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Delavatine A, an unusual isoquinoline alkaloid exerts anti-inflammation on LPS-induced proinflammatory cytokines production by suppressing NF-κB activation in BV-2 microglia

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ABSTRACT

Delavatine A, an unusual isoquinoline alkaloid isolated from *I. delavayi*, was first studied for anti-inflammatory effect using lipopolysaccharide (LPS)-induced BV-2 microglia. In the present study, we found that delavatine A substantially suppressed the LPS-induced pro-inflammatory mediators, nitric oxide (NO), and tumor necrosis factor-a (TNF-a), interleukin-6 (IL-6), interleukin-1β (IL-1β) in BV-2 microglial cells. These effects resulted from the inhibition of their regulatory genes inducible NO synthase (iNOS), cycloxygenase-2 (COX-2) and TNF-a, IL-6, IL-1β. In addition, we examined several pathways related to inflammation. The results revealed that delavatine A significantly decreased LPS-induced the activation of nuclear factor-κB (NF-κB) by suppressing the p65 subunits, and the phosphorylation of IκBα, while not related to PI3K/Akt or MAPK pathways.

1. Introduction

Damage to the brain, such as a range of acute and chronic neurological disorders, is associated with inflammation. Microglia are the major inflammatory cells in the central nervous system (CNS) and can be activated by a broad spectrum of stimuli such as lipopolysaccharide (LPS) and interferon-γ (IFN-γ) in response to chronic neuroinflammation [1,2]. Activation of microglial cells is associated with neuronal dysfunction as a result of pathological processes, injury, or general brain aging, which produce nitric oxide (NO), reactive oxygen species (ROS), and a variety of proinflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β) [3–5]. A number of previous reports have documented that inhibition of microglia activation may attenuate the neuronal death in the animal model of chronic neuroinflammation [3,6,7]. Thus, modulation of microglia activation is an important therapeutic target in the treatment of various neurodegenerative diseases.

NO is a signaling molecule that plays a key role in inflammation by providing an anti-inflammatory effect under normal physiological conditions [8]. During the inflammatory response, NO generated by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) plays an adverse role [9,10]. Therefore, inhibiting production of inflammatory mediators, such as iNOS and COX-2, may ameliorate many inflammatory diseases. TNF-α, IL-6 and IL-1β are multifunctional cytokines that play a key role in host defense, acute phase reactions and immune responses, and play vital roles in the immune system by mediating the induction of apoptosis and development of the humoral immune response [11–13].

It has been well demonstrated that NF-κB, PI3K/Akt and MAPK pathways are known to mediate inflammatory signals [14]. Moreover, activation of other signaling pathways, such as MAPK and PI3K/Akt, are also involved in activation of NF-kB [15]. Increasing reports showed that the activation of NF-κB has significant roles in the transcription of inflammation-associated genes followed by production and secretion of pro-inflammatory or neurotoxic factors such as TNF-α, IL-6, IL-1β, NO and ROS, which simultaneously affect the surrounding neurons and cause neuronal damage [16,17]. Otherwise, NF-κB is activated via inflammatory stimuli, such as bacterial LPS, resulting in a decrease in IκB and an increase in the

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translocation of NF-κB into the nucleus [18,19]. Inflammatory stimuli ultimately lead to phosphorylation of the IKK complex, which composed of a regulatory subunit (IKKγ, also known as NF-κB essential modifier - NEMO) and two similar subunits (IKKα and IKKβ). Typically, the IKK complex is activated by phosphorylation of IKKβ, which in turn phosphorylates IκBα. Phosphorylated IκBα is degraded by the proteasome pathway. The resulting liberated NF-κB such as p50/p65 dimer translocates into the nucleus to regulate the expression of almost 400 different genes including the inflammatory target genes [16,20].

Incarvillea species (Bignoniaceae) are flowering plants growing at high altitudes, which have been used in traditional Chinese herbal medicine for centuries. *Incarvillea delavayi* Bureau et Franchet is native to Yunnan and Sichuan provinces in Southwest China. In Chinese herbal medicine, the roots of *I. delavayi* are commonly used to treat dizziness and anemia and stimulate lactation. A previous study of our group reported delavatine A (named 014 in figures), an unusual isoquinoline alkaloid isolated from *I. delavayi* [21] as well as its weak cytotoxicity against several cancer cell lines.

In the present study, we investigated the effect of delavatine A on the NO production in LPS-stimulated microglia BV-2 cells and examined the effect of delavatine A on the expression of iNOS and COX-2 as well as the proinflammatory cytokines TNF-α, IL-6, IL-1β. In addition, we also analyzed the underling molecular mechanisms by NF-κB, PI3K/Akt and MAPK pathways. Finally, we found that NF-κB pathway through IκBα and NF-κB p65 proteins is the effective pathway.

2. Materials and methods

2.1. Cell culture and treatment

The BV-2 microglia and SH-SY5Y neuroblastoma cells were kindly provided by Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). Respectively, BV-2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, meanwhile, SH-SY5Y cells were maintained in DMEM/F12 (1:1) supplemented with 10% FBS. Cells with medium also containing 100 U/mL penicillin, and 100 U/mL streptomycin were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cells were plated on 100 mm plastic tissue culture dishes (Corning, NY) at a density of 1.2×10^6 cells per dish, and the dishes were incubated until 80% confluence was reached.

Delavatine A was dissolved in dimethyl sulfoxide (DMSO) and freshly prepared each time before use. BV-2 cells and SH-SY5Y cells were separately plated in cell culture plates at a density of 8×10^4 cells/well and 1×10^5 cells/well for 96-well, or 2×10^5 cells/well and 3.5×10^5 cells/well for 6-well and incubated for 12 h before treatment. Then the BV-2 cells were incubated with LPS (1 μg/mL, Sigma, USA) and delavatine A with various concentrations (1, 5, 10 μM) for an additional 24 h, with which pretreated for 1 h before LPS.

2.2. Cell viability analysis using CCK-8 method

The cell viability was assessed by Cell Counting Kit-8 (CCK-8, dojindo, Japan) according to the manufacturer's protocol. Briefly, after treatment, tetrazolium salt-8 (WST-8) solution was added to cultured cells in 96-well plate (10 μL/well), followed by incubation at 37 °C for 2 h. The absorbance (optical density, OD) was measured by spectrophotometry at 450 nm with an ELx-800 microplate reader (Bio-Tek Inc., Winooski, VT).

2.3. Measurement of nitrite production using Griess Reagent System

NO production in the culture supernatant was spectrophotometrically evaluated by measuring nitrite, an oxidative product of NO. Nitrite level was determined with the Griess Reagent System (Beyotime Institute of Biotechnology, Nantong, China) according to the manufacturer's protocol. Briefly, sulfanilamide solution (Griess Reagent I) and N-(1-Naphthyl) ethylenediamine (NED, Griess Reagent II) solution were incubated at room temperature for 15–30 min. Then, 50 μL of each experimental sample and nitrite standard was added to the wells, and 50 μL of the Griess Reagent I was added to all the wells containing either the experimental samples or a dilution series for the nitrite standard reference curve. Next, 50 μL of the Griess Reagent II solution was added to all of the wells and the cells were incubated at room temperature for 5–10 min protected from the light. The fluorescent intensity was then measured using a spectrophotometry at 540 nm with an ELx-800 microplate reader.

2.4. Cytokine determination by enzyme-linked immunosorbent assay (ELISA)

BV-2 microglial cells were pre-stimulated with LPS for 1 h and treated with delavatine A (1, 5, 10 μM) for an additional 12 h or 24 h. The culture supernatants of the cells were collected and the concentrations of TNF-α, and IL-6 were measured by ELISA using monoclonal antibodies and the procedure recommended by the ELISA Ready-SET-Go! (Thermo Fisher Scientific, USA). IL-1β was measured 24 h after LPS treatment. The samples were assessed in triplicate relative to the standards supplied by the manufacturer. And fluorescent intensity was measured using a spectrophotometry at 450 nm with an ELx-800 microplate reader.

2.5. RNA preparation and real-time reverse transcription quantitative polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from cells using the Trizol reagent (Thermo Fisher, USA), according to the manufacturer's instructions. RNA was reversely transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, USA). The cDNA was synthesized from 1 μg mRNA via a 20 μL reverse transcription reaction containing 10 U of ImProm II TM-Reverse Transcriptase, 1 U RNasin ribonuclease inhibitor, 0.5 mM of dNTP mixture, and 2 μM random primer, and incubated in the SimpliAmp™ Thermal Cycler (Life technology, USA). The samples were incubated at 25 °C for 10 min, 37 °C for 120 min, and at 85 °C for 5 min, and then the cDNA was used or immediately stored at −20 °C. The following primers were used for PCR: for TNF-α gene (Forward 5′ -AGGCACTCCCCCAAAAGAT - 3′; and Reverse 5′ -CAGTAGACAGAAGAGCGTGGTG - 3′), for IL-6 gene (Forward 5′ -CGGAGAGGAGACTTCACAGAG - 3′; Reverse 5′ -ATTTCCACGATTTCCCAGAG - 3′), for IL-1β gene (Forward 5′ - GCTGCTTCCAAACCTTTGAC - 3′; Reverse 5′ -AGCTTCTCCACAGCCACAAT - 3′), and for GAPDH gene (Forward 5′ -ATCTTCTTGTGCAGTGCCAGCCTC - 3′; Reverse 5′ -TTTGCCACTGCAAATGGCAGCC - 3′).

The RT-qPCR was carried in a 10 μL final volume containing 1 μL cDNA sample, 0.5 μL primer pairs, 3.5 μL ultra-pure distilled water, and 5 μL SYBR Green PCR Master Mix (Thermo Fisher, USA), and performed by an initial denaturation step at 50 °C for 1 min and 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, and finally 60 °C for 1 min in the Step-One™ Real-time PCR System (Applied Biosystems, USA). Each RNA sample was measured in triplicate. The

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