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Anti-neuro-inflammatory effects of *Nardostachys chinensis* in lipopolysaccharide-and lipoteichoic acid-stimulated microglial cells

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[ABSTRACT] Excessive microglial cell activation is related to the progression of chronic neuro-inflammatory disorders. Heme oxygenase-1 (HO-1) expression mediated by the NFE2-related factor (Nrf-2) pathway is a key regulator of neuro-inflammation. *Nardostachys chinensis* is used as an anti-malarial, anti-nociceptive, and neurotrophic treatment in traditional Asian medicines. In the present study, we examined the effects of an ethyl acetate extract of *N. chinensis* (EN) on the anti-neuro-inflammatory effects mediated by HO-1 up-regulation in *Salmonella* lipopolysaccharide (LPS)- or *Staphylococcus aureus* lipoteichoic acid (LTA)-stimulated BV2 microglial cells. Our results indicated that EN suppressed pro-inflammatory cytokine production and induced HO-1 transcription and translation through Nrf-2/antioxidant response element (ARE) signaling. EN markedly inhibited LPS- and LTA-induced activation of nuclear factor-kappa B (NF-κB) as well as phosphorylation of mitogen-activated protein kinases (MAPKs) and signal transducer and activator of transcription (STAT). Furthermore, EN protected hippocampal HT22 cells from indirect neuronal toxicity mediated by LPS- and LTA-treated microglial cells. These results suggested that EN impairs LPS- and LTA-induced neuro-inflammatory responses in microglial cells and confers protection against indirect neuronal damage to HT22 cells. In conclusion, our findings indicate that EN could be used as a natural anti-neuro-inflammatory and neuroprotective agent.

[KEY WORDS] *Nardostachys chinensis*; Anti-inflammation; Lipopolysaccharide; Lipoteichoic acid; Heme oxygenase-1

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Introduction

Microglial cells are specialized macrophages of the central nervous system (CNS) that play a crucial role in immunological defense against virulence factors [1-2]. Specific forms of stimulation can activate microglial cells, causing them to secrete various inflammatory cytokines [3]. In addition, activation of macrophages leads to phagocytosis of damaged neuronal cells, which protects neuronal tissue and prevents damage to the brain [4]. Therefore, microglial cell activation is important for proper brain development and repair of injured sites of the brain. However, under some disease conditions, microglial cells become continuously over-activated and neurotoxic by releasing cytotoxic agents, including nitric oxide (NO), reactive oxygen species (ROS), pro-inflammatory mediators, and cytokines, resulting in chronic neuronal inflammation [3].

During microglia-induced chronic neuro-inflammation, intracellular responsive cascades such as the nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) become activated, increasing expression of pro-inflammatory products such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and cyclooxygenase-2 (COX-2), as well as genes responsible for regulating cell survival and growth [5-7]. Additionally, the p38 MAPK pathway is associated with several inflammatory diseases such as rheumatoid arthritis, Alzheimer's disease, and inflammatory bowel disease [7]. Another important

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transcription factor involved in inflammatory cytokine induction is the Janus kinase (JAK)-signal transducer, which is associated with the signal transducer and activator of transcription (STAT) signal pathway [8]. The binding of extracellular cytokines to a specific receptor activates the JAK cascade [9]. In turn, JAK phosphorylates the cytoplasmic domain of the receptor, enabling the binding of STATs [10]. Next, the STATs are phosphorylated and form a dimer complex, which is followed by their translocation into the nucleus. The STATs bind to specific DNA sequences to promote the expression of target genes, including several pro-inflammatory cytokines and transcription factors [11].

Therefore, the transcription factor NF-E2-related factor (Nrf)-2/antioxidant response element (ARE) signal pathways are thought to be the central modulators of anti-inflammation and neuroprotection [12]. Under normal conditions, Nrf-2 activation is suppressed by the Keap1 protein dimer, which acts as an adaptor molecule of the Cul3 E3 ligase complex. This suppression leads to the degradation of Nrf-2 [13]. Following Nrf-2 degradation, free Nrf-2 translocates to the nucleus and binds to the ARE sequence located in the promoter region of the oxidative stress response genes NAD (P)H quinone oxidoreductase (NQO)1 and heme oxygenase (HO)-1 [14]. In the field of neuro-inflammation research, the HO-1 gene has been intensely studied for its potential neuroprotective and anti-neuro-inflammatory effects [14]. HO-1 has been suggested as a potential therapeutic target for treating many neuro-inflammatory diseases [14]. The HO-1 gene contains an ARE consensus sequence that responds to Nrf-2 and oxidative and nitrosative stressors such as hypoxia, cytokines, NO, heat shock, and hydrogen peroxide. Following stimulation, HO-1 exerts its anti-neuro-inflammatory effects through the rate-limiting catabolic activity of the toxic free heme [15-16].

The bacterial components LPS and LTA are the major antigenics against innate immune cells such as monocytes and macrophages [17]. LPS is a part of the outer cell membrane of Gram-negative bacteria, while LTA is the smallest bioactive fragment of peptidoglycan (PGN) from the membrane of Gram-positive bacteria [18]. Several types of Toll-like receptors are present on the surface of immune cells and function in the recognition of various bacterial products, including LPS and LTA, which leads to activation of cellular signaling pathways that are involved in cellular defense [19]. There are few reports on LTA than LPS, but both substances trigger activation of immune cells and promote a diverse array of inflammatory responses by inducing the release of various pro-inflammatory cytokines and mediators, including TNF-α, IL-1β, IL-6, and NO (RF). Accumulating evidence suggests that LPS and LTA are recognized by TLR4 and TLR2, respectively (RF). Modulation of cellular signaling pathways in response to either LPS or LTA has been examined, but few reports have compared the effects of these bacterial products [18-19].

The dried root and rhizome of *Nardostachys chinensis* Batal and its relative *Nardostachys jatamansi* DC, commonly known as *Gansongxiang* or *Gansong*, are used in traditional Asian medicine for their sedative, analgesic, antipyretic, antihyperlipidemic, and antihypertensive effects [20]. *N. chinensis* and *N. jatamansi* are rich in guaiane-, aristolane-, and nardosinane-type sesquiterpenoids such as lignan and neolignan, which exert anti-malarial, anti-nociceptive, and neurotrophic activities [26]. Recently, it has been reported that *N. jatamansi* inhibits LPS-induced production of inflammatory factors such as IL-1b, IL-6, TNF-*a*, and IFN-α/β. Several studies have demonstrated the anti-stress and anti-oxidant activities of *N. jatamansi* in models of neurological disorders, mainly *in vivo* [20-21]. However, the anti-oxidant and anti-inflammatory activities of *N. chinensis* are not well established in the context of neurological disorders in comparison with those of *N. jatanamsi*. A recent report has indicated that the constituents of *N. chinensis* inhibit nitric oxide production in RAW 264.7 macrophages [21]. In the present study, we tested the anti-inflammatory effects of *N. Chinesis* in the context of neuro-protection and assessed its molecular mechanisms in microglial cells activated by components of Gram-negative and Gram-positive bacterial cell membranes.

Material and Methods

Reagents and chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Protoporphyrin IX (SnPP) and antibodies against inducible nitric oxide synthase (iNOS), COX-2, HO-1, Nrf-2, NF-κB, extracellular-signal-regulated kinases (ERK), JNK, p38, IκB-α, STAT1, κSTAT3, p-STAT1, p-STAT3, and TATA-binding protein (TBP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipopolysaccharide (LPS, phenol extract of *Salmonella typhimurium*) and lipoteichoic acid (LTA, extract of *Staphylococcus aureus*) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Fractionation of Nardostachys chinensis extract

Dried *Nardostachys chinensis* was purchased from the Pyunghwa Pharmaceutical Factory (Seoul, Korea), who obtained their supply from the local drug market in Xi'an City, China. *N. chinensis* was identified and authenticated based on its microscopic and macroscopic characteristics by Professor Woo Shin Ko, a specialist in traditional oriental herbal medicine at the College of Oriental Medicine, Dongeui University (Busan, Korea). A voucher specimen (number AS-05-04) was deposited at the Department of Molecular Biology, Pusan National University, Busan, Korea. An *N. chinensis* extract was obtained using the following method: approximately 500 g of *N. chinensis* was homogenized, added to 1 L of distilled water, and boiled for 150 min at 100 °C. The resultant solution was filtered through Whatman filter paper (GF/F) to remove the insoluble materials and the supernatants were centrifuged (4 000 r·min^{-1}, 20 min), evaporated using a rotary evaporator, and diluted with 3