Original Article
L-3-n-butylphthalide may attenuate Aβ\textsubscript{1-42} induced neuronal apoptosis and synaptotoxicity in primary cultured cortical neurons possibly via modulating both PI3K/AKT and ERK signaling pathways

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Abstract: Background: Alzheimer’s disease (AD), which is characterized by progressively cognitive decline and neuronal loss, is one of the most common neurodegenerative diseases. The β-amyloid (Aβ)-mediated mitochondrial dysfunction and eventually neuronal apoptosis contribute to the pathogenesis of AD. L-3-n-butylphthalide (L-NBP) has been proven to have significant protective effects in many stroke and AD models. Our study is to investigate the protective effects and mechanisms of L-NBP against Aβ induced cell injury in cortical neurons. Material and method: The primary cortical neurons treated with Aβ\textsubscript{1-42} were used as the AD cell model. Then the cells were treated with or without L-NBP. MTT assay were used to assess the cell viability. Apoptosis of cortical neurons was analyzed by Hoechst 33342 staining. The flow cytometry was used to assess the mitochondrial membrane potential (Δψm). The apoptotic and signaling associated protein expression levels were measured using Western blotting. Results: Pretreatment with L-NBP effectively inhibited Aβ\textsubscript{1-42} induced cytotoxicity, apoptosis, mitochondrial dysfunction, and synaptotoxicity in primary cultured cortical neurons in a dose-dependent way. The protective effects of L-NBP were accompanied by increased phosphorylation of AKT and decreased phosphorylation of the extracellular protein kinase (ERK). The neuroprotective effects could be abolished by LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K)-dependent protein kinase B (AKT). Furthermore, ERK pathway inhibitor U0126 enhanced the beneficial effects of L-NBP in inhibiting Aβ induced apoptosis. Conclusions: L-NBP may exert protective effects on Aβ induced cell injury in cortical neurons through regulating both PI3K/AKT and ERK signaling pathways.

Keywords: Alzheimer’s disease (AD), β-amyloid (Aβ), apoptosis, PI3K/AKT pathway, ERK pathway

Introduction
Alzheimer’s disease (AD) is an age-related neurodegenerative disease featured by extracellular β-amyloid (Aβ) deposition and intracellular neurofibrillary tangles, which is followed by loss of cortex and hippocampus neurons and cognitive dysfunction [1]. The mechanisms underlying AD pathogenesis largely remain elusive. However, increasing evidence suggests that Aβ deposition can initiate a cascade and lead progressively to inflammation, tau hyperphosphorylation, synaptic dysfunction, neuronal apoptosis, and finally dementia [2]. The hippocampi of AD patients and cultured neurons under Aβ exposure show characteristics of apoptosis [3]. However, the mechanism underlying Aβ induced neuronal apoptosis remains to be elucidated.

Aβ might initiate a succession of detrimental changes that ultimately lead to neuronal apoptosis through varying mechanisms, including increased reactive oxygen species production and cytochrome C release, mitochondrial dysfunction, and caspase-3 activation [4]. Aβ may induce neuronal apoptosis via inactivation of phosphoinositide 3-kinase (PI3K)-dependent protein kinase B (AKT), activation of p38 mitogen activated protein kinase (MAPK) or extracellular regulated protein kinase (ERK), nuclear factor-kappa B, and Wnt pathways [5-9]. In pAPPswe-transfected SH-SY5Y cells, neuroglobin attenuated Aβ induced mitochondrial dys-
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L-3-n-butylphthalide (L-NBP) was originally extracted from the seeds of *Apium graveolens* Linn (Chinese celery). Studies have shown that L-NBP protects against ischemic stroke through multiple mechanisms, such as inhibiting platelet aggregation, improving microcirculation in arterioles [12, 13], decreasing oxidative damage and neuronal apoptosis [14], improving mitochondrial function and inhibiting inflammatory response [15]. Furthermore, L-NBP can improve Aβ-induced learning and memory impairments, oxidative damage, and mitochondrial dysfunction [16, 17]. Lei et al. found that L-NBP attenuated Aβ-induced toxicity through modulating mitochondrial apoptosis and MAPK signaling pathway in SH-SYSY cells [18]. However, it is not yet known whether L-NBP can prevent Aβ-induced apoptosis and synaptotoxicity through modulating the PI3K/AKT and/or ERK pathway.

The present study was designed to analyze the protective effects of L-NBP against neuronal apoptosis and synaptotoxicity induced by Aβ in vitro. In addition, the potential mechanisms including mitochondrial function and possible signaling pathway underlying the neuroprotective effects of L-NBP were further analyzed and discussed.

**Materials and methods**

**Animals**

Pregnant Sprague-Dawley rats on gestation day 18 were provided by the Laboratory Animal Research Center of Shandong University, China. All experimental procedures involving animals were carried out in accordance with the Guidance Suggestion for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering.

**Primary cortical neuron isolation and culture**

Primary cortical neurons were cultured as previously described [19]. Briefly, the cerebral cortices were separated from embryonic Sprague-Dawley rat fetuses (E18D), cut into pieces, and then digested with 0.25% trypsin for 20 min in 37°C. After adding DMEM medium (with 10% fetal bovine serum) for 10 min, tissues were dissociated mechanically with a fire-polished pipette. Then after centrifugation and re-suspension, about 1×10⁵ cells in 1 ml were plated on 6-well plates coated with poly-D-lysine. The medium was replaced by Neurobasal medium (Gibco, CA, USA) supplemented with 2% B-27 supplement (Gibco) and 0.5 mM L-glutamine 4 h later. For maintenance, the medium was changed by half every 4 days. Cells were cultured in a humidified incubator containing 5% CO₂ at 37°C for 8 days before experiments.

**Cell treatment**

Aβ1-42 (Sigma-Aldrich Inc, St. Louis, MO, USA) was dissolved in distilled water and aged for 6 days at 37°C. L-NBP (purity >98%), which was synthesized by the Department of Synthetic Medicinal Chemistry, Institute of Materia Medica (Shijiazhuang, China), was dissolved in dimethyl sulfoxide (DMSO) as 200 mM stock solution. The LY294002 and U0126 were from Cell Signaling Technology, Inc. (Beverly, MA, USA) and were dissolved by DMSO. Cells were divided into control group (without treatment), Aβ group (10 μM Aβ1-42 for 24 h), Aβ+L-NBP group (first treated with 0.1, 1.0, or 10 μM L-NBP for 4 h and then treated with 10 μM Aβ1-42 for 24 h), Aβ+L-NBP+LY294002 group (first treated with 60 μM LY294002 for 1 h, then with 10 μM L-NBP for 4 h and finally with 10 μM Aβ1-42 for 24 h), and Aβ+L-NBP+U0126 group (first treated with 10 μM U0126 for 1 h, then with 10 μM L-NBP for 4 h and finally with 10 μM Aβ1-42 for 24 h).

**MTT assay**

Primary cortical neurons (1×10⁴ cells/well) were plated on 96-well plates and treated as above described. After treatment, DMEM and 5 mg/ml of freshly prepared MTT (Sigma-Aldrich Inc, St. Louis, MO, USA) were added to culture plates at a final concentration of 10%, and incu-
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bated for 4 h. DMSO (200 μl) was added to the plates and vibrated for 10 minutes at 37°C to solubilize MTT formazan crystals. Then the treatment wells were quantified spectrophotometrically at 570 nm using a LAS-4000 microplate reader (FUJIFILM, Japan). The cell viability was presented as the ratio of the experimental group to the control group.

**Immunofluorescence assay**

For immunofluorescence analysis, cortical neurons were plated on coverslips coated with poly-L-lysine. Then the cells were fixed with freshly prepared 4% paraformaldehyde in PBS for 30 min, permeabilized with Triton X-100 (0.1%) for 15 min, and rinsed with PBS for 3 times. After blocking with 5% goat serum for 30 min, cells were incubated with the primary antibody of rabbit anti-synaptophysin antibody (1:100, Abcam, UK) overnight at 4°C. After washing with PBS, secondary antibodies of Alexa Fluor 568 goat anti-rabbit IgG (1:500, Abcam, UK) were added and incubated for 1 h under darkness. Then, cells were incubated in 10 μg/ml DAPI for 10 min. After rinsed with PBS for 3 times, the cortical neurons were analyzed on an inverted fluorescence microscopy (Zeiss Jenalumar, Jena, Germany). Cell incubated without primary antibodies was as a negative control. The fluorescence intensity of synaptophysin was quantified using Image J 1.48u software (National Institutes of Health).

**Hoechst 33342 staining**

The cortical neurons were seeded on coverslips. After treatment described above, the cells were fixed with freshly prepared 4% paraformaldehyde for 30 min. Then, Hoechst 33342 (1 μM) was added to the cells and incubated for 30 min in the dark. After washed with PBS for 3 times, the cortical neurons were analyzed on an inverted fluorescence microscopy (UV excitation and emission at 360 and 450 nm, respectively). The cells with condensed nuclear chromatin and fragmentation were confirmed as apoptotic cells. The apoptotic ratio was calculated as the apoptotic cells to the total cells counted.

**Flow cytometry to assess mitochondrial membrane potential (Δψm)**

After treatments, 5 μM of JC-1 was added into the neurons and incubated for 15 min at 37°C under darkness. Then a flow cytometer was applied to analyze the ratio of red (590 nm)/green (529 nm) fluorescence and determine the percentage of cells with Δψm collapse.

**Western blotting**

Cells were harvested and incubated with lysis buffer containing phosphatase and protease inhibitors on the ice. The lysed cells were centrifuged (12,000× g) for 10 min at 4°C. The supernatants were collected and the total protein was qualified by a BCA protein assay kit (Beyotime, Nanjing, China). Then, proteins were electrophoresed on SDS-PAGE and then transferred to PVDF membrane. Membranes were blocked with blocking buffer (Thermo Scientific, Waltham, USA) for 1 h at room temperature and then incubated with primary antibodies against pro-caspase-3, cleaved caspase-3 (c-caspase-3), synaptophysin, phospho-Tau (Ser 199/202), phospho-Akt (Ser 473), phospho-Erk1/2 (Thr202/thr204), Akt, Erk1/2, total Tau and β-actin (Abcam, Cambridge, UK) overnight at 4°C, respectively. After rinsed with PBS, membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature. Finally, the antibody-bound proteins were detected by an ECL Western blot detection kit (Santa Cruz, CA, USA). The densities of protein bands were measured by Image J software.

**Statistical analysis**

All the results are expressed as mean ± SD and statistical analysis was performed using SPSS statistical software (version 17.0). The differences were analyzed by one-way variance analysis combined with Bonferroni-Dunn’s test or Student’s t test. A P value less than 0.05 was considered as statistically significant.

**Results**

**L-NBP can attenuate the cytotoxicity, apoptosis, mitochondrial dysfunction, and synaptotoxicity of primary cultured neurons under Aβ exposure**

Cell viability was measured by the MTT assay. Pilot experiment showed that the survival rate was approximately 50% under exposure to 10 μM Aβ_{1-42} for 24 h (data not shown). Thus, the concentration of Aβ_{1-42} used in this study was determined at 10 μM. We found that 10 μM of Aβ_{1-42} markedly decreased cell viability (Figure 1A), suggesting that Aβ_{1-42} induced cytotoxicity.
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in cortical neurons. Next, the expression of pro-caspase-3, c-caspase-3, p-Tau and synaptophysin was detected by Western Blot. Results showed that 10 μM of Aβ1-42 markedly increased the expression of c-caspase-3 and p-Tau, and decreased the expression of pro-caspase-3, synaptophysin in cortical neurons (Figure 1B and 1C). Immunofluorescence assay also detected the Aβ1-42 induced decreased synaptophysin expression (Figure 2A). The Hoechst 33342 staining showed Aβ1-42 increased the ratio of apoptotic cells significantly (Figure 2B).

This indicates the synaptotoxicity and pro-apoptosis ability of Aβ1-42. Flow cytometry analysis showed that cells with Δψm collapse were increased (Figure 2C), demonstrating that Aβ1-42 induced mitochondrial dysfunction in cortical neurons. However, when the neurons were pre-treated with 0.1, 1, and 10 μM L-NBP, the cytotoxicity, apoptosis, mitochondrial dysfunction, and synaptotoxicity induced by Aβ1-42 in cultured neurons were all attenuated by L-NBP in a dose-dependent manner (Figures 1 and 2). There were significant differences between Aβ...
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The protective effects of L-NBP are accompanied by increased phosphorylation of AKT and decreased phosphorylation of ERK

LY294002 can block the protective effects of L-NBP
function induced by Aβ (Figure 4A-F). Pretreatment with L-NBP could significantly attenuate 10 μM Aβ1-42-induced increased expression of c-caspase-3 and p-Tau, and decreased the expression of synaptophysin and phosphorylation of AKT in cortical neurons. However, Aβ+L-NBP+LY294002 group showed increased expression of c-caspase-3 and p-Tau and decreased phosphorylation of AKT than Aβ+L-NBP group. And the Aβ+L-NBP+LY294002 group showed increased Δψm collapse in contrast with Aβ+L-NBP group. There were significant differences among control group, Aβ group, Aβ+L-NBP group and Aβ+L-NBP+LY294002 group (P<0.05). Additionally, Aβ-induced decreased phosphorylation of AKT in primary cultured neurons could be reversed by LY294002. These results show that L-NBP may protect against Aβ-induced apoptosis by decreasing the phosphorylation of AKT.

**Discussion**

In this study, we used primary cultured cortical neurons under Aβ1-42 exposure as an AD cell model in vitro and the protective effects of L-NBP were assessed from different aspects. First, our results found that the cytotoxicity, apoptosis, mitochondrial dysfunction and synaptotoxicity induced by Aβ1-42 in cultured neurons were all attenuated by L-NBP in a dose-dependent manner. Besides, the protective effects of L-NBP were concomitant with increased phosphorylation of AKT and decreased phosphorylation of ERK. Furthermore, we found that the inhibitor of the PI3K, LY294002, abol-
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Accumulated studies demonstrate that both extracellular and intracellular Aβ_{1-42} can activate caspases, which lead to synaptic and neuronal degeneration [21]. The activation of caspases by Aβ_{1-42} is mediated by both extrinsic and intrinsic pathways [22]. Extracellular Aβ_{1-42} can bind to cell receptors [23], while intracellular Aβ_{1-42} can trigger mitochondrial stress by binding to a mitochondrial alcohol dehydrogenase [24]. Previous studies [5-9] have shown that PI3K/AKT, MAPK, nuclear factor-kappa B and Wnt signaling pathways are involved in Aβ_{1-42} induced neuronal apoptosis. The PI3K/AKT/GSK-3β signaling pathway is critical in the pathogenesis of AD because it can boost tau hyperphosphorylation [25], which is the main component of neurofibrillary tangles. Specifically, decreased activation of PI3/AKT causes increased GSK-3β activity and lead to tau hyperphosphorylation [26, 27]. Our current study demonstrated that the decreased expression of p-AKT, increased expression of p-Tau and

Figure 4. LY294002 can block the beneficial effects of L-NBP. A. Western blots analysis showed the expression of c-caspase-3, p-Tau, p-AKT, and synaptophysin. B-E. The intensities of the indicated protein bands were quantified by densitometry. F. The Δψm collapse in cortical neurons. The data were shown as means ± SD from the three independent experiments. Control, cells without treatment. Cells were treated with Aβ only, Aβ with 10 μM L-NBP, and Aβ with 10 μM L-NBP and LY294002, respectively. #p<0.05 for Aβ vs control, *p<0.05 for Aβ+L-NBP vs Aβ, +p<0.05 for Aβ+L-NBP+LY294002 vs Aβ+L-NBP.
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c-caspase-3 were associated with Aβ1-42 induced neurotoxicity in cortical neurons. The L-NBP pre-incubation can significantly increase the p-AKT level and decrease c-caspase-3 and p-Tau expression. And, these beneficial effects were blocked by LY294002. Our results are partly consistent with previous researches [28, 29]. For instance, curcumin can improve synaptic plasticity and cognitive function in rats via the PI3K/AKT signaling pathway [28]. Furthermore, melatonin protects against Aβ-induced neurotoxicity through decreasing memory deterioration, synaptic disorder, tau hyperphosphorylation, and neurodegeneration through PI3K/AKT/GSK3β signaling in the Aβ1-42-treated mouse model of AD [29]. These results suggest that PI3K/AKT/GSK-3β pathway may be used as an effective therapeutic target in treating AD.

MAPK pathways, the critical mediators that propagate extracellular signals from the membrane to the nucleus, include ERK, JNK/stress-activated protein kinase, and p38. It is generally accepted that ERK activity is typically involved in cell survival, proliferation, differentiation, and memory formation in nervous system [30]. Accumulating evidence [31, 32] have shown that the phosphorylation of ERK is actively involved in AD pathology. Nevertheless, views about the function of ERK under Aβ1-42 exposure are not conclusive. Some studies demonstrate that decreased ERK activity induced by Aβ1-42 can protect against cytotoxicity and apoptosis [33, 34]. However, others studies indicate that Aβ-induced ERK activation leads to neural apoptosis [35, 36]. The current study showed that Aβ1-42-induced increased expression of both p-ERK and c-caspase-3 in cortical neurons and that these increases were attenuated by L-NBP significantly. Meanwhile, the ERK pathway inhibitor U0126 enhanced beneficial role of L-NBP in inhibiting Aβ1-42-induced apoptosis. These results suggest that L-NBP exhibit protective effects through inhibition of ERK phosphorylation in Aβ1-42-induced neuronal apoptosis. Our results are partly consistent with previous investigations, which reported that in isolated hippocampal cell culture, increased phosphorylated ERK by Aβ exposure was in parallel with that in cell death and apoptosis and insulin could inhibit ERK phosphorylation induced by Aβ1-42 [37].

As the source of energy, mitochondria play a crucial role in regulating cell apoptosis and redox balance, and the “mitochondrial cascade hypothesis” has been proposed to be involved in the pathogenesis of AD [38]. Increasing evidence has reported that Aβ1-42 exposure impairs mitochondrial permeability and that mitochon-
drial permeability transition pore openings lead to Δψm collapse and pro-apoptotic factors release from mitochondrial to cytosol [39, 40]. Accumulating evidence suggests that mitochondrial perturbation plays a critical role in synaptic failure and degeneration in AD [41, 42]. The present study further showed that L-NBP significantly attenuated Aβ1-42-induced Δψm collapse and synaptotoxicity, and that LY294002 blocked this effect of L-NBP. The present results are consistent to the study of Zhang Yu et al. [43], which has found that L-NBP can reconstruct synaptic and spine function in aged APP/PS1 AD transgenic mice by suppressing Aβ1-42 plaques deposition and neuroinflammatory response and that Wnt/β-catenin signaling pathway may be involved [43].

In conclusion, our findings indicate that L-NBP has protective effects on Aβ1-42 induced cell injury possibly by regulating both PI3K/AKT and ERK signaling pathways. The PI3K/AKT and ERK pathways may become potential therapeutic targets in AD treatment.

Disclosure of conflict of interest

None.

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