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Anti-inflammatory action of *Athyrium multidentatum* extract suppresses the LPS-induced TLR4 signaling pathway

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ABSTRACT

Ethnopharmacological relevance: The aerial part of *Athyrium multidentatum* (Doll.) Ching (AM) is widely used in the northeastern region of China as an edible wild herb, but its medicinal value, especially its anti-inflammatory effect, has not been fully explored.
Aim of the study: To investigate the anti-inflammatory activity of AM and clarify the anti-inflammatory mechanism involving the TLR4 signaling pathway using a lipopolysaccharide (LPS)-induced inflammatory model.
Materials and methods: AM ethanol extract was used as the experimental material to investigate the effect that the extract has on the production of pro-inflammatory mediators (NO, PGE_2, TNF-α, IL-1β and IL-6); changes in LPS-induced peritoneal macrophages (PMs); and TLR4-mediated intracellular events, including MAPKs (ERK, JNK, and p38) and IκB-α in the MyD88-dependant pathway and IRF3, STAT1, and STAT3 in the TRIF-dependent pathway. In *in vivo* experiments, we established an LPS-induced acute lung injury (ALI) model and investigated the cell count and cytokine (TNF-α, IL-1β and IL-6) levels in bronchoalvelar lavage fluid (BALF) of C57BL6 mice. Histological changes in the lung tissues were observed with H&E staining.
Results: AM extract inhibited NO and PGE_2 by suppressing their synthetase (iNOS and COX-2) gene expression in LPS-induced PMs; the secretion of IL-6, IL-1β, and TNF-α also deceased *via* the down-regulation of mRNA levels. Furthermore, the TLR4-mediated intracellular events involved the phosphorylated forms of MAPKs (ERK, JNK) and IκB-α in the MyD88-dependent pathway and the TRIF-dependent pathway (IRF3, STAT1, STAT3), and the relevant proteins were expressed at low levels in the AM extract groups. In *in vivo* experiments, the cell count and cytokine (TNF-α, IL-1β and IL-6) levels in BALF decreased significantly in a dose-dependent manner in the AM extract groups. The lung tissue structure exhibited dramatic damage in the LPS group, and the damaged area decreased in the AM extract groups; in particular, the effect of 10 mg/kg extract was similar to that of the positive control dexamethasone (DEX).
Conclusion: The findings demonstrate that AM protects against LPS-induced acute lung injury by suppressing TLR4 signaling, provide scientific evidence to support further study of the safety of anti-inflammatory drugs and indicate that AM can be used as an anti-inflammatory and anti-injury agent to prevent pneumonia caused by microbial infection.

1. Introduction

Inflammation is a common and important defense immune response. In general, appropriate inflammatory feedback benefits the body; however, excessive inflammation can lead to kind of diseases such as pulmonary fibrosis (Coker and Laurent, 1998), arthritis (Manzi et al., 2000), pulmonary fibrosis (Coker and Laurent, 1998), and asthma (Kawai et al., 2001). After stimulation with LPS, viruses, and bacterial

Abbreviations: LPS, lipopolysaccharide; ALI, acute lung injury; BALF, bronchoalvelar lavage fluid; NO, nitric oxide; PGE_2, prostaglandin E_2; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α; IL, interleukin; IFN, interferons; qPCR, quantitative PCR; HPLC, high performance liquid chromatography; ELISA, enzyme linked immunosorbent assay; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa B; IκB-α, NF-κB inhibitor alpha; DEX, dexamethasone

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endotoxin, monocytes can differentiate to macrophages at the infection site and release inflammatory factors, such as nitric oxide (NO), prostaglandin E_2 (PGE_2) and other cytokines (Mosser and Edwards, 2008). Therefore, the vital importance treatment of inflammatory diseases is to regulating the levels of inflammatory factors. Toll-like receptor 4 (TLR4) is a cell-membrane receptor for LPS in macrophage signaling pathways. After recognizing LPS, TLR4 can activate different signaling pathways, including MyD88- and TRIF-dependent pathways (Itoh et al., 2003). TLR4 activation by LPS induces the secretion of pro-inflammatory factors in the MyD88-dependent pathway, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-12, and IL-1β. Type I interferons (IFNs) are released in addition to TNF-α, IL-6, IL-12, and IL-1β In the TRIF-dependent pathway (Akira et al., 2006).

In recent years, researchers have paid more attention to the use of plants as both medicine and food because plants can replace synthetic compounds in medicines used to treat disease. *Athyrium multidentatum* (Doll.) Ching (AM) is a perennial fern that belongs to the family Athyriaceae and is mainly distributed in the Changbai Mountain area in China (Yan and Li, 1996). AM is well known as an unparalleled resource of potherb, and its medicinal effects, including soothing the nerves, regulation of blood pressure and pain relief, have been documented (Liu et al., 2012; Qi et al., 2015; Sheng et al., 2011). In addition, studies have indicated that AM possesses high antioxidant activity. For instance, Liu et al. found that total polysaccharides extracted from AM obtained a stronger scavenging capacity on superoxide radicals than vitamin C (Vc) at concentrations from 22.22 to 44.44 μg/mL (Liu et al., 2015); Sheng et al. indicated that the n-butanol fraction from methanol extract of AM exerted high superoxide anion scavenging activity and high reducing power. In addition to the antioxidant property, anti-aging and anti-cancer properties of AM have also been investigated (Sheng et al., 2011). Liu et al. suggested that polysaccharides from AM possessed prominent antioxidant and anti-aging properties and could be promising adjuvant agents for preventing aging (Liu et al., 2016). Qi et al. demonstrated that AM significantly suppressed tumor growth, suggesting that AM may be a novel promising agent for hepatocellular carcinoma treatment (Qi et al., 2017). In our preliminary experiments, we found that AM extract can remarkably inhibit NO generation in PMs. Subsequently, we reviewed the literature, but did not find a publication regarding the anti-inflammatory property of AM. Therefore, the present study used *in vivo* and *in vitro* experimental methods to systematically study the anti-inflammatory property of AM extract to provide a reference for this widely used resource.

2. Materials and methods

2.1. Chemical

FBS, and DMEM were purchased from Gibco Co. (NY, USA). ATP was purchased from InvivoGen Co. (CA, USA). Nitric oxide assay kit and Prostaglandin E2 assay kit were procured from Elabscience Biotechnology Co., Ltd. (Wuhan, China), whereas the mouse tumor necrosis factor α (TNF-α), interleukin (IL) − 6, and IL-1β enzyme-linked immunosorbent assay (ELISA) kits (BD OptEIA Set) and the TMB substrate reagent set were purchased from BD Bioscience (SD, USA). SYBR Premix EX Taq reagent and PrimeScript™ PT reagent kits were purchased from TaKaRa (Shiga, Shanghai). The primers for TNF-α, IL-6 and IL-1β were purchased from Sangon Biotech Co. (Shanghai, China). The iNOS antibody was purchase from EMD Millipore Co. (CA, USA). The COX-2 and IKB-α antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Antibodies against total and the phosphoforms of p38, ERK, JNK, STATs, IRF-3 and β-actin were purchased from Cell Signaling Technology (MA, USA). Lipopolysaccharides (LPS; *Escherichia coli* 0111: B4), MTT, the RIPA buffer, TRIzol reagent and other chemicals were acquired from Sigma Aldrich (MO, USA).

2.2. Preparation of extracts

The dry aerial portions of AM were picked from Dunhua (N43°45′36.11″ E128°28′02.65″Alt. 398 m, June 2015, China), which was authenticated by Dr. Ren-Bo An, a taxonomist from Yanbian University and a voucher specimen of the plant was deposited at the key laboratory of Natural Resources of Changbai Mountain & Functional Molecules, Ministry of Education (023). The 50 g AM sample was ground and extracted thrice with 95% ethanol. The recovery rate reached 5.1%. For further use, the extracts were lyophilized and stored at − 20 °C.

2.3. Animals

Female C57BL/6 and BALB/c mice (22–25 g, 7 weeks old) were purchased from the Changchun Yisi Experimental Animal Co. (Changchun, China) [SPF, SCXK (J) 2016–0003]. The mice were optionally housed with food and distilled water and maintained in controlled conditions with a temperature of 22 ± 2 °C, 60–70% humidity, and a 12/12 h light-dark cycle per day. All experiments were conducted according to the guidelines of the Yanbian University Animal Care Committee of China.

2.4. Cell viability test

C57BL/6 mice were used to obtain peritoneal macrophages (PMs). Mice were intraperitoneally injected with 4% thioglycolate medium 4 d before sacrifice. The peritoneal cavity of mice was washed with 10 mL of PBS and PMs were collected. PMs were cultured in DMEM and supplemented with 10% fetal bovine serum (FBS) and antibiotics and maintained in a humidified incubator with 5% CO_2 at 37 °C. For selection of the non-toxic AM extract, the cell (PMs) viability was determined. In brief, PMs were seeded in 96-well plates (8×10^4 cells/well) and treated with or without LPS for 1 h and serial dilution of extracts for 24 h. Then, the original medium was discarded, 100 μL of DMEM containing MTT (500 μg/mL) was added and cells were incubated for 4 h. To solubilize the formazan crystals, dimethyl sulfoxide was added to each well after the medium was discarded. Measured the optical density at 540 nm and compared with the control group to calculate the cell viability.

2.5. Determination of NO and PGE_2 production

PMs were seeded in 48-well plates (2.5×10^5 cells/well) and treated with or without LPS (0.1 μg/mL) for 1 h and serial dilutions (25, 50, and 100 μg/mL) of extracts for different time periods. NO was evaluated at 24 h, and PGE_2 was examined at 16 h. The culture supernatant was harvested for further detection according to the manufacturer's instruction.

2.6. Enzyme-linked immunosorbent assay

To determine the levels of pro-inflammatory mediators (TNF-α, IL-6, and IL-1β), PMs were seeded in 48-well plates for 12 h. Then, various concentrations of AM extract were added, and cells were incubated for 1 h, and stimulated followed by LPS stimulation for various hours. IL-6 and TNF-α formation was examined at 6 h. To assay IL-1β secretion, PMs were pre-treated with extracts for 1 h after and stimulated by LPS for 3 h, and 5 mM adenosine triphosphate (ATP) was added for 1 h to prime the cells for IL-1β maturation. The level of IL-1β secretion was determined using an ELISA kit, and the optical density was measured at 450 nm. The levels of IL-6, TNF-α and IL-1β secretion were determined using by different kits according to the manufacturer's instructions. Optical densities were measured at 450 nm.