— **Regular Article** —

Resveratrol Attenuates Neuroinflammation-mediated Cognitive Deficits in Rats

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Neuroinflammatory responses are involved in Alzheimer's disease (AD) pathogenesis. In the present study, the protective effects of resveratrol, a polyphenolic compound, on cognitive deficits induced by lipopolysaccharide (LPS) were examined in rats, and the possible mechanisms were explored. The data showed that resveratrol administration by intraperitoneal injection (i.p.) may inhibit cognitive deficits induced by bilateral intracerebroventricular injection of 5 μg of LPS in rats. Subsequently, resveratrol afforded beneficial actions on the inhibition of tumor necrosis factor (TNF)-α, cyclooxygenase (COX)-2, and amyloid-β protein precursor (APP) generation, as well as the inhibition of phospho-transcription factors of nuclear factor κB p65 (p-NF-κB p65) activation followed by the presence of LPS in rat hippocampus. The results of the present study indicate that resveratrol may attenuate LPS-induced direct neuroinflammation in rats, and its mechanisms are, at least partly, due to inhibition of the generation of TNF-α, COX-2, and APP, and the related phosphorylation of NF-κB. These findings suggest that resveratrol might be a potential agent for treatment of neuroinflammation-related diseases, such as AD.

Key words —— resveratrol, lipopolysaccharide, neuroinflammation, cognitive deficit

INTRODUCTION

Alzheimer's disease (AD), the most common age-dependent neurodegenerative disorder, is clinically characterized by progressive memory loss and cognitive decline. The neuropathological hallmarks of AD are neurofibrillary tangles (NFT), intraneuronal filaments composed of aggregated hyperphosphorylated tau protein, senile plaques, and extracellular accumulations of amyloid β protein (Aβ).[1] The etiology of AD in humans is still unknown, but experimental and clinical evidence suggest a close association between neuroinflammation and AD pathogenesis.[2] Neuroinflammation has been known to play a role in the pathogenesis of AD, in which damaged neurons, neurites, highly insoluble Aβ peptide deposits, and NFT provide obvious stimuli for inflammation.[2] Lipopolysaccharide (LPS), a known inducer of neuroinflammation, exacerbates tau pathology by a cyclin-dependent kinase 5-mediated pathway in a transgenic model of AD,[3] and pro-inflammatory conditions promote neuronal damage mediated by amyloid precursor protein (APP) and decrease its phagocytosis and degradation by microglial cells in culture.[4] Likewise, the AD brain shows a chronic inflammatory response characterized by an abundance of reactive astrocytes and activated microglia, complement, and cytokines.[5] These findings strongly suggest that neuroinflammation contributes significantly to AD pathogenesis.

Epidemiological studies suggest that long-term treatment with non-steroidal anti-inflammatory drugs (NSAID) reduces the risk of AD,[6] suppresses inflammation in a transgenic mouse with AD,[7] and reduces Aβ, hyperphosphorylated tau, and memory deficits in Alzheimer mice.[8] These findings further demonstrated that neuroinflammation could be an important causative contributor in the development and/or progression of AD, and anti-inflammatory agents can be effective at reducing the prevalence of AD. However, a harmful side effect of long-term NSAID treatment to prevent AD is the gastrointestinal and occasional liver and kidney toxic-

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ity associated with the inhibition of cyclooxygenase (COX)-1.[9–11] Furthermore, selective COX-2 inhibitors were found to be ineffective.[12] Therefore, it is important to study alternative anti-inflammatory drugs that are safe in long-term treatments.

The polyphenolic compound resveratrol (*trans*-3,4′,5-trihydroxystilbene, Res) is a naturally-occurring phytochemical which has been found in more than 70 plant species, including human food products like grapes, peanuts, berries, and some herbs. The physiological function of this polyphenol is thought to serve as a phytoalexin, protecting plants against environmental stress or pathogenic attack, and as a strong antioxidant to reduce the oxidation of lipoproteins in animals.[13,14] Recently, several animal studies have focused on the neuroprotective effects of Res, showing it to slow the neuropathology associated with AD[15] and to protect against injury from brain trauma and cerebral ischemia.[16] It was further shown to modulate cholinergic neurotransmission and improve cognition in diabetic rats.[17] One of our previous studies also demonstrated that resveratrol may attenuate rat cortical neuronal apoptosis induced by oxygen-glucose deprivation/reperfusion[18] and slow the neuropathology associated with Parkinson disease (PD).[19] In addition, resveratrol may be useful for attenuating acute cognitive disorders in elderly individuals with an infection.[20] However, there have been no studies investigating the potential for resveratrol to reduce LPS-induced direct neuroinflammation.

Therefore, the purpose of the present study was to investigate the effects of resveratrol on LPS-induced direct neuroinflammation and explore the possible protective mechanisms.

MATERIALS AND METHODS

Drugs and Reagents —— Res (purity > 98% by HPLC) was provided by Hunan Huaguang Biological Products (Huaihua, China). It was dissolved in normal saline (NS) before use. LPS (*Escherchia coli*; 055:B5) and ibuprofen (IBU) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Both LPS and IBU were dissolved in NS. A Power SYBR Green Master Mix obtained from Applied Biosystems (Foster City, CA, U.S.A.) was used. The reverse transcription kit was purchased from TaKaRa Biotech Co. (Dalian, China). Trizol was purchased from Huashun Bioengineering Co. (Shanghai, China), RNeasy Mini Kit from Qiagen Co. (Valencia, German), and Primer Express software from ABI Co. (Foster City, CA, U.S.A.). The Power SYBR Green Master Mix (Applied Biosystems, Cheshire, U.K.) was used for real time PCR analysis. The BCA protein assay kit was purchased from Biocolor Biotechnology (Shanghai, China), and both antibodies of tumor necrosis factor (TNF)-α, cyclooxygenase (COX)-2, phospho-nuclear factor (NF)-κB p65, and glyceraldehyde phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology, Inc. (Boston, MA, U.S.A.). The antibodies of APP and secondary horseradish peroxidase-conjugated anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology, Inc. (Richmond, CA, U.S.A.).

Apparatus —— The Morris water maze and MT-200 analysis system were purchased from Chengdu Taimeng Co. (Chengdu, China), and the quantitative real-time reverse transcriptase (RT)-PCR instrument was obtained from BIO-RAD (Richmond, CA, U.S.A.).

Animals —— Male specific pathogen-free Sprague-Dawley (SD) rats (8–10 weeks old weighing 250–350 g) were used in the present study. The rats were obtained from the Animal Center of the Third Military Medical University (Chongqing, China, Certificate No. SCXK 20020003). The animals were acclimatized for 4 days at 22 ± 1°C with a 12-hr light-dark cycle, and were allowed free access to food and tap water throughout the experiment. All efforts were made to minimize the number of animals used, and all animal studies were performed in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China (November 14, 1988) and the Institutional Review Committee for the Use of Animals.

Drug Administration and Surgery —— Sixty rats were randomly assigned to six groups: sham, LPS, LPS + ibuprofen (IBU, 40 mg/kg as a positive control), and LPS + Res 10, 20, and 40 mg/kg groups (n = 10, respectively). Rats in the LPS + IBU groups were administered IBU by gavage at the dose of 40 mg/kg, and rats in the LPS + Res 10, 20, and 40 mg/kg groups were administered Res by gavage at doses of Res 10, 20, and 40 mg/kg once per day three days before surgery and thereafter continuously for 9 days. The sham and LPS groups received the same volume of saline as that of the Res.

Three days after treatment with Res, IBU or

Table 1. Primer Sequences for Real Time RT-PCR Analysis

Gene	GenBank Accession#	Forward	Reverse
TNF-α	L19123	GTGATCGGTCCCAACAAGGA	CTCCCACCCTACTTTGCTTGTG
COX-2	NM_017232	GCTGGCCTGGTACTCAGTAGG	CGAGGCCACTGATACCTATTGC
APP	BC062082	GCATGGTGGACCCCAAGA	TGGTTCATGCGCTCGTAGATC
GAPDH	NM_017008	CAGTGCCAGCCTCGTCTCA	TAACCAGGCGTCCGATACG

NS, the rats were anesthetized with 40 mg/kg chloral hydrate by intraperitoneal injection (i.p.), and secured in a stereotaxic apparatus with their rectal temperature maintained at 37°C using a heating pad. An area of skin on top of the skull was shaved and sterilized. Two small holes for needle insertion were drilled in the parietal bone posterior to the bregma on either side of the midline (coordinates: posterior −0.8 mm, medial/lateral ± 1.5 mm relative to bregma, dorsal/ventral −3.8 mm below dura).[21] LPS (5 μg in 5 μl sterile NS) was injected into the bilateral intracerebroventricular (b.i.c.v.) region in the LPS, LPS + IBU, and Res groups via a stainless steel needle using a microinjector. LPS solution was injected into each of the lateral cerebral ventricles over a 5-min period with a 5-min waiting period between the two injections. The sham group underwent all surgical steps, with the exception that saline was administered rather than LPS. Following wound suturing, all rats received an intramuscular injection of 40000 units/0.25 ml of the antibiotic penicillin. The rats were closely monitored during recovery and kept in a room at 22°C. Animal body weights were recorded daily, and their general behaviors were monitored. The rectal temperature was monitored by a clinical thermometer after surgery daily to ensure the rats were healthy.

Morris Water Maze Test —— Spatial learning and memory were evaluated by the Morris water maze. The apparatus and test procedure have been described elsewhere.[22, 23] The Morris water maze test was started on the 5th day after surgery. The procedure included two steps. The first step was the place navigation test from Day 1 to 4, in which the escape latency (the time required to escape onto the hidden platform) was used to evaluate learning and memory function. Rats that found the platform were allowed to remain on the platform for 20 s and were then returned to the home cage. If a rat did not reach the platform within 120 s, it was gently guided to the platform by the experimenter, where it remained for 20 s. The last trial was regarded as the probe test. The second step was the spatial probe test on Day 5 after removal of the platform and after the space navigation test, which was performed to test the ability of rats to find the removed platform by memory.

Real-time RT-PCR Analysis —— After the behavioral experiments, 4 rats extracted randomly from each group were anesthetized by an overdose of 7% chloral hydrate (50 mg/kg), and the hippocampi were removed and placed into tubes containing Trizol. Total RNA was isolated and purified with an RNeasy Mini Kit. The forward and reverse primer sequences for selected genes were designed with ABI Primer Express software and are listed in Table 1. A Power SYBR Green Master Mix was used for real time PCR analysis. The relative differences in expression between groups were expressed using cycle time (Ct) values as follows: the Ct values of the interested genes were first normalized with GAPDH of the same sample, and then the relative differences between control and treatment groups were calculated and expressed as relative increases, setting control as 100%.

Western Blot Analysis[24] —— The remaining rats from each group after the behavioral experiments were euthanized by rapid decapitation, and the right hippocampi were removed and rapidly frozen at −80°C. The frozen tissues were cut into small pieces, homogenized in 0.5 ml of radio immunoprecipitation assay (RIPA) buffer [150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% deoxycholate, 0.1% sodium dodecylsulfate (SDS), 50 mM Tris-hydrochloric acid, and 2 mM phenylmethylsulfonyl fluoride pH 7.4], and incubated at 4°C overnight. The dissolved proteins were collected after centrifugation at 10000 *g* for 30 min, and the supernatant was then collected. Protein concentrations were determined using an enhanced bicinchoninic acid (BCA) protein assay kit. The proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane, the membrane was incubated with a primary antibody (Ab) against TNF-α (1:1500), phospho-transcription factors of the NF-κB p65 (p-NF-κB p65, 1:1500), APP

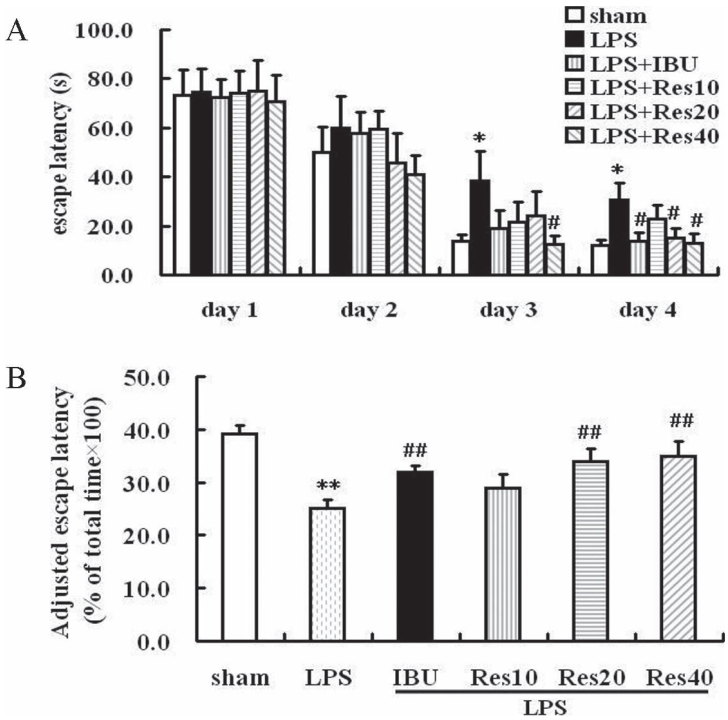

Fig. 1. Effect of Res on LPS-induced Cognitive Deficits

LPS (5 μg/5 μl) or NS was slowly injected by b.i.c.v., and then the rats were subjected to the Morris water maze test 5 days later. A) Mean escape latency in the navigation test; B) adjusted escape latency in the spatial exploring test. Data are presented as mean ± S.E., $n = 10$. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs*. sham; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs*. LPS alone.

(1:2000), and GAPDH (1:50000), and then incubated with the appropriate secondary horseradish peroxidase-conjugated anti-rabbit IgG antibodies (1:40000). The membranes were visualized using an electrochemiluminescence (ECL) system and then developed on Hyperfilm (Amersham). Immunoreactive proteins were visualized using an ECL Western blotting detection kit.

Statistical Analysis —— All values are presented as the mean and standard error. One-way analysis of variance was used to examine statistical comparisons between groups. The statistical significance of differences between two groups was determined using two-tailed Student's *t*-test. All analyses were performed using SPSS 12.0. $p < 0.05$ was considered as significant.

RESULTS

Effect of Res on LPS-induced Cognitive Deficits

LPS administration by b.i.c.v. was tolerated by the majority of rats, with less than 5% mortality. Initially, all the rats lost a few grams of body weight within 2 days after surgery. However, all of the rats had regained weight and continued to grow normally for the duration of the study. Five days after surgery, all of the rats underwent the 5-day Morris water maze test. The results indicated that the learning and memory abilities of LPS-treated rats were significantly impaired compared with the sham group. In the navigation test, the mean escape latency was tripled on the 3rd and 4th days ($p < 0.05$, Fig. 1A), while in the spatial exploring test, the adjusted escape latency was reduced by more than 35% ($p < 0.01$, Fig. 1B), indicative of cognitive impairment. However, LPS-induced learning and memory deficits were significantly alleviated by treatment with resveratrol at the dose of 40 mg/kg on the 3rd day ($p < 0.05$, Fig. 1A), and by treatment with Res at the doses of 20 and 40 mg/kg as well as IBU compared with the LPS group on the 4th day ($p < 0.05$, Fig. 1A). Meanwhile, the adjusted escape latency was significantly increased by treatment with Res at doses of 20 and 40 mg/kg as well as IBU compared with LPS alone ($p < 0.01$, Fig. 1B).

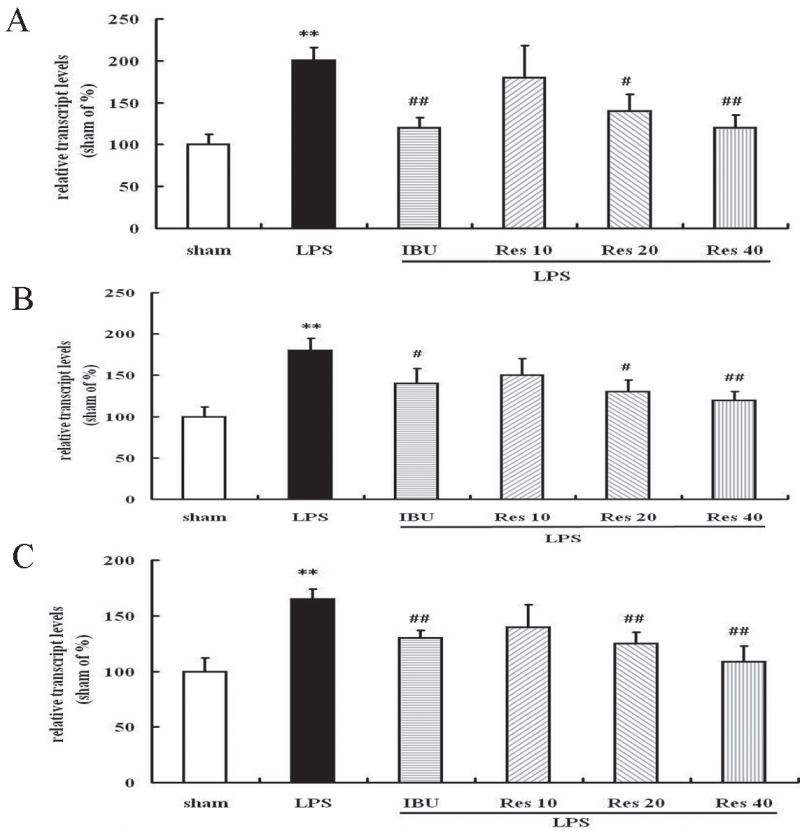

Fig. 2. Effect of Res on the Expressions of TNF-α, COX-2 and APP mRNA

The expressions of TNF-α, COX-2 and APP mRNA in hippocampus were detected by real time RT-PCR. A) Expression of TNF-α mRNA; B) expression of COX-2 mRNA; C) expression of APP mRNA. Data are mean ± S.E., $n = 4$. $^{**}p < 0.01$ *vs*. sham; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs*. LPS.

Effect of Res on the Expressions of TNF-α, COX-2 and APP mRNA

The expressions of TNF-α, COX-2 and APP mRNA in hippocampus were detected by real time RT-PCR. It was found that administration by b.i.c.v. significantly increased the expressions of TNF-α, COX-2 and APP mRNA; the values were 2.01, 1.80- and 1.65-fold compared with the sham group, respectively ($p < 0.01$, Fig. 2A–2C). However, compared with the LPS group, treatments with Res at doses of 20 and 40 mg/kg as well as IBU significantly decreased the mRNA expressions of these selected genes ($p < 0.05$, 0.01, Fig. 2A–2C).

Effect of Res on the Expressions of TNF-α, COX-2 and APP Proteins

To explore the mechanism of action of resveratrol on the attenuation of LPS-induced cognitive deficits, the expressions of TNF-α, COX-2 and APP proteins were detected by Western blot. Consistent with the mRNA expressions, LPS administration by b.i.c.v. significantly increased the expressions of TNF-α, COX-2 and APP proteins ($p < 0.01$, Fig. 3A–3D). However, after treatment with IBU or Res at the doses of 20 and 40 mg/kg, the TNF-α and COX-2 expressions were significantly decreased compared with the LPS group ($p < 0.05$, $p < 0.01$, Fig. 3A–3C). Likewise, after treatment with IBU or Res at 10–40 mg/kg, the APP expression was significantly decreased compared with the LPS group ($p < 0.05$, $p < 0.01$, Fig. 3A and 3D).

Effect of Res on the Phosphorylation of NF-κB

To further explore the mechanisms underlying the inhibitory effect of Res on the expressions of TNF-α, COX-2 and APP, phosphorylation of NF-κB p65 was analyzed by Western blot. NF-κB is inactive in the cytosol because it is bound to inhibitory κB (IκB), and becomes active after IκB has been phosphorylated and subsequently degraded. RelA/p65 is a subunit of the NF-κB transcription complex, which plays a crucial role in inflammatory and immune responses. We found that LPS administration by b.i.c.v. enhanced the phosphorylation of NF-κB p65 ($p < 0.01$, Fig. 3A and 3E). However, treatment with IBU or resveratrol at the

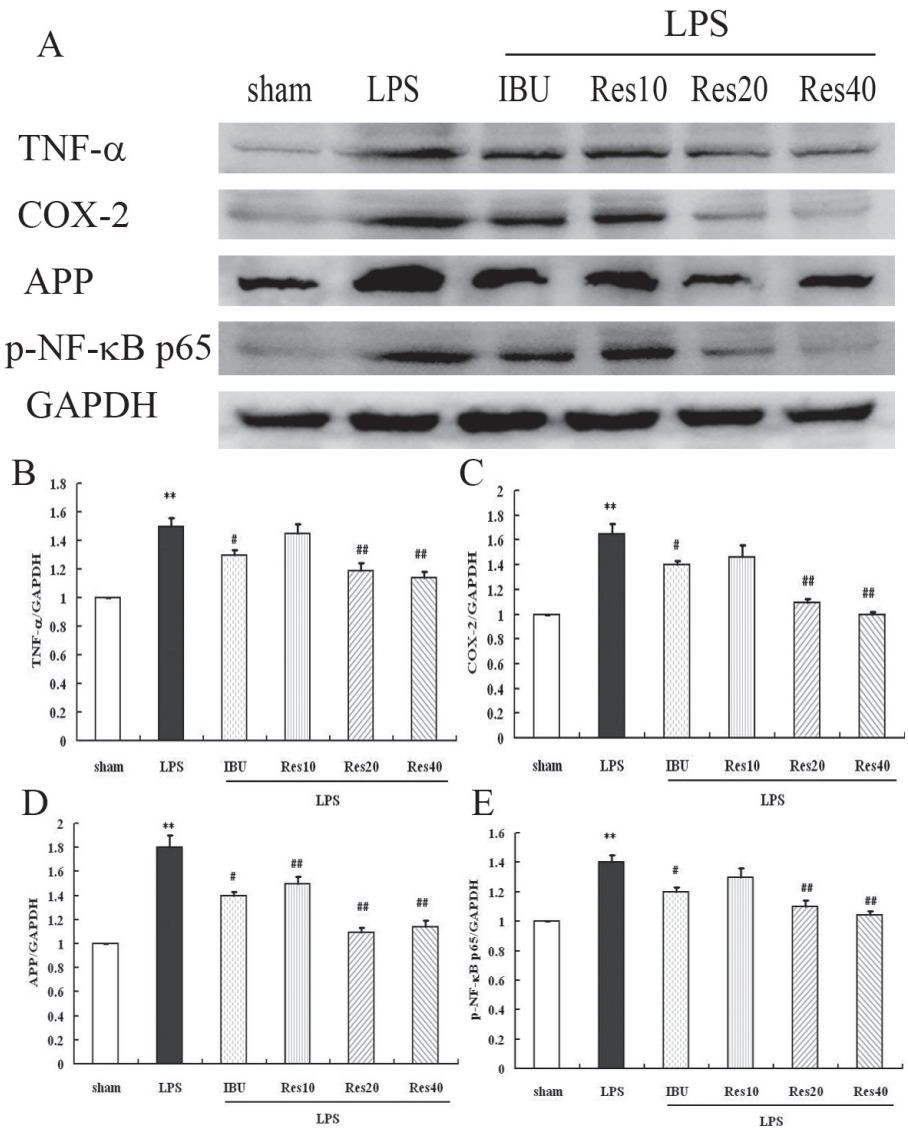

Fig. 3. Western Blotting Analysis of Relative Protein Content

A) Western blot of various protein contents. Protein contents were plotted for Sham, LPS, LPS + IBU, and LPS + various concentrations of Res. B) TNF-α, C) COX-2, D) APP proteins, and E) NF-κB p65 phosphorylation. The relative optical density was normalized to GAPDH. Data are mean ± S.E., $n = 4$. $^{**}p < 0.01$ *vs*. sham; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs*. LPS.

doses of 20 and 40 mg/kg was found to significantly decrease the LPS-induced phosphorylation of NF-κB p65 ($p < 0.01$, Fig. 3A and 3E). Taken together, these findings indicate that the inhibition of LPS-induced production of TNF-α, COX-2 and APP is mediated by suppressing LPS-induced activation of NF-κB.

DISCUSSION

In the present study, we have clearly demonstrated that LPS administration by b.i.c.v. significantly produced AD-like neuroinflammation, including cognitive deficits, the expressions of LPS-medicated TNF-α, COX-2 and APP mRNA and proteins, as well as the phosphorylation of NF-κB in the hippocampus; and Res significantly ameliorated cognitive deficits induced by LPS, and attenuated LPS-induced overexpressions of TNF-α, COX-2 and APP mRNA and proteins, as well as the phosphorylation of NF-κB in the hippocampus.

LPS, a bacterial endotoxin, is widely used to produce neuroinflammation, either by systemic injection, by intracentric microinjection or chronic infusion, or by incubation with brain cells.[3, 24, 25] The

susceptibility of different brain regions to LPS exposure varies greatly, with the substantia nigra being the most sensitive.[25] The substantia nigra has the highest density of microglia, and is highly vulnerable to LPS-induced neuroinflammation and subsequent loss of dopaminergic neurons.[25] LPS has been used as an inflammation tool to study PD.[26,27] However, LPS-induced neuroinflammation is long-lasting,[27] and hippocampal function can also be altered following acute or chronic LPS infusion into rat brain.[28,29] Learning and memory deficits occur when neuroinflammation affects hippocampal function,[28,29] even under conditions of no apparent neuron death in the hippocampus.[29] In early AD, the activated microglia is evident in hippocampus, indicating that LPS can also affect the hippocampus, and thereby contributing to the pathogenesis of AD.[6,28] The present study found that LPS administration by b.i.c.v. significantly produced cognitive deficits, but Res, which may cross the blood-brain barrier,[30] as well as IBU treatment significantly attenuated LPS-induced cognitive deficits, as evidenced by the Morris water maze test. Hence, resveratrol is effective against LPS-induced direct neuroinflammation via b.i.c.v. administration.

To explore the mechanism of action of Res on the attenuation of LPS-induced cognitive deficits, the expressions of TNF-α, COX-2 and APP proteins were detected by Western blot. As mentioned in previous similar report,[31] LPS administration by injection may cause an increase in the expressions of TNF-α and COX-2 mRNA and protein. However, the present study demonstrated that resveratrol significantly attenuated the expressions of TNF-α and COX-2 mRNA and protein. Thus, the suppression of TNF-α and COX-2 production by resveratrol might partially explain the anti-inflammatory action, and provide a mechanistic basis for resveratrol in the treatment of neuroinflammation-related disease.

The current study also revealed that administration by b.i.c.v. significantly enhanced the expressions of APP mRNA and protein, and Res treatments dramatically repressed LPS-induced APP production. The abnormal processing of APP by β and γ-secretase protease enzymes is a key event in the development of AD pathology, resulting in an increase in the generation of the 42 amino acid form of the Aβ peptide which aggregates to form the insoluble amyloid plaques.[32] The role of the inflammatory process has been underlined by the report that microglial cells derived from transgenic animals expressing the mutant form of the presenilin protein PS1 associated with the familial form of the disease are more susceptible to the actions of pro-inflammatory factors such as LPS, which also acts to potentiate the neurotoxic effects of the Aβ peptide.[33] In neuroinflammation, microglial scavenger function is impaired and reactivity against APP is enhanced as an initial step for neurodegeneration.[4] Hence, the inhibition of APP production by Res may result from its indirect action, and the exact mechanism needs to be further investigated.

To further explore the mechanisms underlying the inhibitory effect of Res on the expressions of TNF-α and COX-2, the phosphorylation of NF-κB p65 was also analyzed by Western blot. Among the several transcriptional factors activated by inflammatory responses during viral and bacterial infections, NF-κB is known to up-regulate the expressions of cytokines, chemokines, adhesion molecules, acute phase proteins, and inducible effector enzymes that are involved in innate immune responses.[34] NF-κB usually exists as a homodimer or heterodimer, and a heterodimer of p50 and one of Rel family p65 is known to mediate the expression of genes related to innate immune responses.[35] In unstimulated cells, NF-κB dimers are bound to IκBs, and as a result are retained in the cytoplasm. However, when the cells are stimulated with pro-inflammatory stimuli, such as LPS, IκBs are rapidly phosphorylated and degraded via IκB kinase complex, and the free NF-κB is translocated to the nucleus, where it binds to target sites and induces the transcriptions of pro-inflammatory mediators.[36] Therefore, it can be concluded that the free NF-κB is translocated to the nucleus, where it binds to target sites and induces the transcriptions of pro-inflammatory mediators, including TNF-α and COX-2. Thus, it is concluded that the inhibitions of resveratrol on the phosphorylation of the p65 subunit of NF-κB, and on subsequent transcriptions of TNF-α and COX-2 mRNA, are involved in its attenuation of LPS-induced cognitive deficits.

In summary, the present study evaluated the effect of resveratrol on LPS-induced direct neuroinflammation in rats. We also investigated the mechanisms of resveratrol inhibition of TNF-α, COX-2 and APP generation, and the related phosphorylation of NF-κB.

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