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Nauclea officinalis inhibits inflammation in LPS-mediated RAW 264.7 macrophages by suppressing the NF-κB signaling pathway

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Chemical compounds studied in this article::
Pumiloside (PubChem CID: 10346314)
3-epi-pumiloside (CAS no: 126722-26-7)
Strictosamide (PubChem CID: 11969629)
Vincosamide (PubChem CID: 44567197)
DMSO (PubChem CID: 679)
$NaNO_2$ (PubChem CID: 23668193)
EtOH (PubChem CID: 702)
Acetonitrile (PubChem CID: 6342)
Methanoic acid (PubChem CID: 284)
3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide

ABSTRACT

Ethnopharmacological relevance: *Nauclea officinalis* has been traditionally used in China for the treatment of fever, pneumonia and enteritidis etc. This study aims to investigate effects of *N. officinalis* on the inflammatory response as well as the possible molecular mechanism in LPS-stimulated RAW 264.7 murine macrophage cells.

Materials and methods: Anti-inflammatory activity of *N. officinalis* (10, 20, 50, and 100 μg/mL) was investigated by using LPS-induced RAW 264.7 macrophages. The NO production was determined by assaying nitrite in culture supernatants with the Griess reagent. The levels of TNF-α, IL-6 and IL-1β in culture media were measured with ELISA kits. Real time fluorescence quantitative PCR was detected for mRNA expression of iNOS, TNF-α, IL-6 and IL-1β. Western blot assay was performed to illustrate the inhibitory effects of *N. officinalis* on phosphorylation of IκB-α and NF-κB p65.

Results: Treatment with *N. officinalis* (10–100 μg/mL) dose-dependently inhibited the production as well as mRNA expression of NO, TNF-α, IL-6 and IL-1β in RAW 264.7 macrophages. Western blot assay suggested that the mechanism of the anti-inflammatory effect was associated with the inhibition of phosphorylation of IκB-α and NF-κB p65.

Conclusions: The results indicated that *N. officinalis* potentially inhibited the activation of upstream mediator NF-κB signaling pathway via suppressing phosphorylation of IκB-α and NF-κB p65 to inhibit LPS-stimulated inflammation.

1. Introduction

Inflammation is an important part of the complex biological interactions that arise in any tissue in the response to bacterial, chemicals or physical injury (Song et al., 2014). It is well known that many systemic diseases generate and develop along with serious inflammatory reaction including inflammatory bowel disease, atherosclerosis multiple sclerosis, hyperlipidemia and bacterial pneumonia (Cho et al., 2014). The inflammatory process is normally elicited by numerous stimuli such as physical, noxious chemical stimuli or microbiological toxins (Shin et al., 2010), which leads to production of various molecules. Among them, the nitric oxide (NO) and various pro-inflammatory cytokines including tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) plays a crucial role (Xu et al., 2014; Nguyen et al., 2015). Excessive production of these inflammatory mediators and cytokines cause inflammatory activities, tissue necrosis, even inflammatory diseases.

Nuclear factor-kappa B (NF-κB) represents an essential family of eukaryotic transcription factors which are involved in regulating the expression of numerous genes in immune response and cell growth (Xu et al., 2003). Extensive studies have shown that phosphorylation and degradation of IκB-α can activate the downstream NF-κB signaling pathway via p65 translocation into the nucleus to change the expression of related genes in response

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to LPS stimulation (Kwon et al., 2014; Fan et al., 2015). Overproduction of TNF-α, IL-1β and IL-6, which are regulated by NF-κB signaling pathway, aggravates the early immune response and inflammatory reaction after NF-κB activation. Therefore, restraining NF-κB activation has potential application in reducing inflammatory status in organism (Bocchini et al., 1992).

Nauclea officinalis, one of the commonly used traditional medicine in China (Wang et al., 2012a, 2012b), is the only species of genus *Nauclea* in China (Sun et al., 2007). It is widely used for the treatment of cold, fever, throat swelling, pink eyes, etc (Chen et al., 2014a, 2014b). It is reported that *N. officinalis* exhibits various biological properties such as antimalarial (Kahunu et al., 2010), antibacterial (Hu et al., 2009) and anti-inflammatory effect (Fu et al., 2002). The in vivo anti-inflammatory activity indicates that *N. officinalis* can inhibit early symptoms of acute inflammation such as seepage and swelling (Fu et al., 2002). However, up to now no more detail regarding the inhibitory effects of *N. officinalis* on the inflammatory response has been reported and the molecular mechanism by which *N. officinalis* enhances pro-inflammatory cytokine production remains unclear.

In this paper, the production and relative mRNA expression of NO, iNOS, TNF-α, IL-6 and IL-1β in cell supernatant were determined. Besides, the phosphorylation of IκBα and p65 was also determined to explore the possible anti-inflammatory molecular mechanism.

2. Materials and methods

2.1. Chemicals and reagents

N. officinalis (110101) was provided by Hainan Pharmaceutical Factory Co., Ltd. (Wuzhishan, China). Pumiloside, 3-epi-pumiloside, strictosamide and vincosamide (HPLC ≥ 98%) were separated from *N. officinalis* and their chemical structures were analyzed by MS, ^{1}H- and ^{13}C-NMR (Zhu et al., 2013). Lipopolysaccharide (LPS), Griess reagent and dimethyl sulfoxide (DMSO) were obtained from Sigma (California, USA). MTT and $NaNO_2$ were obtained from Nanjing Naoao Science and Technology Co., Ltd. (Nanjing, China). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and trypsin were obtained from Nanjing KeyGEN Biotech. Co., Ltd. (Nanjing, China) and the Trizol reagent was purchased from Invitrogen (California, USA). Mouse TNF-α, IL-1β and IL-6 ELISA kits were acquried from MultiSciences Biotech. Co. Ltd. (Hangzhou, China). Mouse monoclonal TNF-α antibody, IL-1β antibody, IL-6 antibody, β-acitn antibody, mouse monoclonal phospho-NF-κB p65 antibody and mouse polyclonal IκBα antibody were obtained from Cell Signaling Technology (Massachusetts, USA). RAW 264.7 mouse macrophage cells were purchased from Shanghai cell bank, Chinese academy of sciences (Shanghai, China). All other chemicals were of reagent grade.

The sample of *N. officinalis* (50 g) was extracted under reflux in boiling 80% EtOH (1000 mL) for 2 h and the extraction was repeated three times. After solvent removal, 80% EtOH crude extract (2.92 g) was obtained.

2.2. UPLC-PDA analysis

UPLC-PDA (Massachusetts, USA) was used to analyze the *N. officinalis* extract and a standard solution including pumiloside, 3-epi-pumiloside, strictosamide and vincosamide. Waters Acquity BEH C_{18} column (2.1 mm × 100 mm, 1.7 μm) was eluted with the mobile phases of acetonitrile (A) and methanoic acid/water (1:1000, v/v) (B) in gradient mode. The flow rate was 0.4 mL/min with the following gradient program: 0–0.5 min, 2% A; 0.5–6 min, 2–10% A; 6–20 min, 10–33% A. The column temperature was 40 °C. The injection volume was 0.3 μL. The detected wavelength was set at 245 nm for pumiloside and 3-epi-pumiloside, 226 nm for strictosamide and vincosamide. The concentrations of four compounds in the exact were calculated with reference to standard curve of the corresponding compound.

2.3. Cell culture

RAW 264.7 cells were cultured in DMEM which was supplemented with 10% FBS, 100 EU/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Experiments performed in *N. officinalis* were repeated three times independently.

2.4. MTT assay for cell viability

RAW 264.7 cells were seeded in 96-well plates at a density of 4×10^6 cells/mL and incubated for 24 h. The cells were then treated with 0.05% DMSO, or *Nauclea officinalis* (10, 20, 50, 100, 200, and 500 μg/mL) in the absence or presence of 1 μg/mL LPS for 24 h. MTT reagent (5 mg/mL) which was dissolved in PBS was added to each well. After incubation at 37 °C for 4 h, the culture medium was discarded and then 150 μL of DMSO was added to dissolve the crystals. Optical density was measured at 570 nm using a microplate reader.

2.5. NO assay

RAW 264.7 cells (4×10^6 cells/mL) were plated in 96-well plates and subsequently treated with or without LPS (1 μg/mL) in the presence of different concentrations of *N. officinalis* (0, 10, 20, 50, and 100 μg/mL) for 24 h. Each culture supernatant (100 μL) was mixed with Griess reagent (50 μL) for 10 min at room temperature. The absorbance values were detected at 550 nm. The NO production was determined with reference to standard curve of sodium nitrite.

2.6. Enzyme-linked immunosorbent assay

RAW 264.7 cells were divided into six groups randomly and plated at a density of 4×10^6 cells/mL in 96-well plates. The control group was only treated with DMEM which contained 0.05% DMSO and the other five groups were subsequently treated with LPS (1 μg/mL) in the presence of various concentrations of *N. officinalis* (0, 10, 20, 50, and 100 μg/mL) for 24 h. The assay was performed according to the manufacturer's instruction. The OD of the microplate was read at 570 nm.

2.7. Real time fluorescence quantitative PCR

RAW 264.7 cells (4×10^6 cells/mL) were treated with 0.05% DMSO (control), or *N. officinalis* (0, 10, 20, 50, and 100 μg/mL) in the absence or presence of 1 μg/mL LPS for 24 h. Total RNA was isolated using Trizol. The quantity of total RNA were determined at 260 nm and 280 nm. cDNA was generated using the 1st strand cDNA synthesis Kit. cDNA was used as a template for real-time PCR in triplicates with Fast SYBR Green Master Mix (TOYOBO, Japan) and gene-specific primers. PCR was performed for 40 cycles in 20 μL reaction volumes by real-time qPCR using the DA7600 Real-Time PCR System. GAPDH was used as the internal control for normalization. The upstream and downstream primer sequences were as follows: for iNOS: sense primer: 5-AGCAACTACTGCTGGTGGTG-3, antisense primer: 5-TCTTCAGAGTCTGCCCATTG-3; for TNF-α: sense primer: 5-ATGAGAAGTTCCCAAATGGC-3, antisenseprimer: 5-CTCCACTTGGTGGTTTGCTA-3; for IL-1β: sense primer: 5-GAAGAAGAGCCCATCCTCTG-3, antisenseprimer: 5-TCATCTCGGAGCCTGTAGTG-3; for IL-6: sense primer: