Inhibitory Effects of Dendrobium Alkaloids on Memory Impairment Induced by Lipopolysaccharide in Rats

Abstract

Dendrobium alkaloids (DNLA), extracted from Dendrobium nobile Lindl. whose botanical name is Dendrobium moniliforme, Orchidaceae family, were studied for their effect on lipopolysaccharide (LPS)-induced memory impairment in rats. SD rats were pretreated with DNLA (40, 80, 160 mg/kg/d for 7 d), followed by LPS (50 µg) injection into the right lateral ventricle to produce memory impairment. DNLA treatment continued for another 13 days. The spatial behavior was tested by the Morris water maze; the level of tumor necrosis factor receptor 1 (TNFR1) mRNA was detected by real time RT-PCR, and the protein level of TNFR1, nuclear factor kappa-B (NF-κB) and phosphorylated p38 mitogen-activated protein kinases (p-p38 MAPK) by Western blotting. The results showed that DNLA significantly improved the neurobehavioral performance and prevented LPS-induced elevation in TNFR1 mRNA and protein levels. LPS-induced activation of p38 MAPK and NF-κB pathway was also suppressed. In conclusion, DNLA is effective in protecting against LPS-induced brain impairment, and this effect is due, at least in part, to prevent overexpression of TNFR1 via inhibition of p-p38 MAPK and the downstream NF-κB signal pathway.

Abbreviations

AD: Alzheimer’s disease
Aβ: amyloid β peptide
DNLA: Dendrobium nobile Lindl. alkaloids
LPS: lipopolysaccharide
NF-κB: nuclear factor kappa-B
NSAIDs: anti-inflammatory drugs
p-p38 MAPK: phosphorylated p38 mitogen-activated protein kinases
TNFR1: tumor necrosis factor receptor 1

Introduction

Alzheimer’s disease (AD) is an age-related neurodegenerative disease characterized by the buildup of two aberrant protein aggregates in the brain, i.e., the amyloid plaques composed of amyloid β peptide (Aβ) and neurofibrillary tangles consisted of hyperphosphorylation tau protein [1]. AD brain also shows a number of pathological abnormalities including a profound loss of synapses, microglial activation, and inflammatory processes [2]. Aβ deposition is associated with microglial activation and triggers neuroinflammatory response, leading to the loss of neurons and the decline of cognitive functions [3]. Thus neuroinflammation is believed to play an important role in the pathogenesis and clinical symptoms of AD. Lipopolysaccharide (LPS), an inflammation inducer, has been reported to influence Aβ deposition [4], and LPS injection into the mouse brain ventricle caused memory deficiency and Aβ accumulation [5]. Neuroinflammation is characterized by microglia and astrocyte activation resulting in overexpression of inflammatory factors in the AD brain and is evident in AD transgenic animal models [6,7]. Epidemiological studies suggest that long-term treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) reduces the risk of AD, and protects against AD associated cognitive impairment [8–10]. However, long-term NSAID treatment causes many side effects, such as gastrointestinal distress and occasionally liver and kidney toxicity associated with inhibition of cyclooxygenase 1 [11, 12]. Thus, a search for alternative anti-inflammatory drugs for AD treatment is warranted. Dendrobium nobile (family Orchidaceae), a traditional Chinese herbal medicine, has been used as a tonic and a remedy for cataracts and throat in-
flammation. The major active ingredients are alkaloids, stilbenoids, glycosides, and polysaccharides [13]. Our previous researches indicated that Dendrobium alkaloids (DNLA) have protective effects against neuronal damages induced by LPS, oxygen-glucose deprivation, and reperfusion, resulting in decreases in neuron apoptosis and AP deposition in rat hippocampus [14, 15]. DNLA has high solubility in lipids and thus has the potential to penetrate the blood brain barrier. The goal of the current study was to further investigate the protective effects of DNLA on LPS-induced neuroinflammation in rats, focusing on cognitive impairment and the expression of neuroinflammation mediators, such as TNFR1, NF-κB and p-p38 MAPK in the rat hippocampus.

Materials and Methods

Animals and drugs
Male Sprague-Dawley (SD) rats, 250–300 g, were purchased from the Animal Center of the Third Military Medical University (Chongqing, China; certificate number SCKX 2002008). Ibuprofen was purchased from Tianjin SmithKline & French Laboratories, Ltd. (No. 07010371). One capsule contained 300 mg ibuprofen (purity ≥98%). The animal experimental protocol was approved by the Institutional Animal Use Committee, and all procedures were complied with Animal Care and Use Guidance in China.

Isolation of DNLA
DNLA was isolated from Dendrobium nobile Lindl. as previously described [14]. A voucher specimen (GZ-CAS189) was deposited in the Key Laboratory of Natural Products of Guizhou Province, Chinese Academy of Sciences. The procedure of isolation was as briefly described: the dried stems were soaked in MeOH for 2 days, followed by four extractions using boiling MeOH. The extracts were concentrated under reduced pressure. The concentrated extracts were extracted several times with HCl until a negative reaction with Mayer’s reagent was evident. The acidic extracts were then filtered, shaken thoroughly with ethyl ether to remove acidic and neutral substances and adjusted to pH 11.0 with ammonium hydroxide. The resultant solution was further extracted exhaustively using ethyl ether and dried over anhydrous Na₂SO₄, which mainly contained alkaloids, DNLA.

DNLA analysis
DNLA was dissolved in CH₂Cl₂ and applied to a GC apparatus (Agilent 6890 N) equipped with a hydrogen flame ionization detector. The conditions of capillary GC analysis were capillary column HP-5 (0.32 mm i.d. and 0.25 μm film thickness; Agilent), detector temperature 280°C, injector temperature 250°C, carrier gas helium (0.7 mL/min), and injection volume (1 μL). GC oven temperature was kept at 150°C for 5 min, programmed to 250°C at a rate of 7°C/min and kept constant at 250°C for 5 min. Relative percentage of alkaloids was determined using the area under peaks from corresponding standard ion chromatography, using Agilent software.

Morris water maze
Morris water maze is an open-field water-maze procedure in which animals learn to search for a hidden platform from opaque water and to memorize the location of the platform [16]. It is a widely used model in learning and memory studies [17, 18]. The water pool is circular (120 cm in diameter, 38 cm in height) and the clear Perspex Platform (12 cm in diameter, 28 cm in height) is placed 2 cm below the water, fixed in the middle of SE quadrant. The rats were gently placed in the pool from one of four quadrants (SW, NW, NE, and SE), and the placed position was altered on each test. The time taken to escape from water (escape latency) and the path crossing water (searching distance) were monitored by a digital camera and a computer system, and were used for the evaluation of spatial learning and memory functions. The rats were trained twice per day for 5 days before treatment. The water temperature was adjusted to 24 ± 1°C. Approximately 0.75 kg milk powder was dissolved in the pool daily to make the water opaque. The qualified rats were defined by the criteria that they reached the hidden platform between 5–50 s and remained on the platform for about 10 s.

Study design
The study design was a modification of the literature in which minocycline, administered 12 h before injection of LPS into the cerebrum, and the administration continued for several days, attenuated the LPS-induced brain injury and neurobehavioral performances [19]. In the present study, 42 qualified (60% of trained rats) were randomly divided into 6 groups (n = 7): control group (Control); LPS group (LPS); DNLA 40 mg/kg/d + LPS (DNLA-40); DNLA 80 mg/kg/d + LPS (DNLA-80); DNLA 160 mg/kg/d + LPS (DNLA-160); and ibuprofen 40 mg/kg/d + LPS (Ibuprofen). DNLA and ibuprofen were dissolved in distilled water in suspension by ultrasonic instruments. All groups were pretreated for 7 days by gavage, followed by injection of LPS (from Escherichia coli 055:B5; Sigma) into the right lateral ventricle; the control group was given the same volume of sterile normal saline. DNLA and ibuprofen treatments continued for another 13 days.

All rats were anesthetized with 350 mg/kg, i.p. chloral hydrate (Sinopharm Chemical Reagent Co., Ltd.) and secured in a stereotaxic apparatus (SR-6N). The skin on top of the skull was shaved and sterilized. A small hole was drilled in the right parietal bone, posterior to bregma ~0.8 mm, right lateral ~1.5 mm relative to midline, depth ~3.5 mm below dura [20]. LPS (50 μg in 5 μL sterile normal saline) or saline was injected into the right lateral cerebral ventricle via a stainless steel needle with a micro-injector. The injection lasted 5 min and the needle remained for another 5 min. At the 13th day of post-injection of LPS, the spatial memory was tested by the Morris water maze, and then all animals were anesthetized and decapitated. The right hippocampus was immediately dissected on an ice-plate and snap frozen in liquid nitrogen.

Real-time RT-PCR
Total RNA was extracted from the right hippocampi using Trizol reagent (Invitrogen) and purified with the RNeasy mini kit (Qiagen). Total RNA was dissolved in 30–50 μL DEPC water and adjusted to a final concentration of 50 ng/μL. Then total RNA was reverse transcribed with MuLV reverse transcriptase and Oligo-(dT) primers and subjected to real-time RT-PCR to quantify expression of TNFR1 using SYBR green DNA PCR Master Mix (Applied ABI Company). Primers were designed according to the GeneBank sequence. TNFR1 (NM_013091, sense 5′-GCCAGGATGCGTGGTAC-3′, antisense 5′-TCA CAC ACC TCG CAG ACT GTT TC-3′) and -actin (NM_031144, sense 5′-TGA CAG GAT GCA GAA GGA GA-3′, antisense 5′-TAG AGC CAC CAC CAA TTC ACA CA-3′). The expression was calculated using cycle time (Ct) values, and the TNFR1 expression was determined as relative to β-actin mRNA level, setting the control as 100%.
**Western blotting**

The hippocampi were homogenized in RIPA lysis buffer in the presence of protease inhibitor and phosphatase inhibitor mixtures and centrifuged at 20000 g for 15 min at 4°C. Supernatants were used for protein quantification by the BCA protein assay, and total protein (80 µg/20 µL) was loaded on SDS-PAGE gels. After electrophoresis, proteins were transferred to the PVDF membrane at a constant voltage of 35 V for 1 h. Blots were blocked with 5% nonfat dry milk in PBS buffer for 2 h at room temperature with constant shaking and then incubated with primary antibodies against NF-κB (1:600), TNFR1 (1:400), p-p38 MAPK (1:1000), and β-actin (1:600) in 5% nonfat dry milk in PBS buffer at 4°C overnight. All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz). After washing three times with PBST, blots were incubated with HRP-labeled goat anti-rabbit IgG (1:1000; Zhongshan Goldenbridge Biotechnology) at room temperature for 2 h with gentle rotation. After washing, blots were developed using enhanced chemiluminescence reagent BeyoECL Plus. The image was scanned, and band intensity was quantified using Quantity One software v4.52 (BioRad).

**Statistical analysis**

All results are expressed as mean ± standard error and were subjected to one-way ANOVA followed by Dunnett’s multiple-comparisons post hoc test with SPSS 13.0 software, with significance taken at p < 0.05.

**Results**

The alkaline solution extracted from the dried stems of *Dendrobium nobile* Lindl. contains alkaloids (96.1%) and polysaccharides (1.2%). Relative percentage of alkaloids was determined through GC analysis with dendrobine (C16H25O2N, 90.7%) as the main alkaloid, followed by nobilonine (C17H27O3N, 4.47%), dendramine (C16H25O3N, 2.31%), and 3-hydroxy-2-oxodendrobine (C16H23O4N, 1.29%). Chemical structures of these ingredients are shown in Fig. 1 [14].

To evaluate the spatial memory function of rats, the Morris water maze was performed 20 days after administration of DNLA and LPS. The escape latency and searching distance, two commonly used tests, were performed. All the performances were conducted twice and the average values were calculated for evaluation. The LPS group remarkably retarded the acquisition of the task, the time taken to find the hidden platform (escape latency for LPS: 30.2 s vs. control: 9.41 s), and the distance traveled for rats to reach the platform (searching distance for LPS: 775 cm vs. control: 259 cm) were significantly prolonged. DNLA treatment prevented the increased escape latency and searching distance produced by LPS in a dose-dependent manner (p < 0.05) (Fig. 2A, B). For example, at the dose of DNLA 80 mg/kg/d, the escape latency (DNLA: 11.3 s vs. LPS: 30.2 s) and searching distance (DNLA: 252 cm vs. LPS: 775 cm) were significantly reduced, respectively. The expression of TNFR1 in the hippocampus, a key receptor mediating LPS-induced neuroinflammation, was determined by real-time RT-PCR. LPS treatment resulted in 15-fold increases in TNFR1, which was significantly prevented by 70% with the treatment of DNLA 80 mg/kg/d, and by 90% with DNLA 160 mg/kg/d (Fig. 3). The effect of DNLA was comparable with that of ibuprofen (DNLA + LPS: 119; ibuprofen + LPS: 147 vs. LPS alone: 1540).

To further elucidate the mechanism of action by which DNLA protected against LPS-induced memory deficiency, we determined the protein expressions of TNFR1, NF-κB, and p-p38 MAPK with Western blotting. The protein expressions of TNFR1, NF-κB, and p-p38 MAPK of the hippocampus in LPS treated rats were obviously higher than those in the control group (LPS: 1.35, 1.37, 0.44 vs. control: 0.4, 0.35, 0.1, respectively). DNLA treat-
The inflammatory effects of ibuprofen and/or the Aβ-LPS [15,27], and this effect may be mediated through anti-inflammatory mechanisms. Previous studies showed that certain NSAIDs such as ibuprofen produced an anti-inflammatory effect against LPS-induced neuroinflammation and the produced beneficial effects on cognitive function. In the previous study, DNLA treatments significantly protected against LPS-induced cognitive deficits in rats, resulting in decreases in caspase 3 and caspase 8 mRNA expressions and Aβ, deposition in the hippocampus [15], indicating that DNLA has protective effects on LPS-induced rat brain damage. The present study further verified the protective effect of DNLA, focusing on neuroinflammation as a potential mechanism. The results clearly showed that DNLA was effective in preventing overproduction of TNFR1, possibly through inhibition of p-p38 MAPK and the downstream Aβ signal pathway.

Inflammation is now believed to play a fundamental role in the progression of neuropathological changes of AD [21]. Various stimuli including LPS can activate microglia to release toxic inflammatory factors, such as TNF-α, interleukin-6, and interleukin-1β [22], predominantly through activation of the NF-κB pathway. An overexpression of these mediators may trigger signaling cascades in neurons to cause neuropa-thology alternations, such as Aβ deposition, neurofibrillary tangle, inflammation, neuronal loss, synaptic loss, and neuronal dysfunction [23]. The LPS-induced rat neuroinflammation model is now widely used to evaluate the anti-inflammatory agents. To date, there are few reports about the effects of DNLA on this model. Therefore, the present study adopted the LPS-induced cognitive deficit model to define the relationship between anti-inflammatory effects of DNLA against LPS-induced neuroinflammation and the produced beneficial effects on cognitive function.

Classical targets of NSAIDs include cyclooxygenase, NF-κB, and peroxisome proliferator-activated receptors [24]. Previous findings showed that certain NSAIDs such as ibuprofen produced an anti-inflammatory effect through inhibition of NF-κB [25,26]; ibuprofen could also alleviate rat behavioral deficits induced by LPS [15,27], and this effect may be mediated through anti-inflammatory effects of ibuprofen and/or the Aβ-lowering properties of ibuprofen [23]. Therefore, we selected ibuprofen as the positive control in the present study and the results clearly showed that ibuprofen produced significant inhibition of TNFR1 overexpression, consistent with its anti-inflammatory actions.

Our research suggested that DNLA could reduce the overproduction of TNF-α in the cerebral cortex and hippocampus of LPS-treated rats (manuscript in preparation). TNFR1 triggers the NF-κB signaling pathway, and activated NF-κB can be translocated to the nucleus to regulate inflammatory processes that could exacerbate AD [28–30]. Therefore, the inhibition of the NF-κB signaling pathway by ibuprofen and DNLA could play an important role in their beneficial effects against LPS-induced neurodysfunction.

Another important inflammatory signaling is the activation of the p38 MAPK pathway, which is one of the important signal transduction pathways in the course of neuroinflammatory response [31]. Previous studies have shown that the expression of p-p38 MAPK in an AD brain was significantly increased, and the activation of p38 MAPK was limited in senile plaques and tangles fibers located in the hippocampus and cortical areas of an AD brain [32]. However, little is known about the effects of DNLA on p38 phosphorylation in the brain. This is the first report showing that DNLA is very effective to inhibit LPS-induced p-p38 activation. This finding is novel, and may add to our understanding of DNLA-induced anti-inflammatory effects in the rat hippocampus. In summary, the present study clearly demonstrated that DNLA treatment significantly protected the rat brain from LPS-induced neuroinflammation and cognitive dysfunction. This effect appears to be due, at least in part, to suppression of LPS-induced overexpression of hippocampus TNFR1, NF-κB, and p-p38 MAPK, thus providing a potential strategy for AD treatment.
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