated mice

The spatial learning abilities of Aβ oligomer-treated mice were evaluated with MWM. The results showed that over the 5 training days, the time to find the hidden platform progressively declined in all groups. However, Aβ oligomer-treated mice showed significantly higher escape latencies than the control group at day 4 and 5 (P<0.01), implying learning deficits in the Aβ oligomer-induced mice. From the fourth day, the high-dose of honokiol-treated mice exhibited a significantly

Table 1. Primary antibody for Western blotting

Antibody	Dilution rate	Company	Cat No.
Bax	1:400	Boster, Wuhan, China	BA0135
Bcl-2	1:400	Boster	BA0412
Cleaved-caspase-3	1:1000	CST	9664
Cleaved-caspase-9	1:1000	CST	9509
CytC	1:400	Boster	PB0291
IkBα	1:400	Boster	PB0303
p-p65	1:500	Bioss, Beijing, China	bs-0982R
NF-κB	1:400	Boster	PB0073
COX IV	1:400	Beyotime	BM1759
Histone H3	1:500	Bioss	bs-17422R
β-actin	1:1000	Santa cruz	sc-47778

better performance than mice in the A β group. At day 5, the middle-dose of honokiol-treated mice also showed significantly lower escape latencies than the A β group (Fig. 1A). In the spatial probe test, as shown in Fig. 1B, compared with the control group, the time that the mice in the A β group spent in the target zone was significantly shorter ($P < 0.01$). The treatment with honokiol dose-dependently increased the percentage of time in the target zone in A β oligomer-induced mice. The representative pathways of mice in the spatial probe test are shown in Fig. 1C. These results indicate that honokiol treatment significantly attenuates spatial learning impairments in A β oligomer-induced mice.

Honokiol improves hippocampal neuron survival in A β oligomer-treated mice

In the Nissl staining, obvious neuronal loss and nucleus disappearance were observed in the CA1 region of hippocampus A β oligomer-treated mice (Fig. 2A). The number of neurons was significantly decreased in the CA1 region in the A β group compared with the control group ($P < 0.01$). However, the decreased neurons were dose-dependently increased by the treatment of honokiol. In the NeuN-TUNEL double staining, the number of apoptotic neurons was dramatically increased after A β_{1-42} oligomer injection, which was significantly decreased by the treatment of honokiol (Fig. 2B).

Honokiol attenuates neuron apoptosis through regulating mitochondrial functions in the hippocampus of A β oligomer-treated mice

We then investigated the expressions of the proteins involved in mitochondrial-apoptosis. As shown in Fig. 3A, a significant decrease in Bcl-2 expression and increase in Bax expression were found in the hippocampus of A β oligomer-treated mice compared with the control mice, and the expressions of cleaved-caspase 9 and 3 were also increased notably compared with the control mice. Moreover, cytochrome C (Cyt C) leakage was found, as evidenced by the upregulated cytoplasm Cyt C and the downregulated mitochondrial Cyt C. Treatment with honokiol dose-dependently enhanced the expression of Bcl-2 and reduced that of Bax, and the expressions of cleaved-caspase 9 and 3 were also reversed compared with the A β oligomer-treated mice. The abnormal expressions of cytoplasm mitochondrial Cyt C were also restored by the treatment of honokiol.

To confirm the effects of honokiol on mitochondrial functions, MMP and ROS generation were also evaluated in the hippocampus of A β oligomer-induced mice. Compared with the control group, the MMP was significantly reduced and the ROS production was significantly

Fig. 1. Morris water maze test. The spatial learning and memory ability of mice were tested by Morris water maze. (A) The time mice spent to find the platform during 5 training days. (B) The percentage of time mice spent in the target zone in the spatial probe test. (C) The representative pathways of mice in the spatial probe test. Results are presented as the mean $\pm$ SD ($n=6$). ** $P < 0.01$ vs. the control group, ## $P < 0.01$ vs. the A β group.

Fig. 2. Neuron survival and apoptosis in the hippocampus of A β oligomer-treated mice. (A) Nissl staining of CA1 region of hippocampus and quantitative analysis of survival neuron numbers. (B) NeuN-TUNEL double-staining of CA1 region of hippocampus and quantitative analysis of NeuN-TUNEL double-positive cells. Scale bar: 100 μ m. Results are presented as the mean $\pm$ SD (n=6). **P<0.01 vs. the control group, ##P<0.01 vs. the A β group.

Fig. 3. Effects of honokiol on mitochondrial function in the hippocampus. (A) Western blotting analysis of the expressions of mitochondrial-apoptosis-associated proteins, including Bcl-2, Bax, Cyt C and cleaved-caspase 3 and 9. (B) Histogram showing the red/green fluorescence of JC-1 intensity ratio (% of control) in each group. (C) Histogram showing the ROS generation (% of control) in each group. Results are presented as the mean $\pm$ SD (n=6). **P<0.01 vs. the control group, #P<0.05 vs. the A β group, ##P<0.01 vs. the A β group.

increased in the A β group (Fig. 3B and C). Treatment with high-dose of honokiol effectively enhanced the MMP and suppressed the ROS production in the hippocampus, indicating the protective effects in mitochondrial of honokiol.

Honokiol inhibits the NF- κ B signaling pathway in the hippocampus of A β oligomer-treated mice

The NF- κ B signaling pathway, which is involved in the pathological mechanisms of A β -associated AD [19], was also investigated in the present study. Compared with the control mice, the expression of I κ B α was downregulated and the expression of p-p65 was upregulated in the hippocampus of A β oligomer-treated mice (Fig. 4A and B). In addition, nuclear p65 expression was also upregulated, and the activity of NF- κ B-DNA binding was increased (Fig. 4C and D). Honokiol dose-dependently reversed these changes. Moreover, the protein expressions of two target genes of NF- κ B, amyloid precursor protein (APP) and β -site APP cleaving enzyme (BACE1) [20-22], were also upregulated in the hippocampus after A β oligomer-stimulation and were restored after the treatment of honokiol (Fig. 4E and F).

Honokiol attenuates A β oligomer-induced primary neuron apoptosis through regulating mitochondrial functions

To investigate the cellular target of honokiol, hippocampal neurons were primary cultured and the associated mechanisms were studied. Fig. 5A shows the effects of honokiol on the neuronal apoptosis. A β oligomer induced obvious apoptosis in primary neurons, as evidenced by the significantly increased number of condensed nuclei and high-stained neurons. The treatment of honokiol reduced the number of apoptotic neurons in a dose-dependent manner (Fig. 5A). Consistently, A β oligomer also induced the expressions of mitochondrial-apoptosis-associated proteins made a pro-apoptotic shift. The expression level of the anti-apoptotic protein Bcl-2 was downregulated and the pro-apoptotic proteins

Fig. 4. Honokiol inhibits the NF- κ B signaling pathway in the hippocampus of A β oligomer-treated mice. (A, B and C) Western blotting analysis of I κ B α , p-p65 and nuclear p65 expressions. (D) EMSA of NF- κ B-DNA binding. (E and F) Western blotting analysis of APP and BACE1 protein expressions. Results are presented as the mean $\pm$ SD (n=6). **P<0.01 vs. the control group, #P<0.05 vs. the A β group, ##P<0.01 vs. the A β group.

Fig. 5. Effects of honokiol on Aβ oligomer induced apoptosis and mitochondrial functions in primary neurons. (A) Hoechst staining of primary hippocampus neurons. Scale bar: 50 μm. (B) Western blotting analysis of apoptosis-associated proteins Bcl-2, Bax, Cyt C, and cleaved-caspase-3 and 9. (C) Histogram showing the ROS generation (% of control) in each group. Results are presented as the mean ± SD (n=3). **P<0.01 vs. the control group, *P<0.05 vs. the Aβ group, ##P<0.01 vs. the Aβ group.

Bax, cleaved-caspase 9 and 3 were upregulated (Fig. 5B). In addition, the expression level of Cyt C was increased in the cytoplasm and decreased in the mitochondrial, indicating the Cyt C leakage in the neurons. Honokiol effectively restored these changes. These findings are similar to that in the hippocampal tissues and confirm that honokiol attenuates Aβ oligomer-induced neuronal apoptosis.

MMP and ROS were also examined to reflect the mitochondrial function in primary hippocampal neurons (Fig. 5C and D). In consistent with the observation *in vivo*, Aβ oligomer leads to reduced MMP and enhanced ROS and pretreatment with honokiol dose-dependently reversed these changes.

Honokiol inhibits the NF-κB signaling pathway in the Aβ oligomer-induced hippocampal neurons

The effect of honokiol on the NF-κB signaling pathway was also investigated in primary hippocampal neurons. In line with the *in vivo* study, the expression of IκBα (Fig. 6A) was

Fig. 6. Honokiol inhibits the NF-κB signaling pathway in the Aβ oligomer-induced hippocampal neurons. (A, B and C) Western blotting analysis of IκBα, p-p65 and nuclear p65 expressions. (D) EMSA of NF-κB-DNA binding. (E and F) Western blotting analysis of APP and BACE1 protein expressions. Results are presented as the mean ± SD (n=3). **P<0.01 vs. the control group, #P<0.05 vs. the Aβ group, ##P<0.01 vs. the Aβ group.

inhibited and the p65 phosphorylation was enhanced by Aβ oligomer (Fig. 6B), thereby promoting the p65 nuclear translocation, as evidenced by the increased expression of nuclear p65 (Fig. 6C). Meanwhile, NF-κB-DNA binding activity was increased after Aβ oligomer exposure (Fig. 6D). In addition, the protein expressions of APP (Fig. 6E) and BACE1 (Fig. 6F) were also upregulated in the Aβ group. The pretreatment with honokiol effectively inhibited the activation of the NF-κB signaling pathway and the protein expressions of APP and BACE1.

Discussion

In the present study, we found that honokiol improved AβO-induced cognitive dysfunction in mice. In addition, honokiol attenuated mitochondrial dysfunction and mitochondrial-pathway apoptosis in hippocampus of AβO-treated mice and AβO-stimulated hippocampal neurons. Furthermore, honokiol inhibited AβO-activated NF-κB signaling pathway and therefore downregulated the expressions of APP and BACE1. These mechanisms may contribute to the neuroprotective effects of honokiol.

Hippocampal neurogenesis is essential for learning and memory. The cognitive deterioration in AD is closely associated with hippocampal injury induced by Aβ [23]. In the present study, we observed that mice with AβO intrahippocampal injection had the delayed escape latency in MWM. In addition, Nissl staining and TUNEL-NeuN double staining showed

the neuronal damage and apoptosis in CA1 region where A β O had been injected. Apoptosis plays a critical role in neurologic disorders including AD. Many factors will induce neuronal apoptosis during AD. Although the underlying mechanism is elusive, accumulating evidence shows that A β O induces neuronal apoptosis and contributes for the pathophysiology of AD [24-26]. Honokiol effectively protected neurons against A β O *in vivo* and *in vitro*, as evidenced by the markedly improved neuronal survival in hippocampus and the reduced apoptotic neurons in hippocampus and primary neurons exposed to A β O. This neuroprotective effects may contribute to the learning and memory improvement in mice.

In general, cellular apoptosis is triggered via two major pathways, the mitochondrial (intrinsic) pathway and the death receptor-mediated (extrinsic) pathway. Here, we focused on the mitochondrial pathway, which is mediated by Cyt C, the Bcl-2 family and caspases. In this apoptotic pathway, Cyt C is released from mitochondria, assembles with Apaf-1 and procaspase 9, and activates caspase-3 to trigger apoptosis [27]. Bcl-2 family is involved in the regulation of the Cyt C mediated mitochondrial apoptotic pathway [28, 29]. BH3-only proteins (apoptosis initiator), Bcl-2 (anti-apoptotic protein) and Bax (pro-apoptotic proteins) and Bak (Bcl-2 antagonist) form the tripartite apoptotic switch to control the release of apoptogenic factors, particularly Cyt C [30]. In our study, honokiol dose-dependently inhibited A β O-induced downregulation of Bcl-2, upregulation of Bax, Cyt C leakage and elevated levels of cleaved caspase-9 and 3 in hippocampus and cultured neurons, which indicates that honokiol attenuates neuronal apoptosis by modulating mitochondrial apoptotic pathway-associated proteins in A β O-exposed hippocampus.

As the finding of honokiol inhibited mitochondrial Cyt C leakage in the A β O-exposed hippocampal neurons, we evaluated the effects of honokiol on the MMP and ROS generation, two typical parameters that reflect mitochondrial functions, in A β O-exposed hippocampus and neurons. Mitochondria are the main source of cellular ATP production via oxidative phosphorylation (OXPHOS). Besides, mitochondria also produce ROS as signaling molecules and participate in diverse cellular processes [31]. However, stress leads to excessive ROS production in mitochondria, which in turn damages the ROS-produced cells and exacerbates the stress. During AD, A β has deleterious effects on mitochondrial function and contributes to energy deficiency and overproduction of ROS in brain [32]. In agreement with the previous studies, we found that A β O results in reduced MMP and elevated ROS generation in the A β O-exposed hippocampus and primary neurons. Predictably, honokiol improved mitochondrial functions by increasing MMP and reducing ROS production. Based on our findings and the accepted knowledge [32-34], A β O interfered with the OXPHOS and respiratory chain, destroyed the proton gradient, decreased the MMP and increased the permeability of mitochondrial membrane, which led to Cyt C leakage and excessive ROS generation. Meanwhile, the Bcl-2 was inhibited and the Bax was activated, which finally induced apoptosis together with the Cyt C and ROS. Although our findings did not reveal a definite molecular target of honokiol, we can conclude that honokiol can protect neurons from A β O-induced mitochondrial dysfunction, which may be involved in its anti-apoptotic effects.

NF- κ B is a key transcriptional regulator of various physiopathologic processes including learning and memory [19]. In normal state, NF- κ B is inhibited in cytoplasm by I κ B α . When I κ B α is phosphorylated by I κ B kinase (IKK), NF- κ B is released and translocates to the nucleus, where it binds to the target DNAs and regulates the gene expressions [35]. Several factors can activate NF- κ B in AD brain [19]. Here we found that A β O induced markedly nuclear translocation of NF- κ B and this should be, at least partly, resulted from the increased ROS [36]. In addition, APP and BACE1, two target genes of NF- κ B [21, 37], were found to be upregulated in the hippocampus and primary neurons treated by A β O. Based on the previous reports, the effects of NF- κ B activation on APP and BACE1 are controversial. Yang et al. found that TNF- α -induced NF- κ B activation did not affect APP in primary hippocampal neurons [38], while others reported that NF- κ B activation upregulates promoter activity of APP in HEK293 cells [22]. Both Chami and Chen discovered that NF- κ B mediated elevated BACE1 expression in HEK293 cells [22, 37], while Bourne et al. reported that factor NF- κ B

served as a suppressor in neuronal cell lines but as an activator of BACE1 transcription in astrocytes [39]. These discrepancies may due to the difference of the cells and stimulation factors used in the studies. However, it is noteworthy that the effect of A β O-induced NF- κ B activation on APP and BACE1 expressions has not been reported. In our study, NF- κ B activation and upregulation of APP and BACE1 were found in A β O-treated hippocampus and primary neurons, honokiol treatment restored these changes. These findings may lead to a hypothesis that the A β O-induced NF- κ B activation upregulates APP and BACE1 expressions, thereby resulting in elevated A β generation, which forms a vicious cycle and exacerbates the neuronal damage. Honokiol blocked the cycle by inhibiting the NF- κ B signaling. Unfortunately, the present study did not directly reveal the regulatory effect of NF- κ B on APP and BACE1 under A β O stimulation, which is not the aim of this study and should be further studied in the future. Our study indicates that honokiol may protect the hippocampal neurons against A β O by inhibiting NF- κ B activation.

Conclusion

Honokiol attenuates A β O-induced learning and memory dysfunction in mice. The neuroprotective effects of honokiol may be associated with the neuronal apoptotic attenuation, mitochondrial functions preservation and NF- κ B inactivation. Our findings provide evidence for the utility of honokiol in treating A β O-based cognitive dysfunction.

Acknowledgements

This study was supported by a grant from the Natural Science Foundation of Liaoning Province (No. 2015020302).

Disclosure Statement

The authors declare no conflict of interest.

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