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Alpinetin attenuates inflammatory responses by interfering toll-like receptor 4/nuclear factor kappa B signaling pathway in lipopolysaccharide-induced mastitis in mice

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ABSTRACT

Alpinetin, a novel plant flavonoid derived from *Alpinia katsumadai Hayata*, has been reported to exhibit anti-inflammatory properties. However, the effect of alpinetin on mastitis has not been investigated. The aim of this study was to investigate the protective effect of alpinetin against lipopolysaccharide (LPS)-induced mastitis and to clarify the possible mechanism. In the present study, primary mouse mammary epithelial cells and an LPS-induced mouse mastitis model were used to investigate the effect of alpinetin on mastitis and the possible mechanism. In vivo, we observed that alpinetin significantly attenuated the infiltration of neutrophilic granulocytes, and the activation of myeloperoxidase; down-regulated the level of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6; inhibited the phosphorylation of IκB-α, NF-κB p65 and the expression of TLR4, caused by LPS. In vitro, we also observed that alpinetin inhibited the expression of TLR4 and the production of TNF-α, IL-1β and IL-6 in LPS-stimulated primary mouse mammary epithelial cells. However, alpinetin could not inhibit the production of IL-1β and IL-6 in TNF-α-stimulated primary mouse mammary epithelial cells. In conclusion, our results suggest that the anti-inflammatory effects of alpinetin against LPS-induced mastitis may be due to its ability to inhibit TLR4-mediated NF-κB signaling pathways. Alpinetin may be a promising potential therapeutic reagent for mastitis treatment.

1. Introduction

Mastitis, an infection of the mammary gland, is a highly prevalent and important infectious disease. The disease causes a decline in milk production and quality and results in impaired infant growth and development [1]. Some reports have shown that approximately 30% of women suffered at least one case of infectious mastitis [2]. Many microbial, host, and environmental factors can induce the development of mastitis [3,4]. Lipopolysaccharide (LPS), a main component of the outer membrane of Gram-negative bacteria, has been identified as an important risk factor for mastitis [5]. LPS can activate the host receptor TLR4 and trigger an inflammatory response, resulting in mastitis. Injection of LPS through the duct of the mammary gland has gained wide acceptance as a clinically relevant model of mastitis [6,7]. Several candidate therapy strategies have been applied to treat mastitis in the last decade. However, the incidence of mastitis remains high. Recently, treatments aimed at modulating TLR4 signaling and alleviating nonspecific inflammatory reactions may have potential therapeutic advantages for mastitis. Therefore, the development of novel therapies for mastitis is urgently needed.

Alpinetin (Fig. 1), a novel plant flavonoid isolated from *Alpinia katsumadai Hayata*, is responsible for the plant's pharmacological activities. Alpinetin has been reported to have antibacterial, anti-tumor and other important therapeutic activities [8–10]. Recently, it has been reported that alpinetin has an anti-inflammatory effect [11]. Alpinetin was found to inhibit inflammatory cytokine production in LPS-activated macrophages and to attenuate LPS-induced acute lung injury in mice [11]. Recently, it has been reported that the anti-inflammatory effects of alpinetin are mediated by blocking the activation of NF-κB and MAPKs signaling pathways [11]. Although a number of studies have addressed the therapeutic potential of alpinetin, its ability to protect against bacterial endotoxin-induced mastitis remains poorly understood. In this study, we sought to assess the preventive effects of alpinetin in an LPS-induced mouse mastitis model and elucidate the potential anti-inflammatory mechanism.

2. Materials and methods

2.1. Animals

Seventy-two female and thirty-six male BALB/c mice were housed in microisolator cages and received food and water. The laboratory

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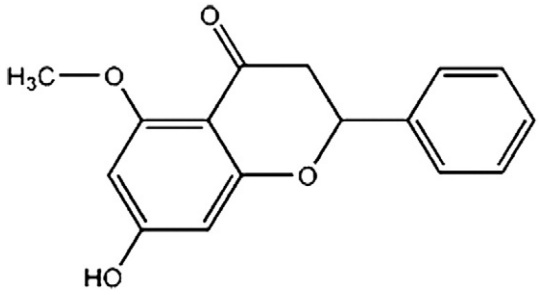

Fig. 1. Chemical structure of alpinetin.

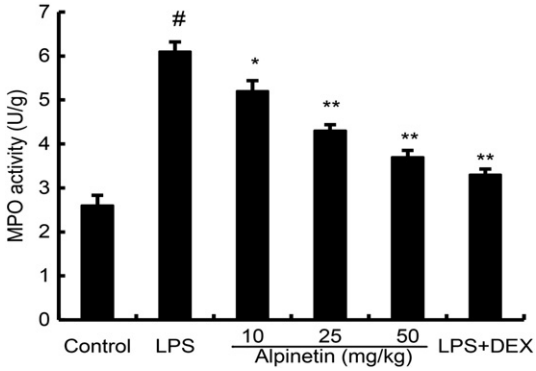

Fig. 2. Effects of alpinetin on MPO activity in mammary gland of LPS-induced mastitis. Mice were given an intraperitoneal injection of alpinetin (10, 25 and 50 mg/kg) 1 h before and 12 h after LPS instillation respectively. MPO activity was determined at 24 h after LPS administration. The data presented are the means ± SEM. p# < 0.01 vs. control group, p* < 0.05, p** < 0.01 vs. LPS group.

temperature was 24 ± 1 °C, and the relative humidity was 40–80%. All experimental protocols were approved by the regional Animal Ethics Committee.

2.2. Reagents

Alpinetin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Mouse TNF-α, IL-6 and IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biolegend (CA, USA). Mouse mAb phospho-NF-κB p65, mouse mAb phospho-IκBα and rabbit mAb IκBα were purchased from Cell Signaling Technology Inc. (Beverly, MA). Mouse mAb TLR4 was purchased from GeneTex. HRP-conjugated goat anti-rabbit and goat-mouse antibodies were provided by GE Healthcare (Buckinghamshire, UK). The myeloperoxidase (MPO) determination kit was provided by the Jiancheng Bioengineering Institute of Nanjing (Nanjing, Jiangsu, China). Dulbecco's modified Eagle's medium (DMEM: F12/1:1) and fetal calf serum (FCS) were purchased from Hyclone (Logan, UT, USA). Collagenase I and II were purchased from Invitrogen Corp. (Carlsbad, California, USA). Epidermal growth factor (EGF) and transferrin were purchased from PeproTech. All other chemicals were of reagent grade.

2.3. Animals and treatment

All of the mice were randomly divided into six groups: control group, LPS group, alpinetin (10, 25 and 50 mg/kg) + LPS group, and DEX + LPS group. Alpinetin (10, 25 and 50 mg/kg) and DEX were given by intraperitoneal injection 1 h before and 12 h after LPS stimulation respectively. After infusion of LPS for 24 h, the mice were sacrificed using CO_2 inhalation. The mammary glands were collected.

2.4. Cell culture and treatment

Primary mouse mammary epithelial cells were prepared as previously described by Smalley [12]. Briefly, the mammary tissues were removed aseptically from 15 day-gravidity BALB/c mice and minced into paste. The minced tissues were digested by collagenase I/II/trypsin mixture (Invitrogen, Carlsbad, California, USA) at 37 °C. After filtration, which removed undissociated tissues and debris, the cells were collected by centrifugation at 250 g for 5 min 3 times. The cell pellets were resuspended in DMEM/F12 containing 10% FCS, incubated for 1 h at 37 °C, and then the supernatant was collected. This step was repeated 3 times to clear away fibroblasts. After the last incubation, the cells were resuspended in DMEM/F12 containing 10% FCS, 0.5% transferrin, 0.1% T3 and 0.5% EGF and cultured at 37 °C with 5 % CO_2. The media was changed once every 48 h. In all of the experiments, primary mouse mammary epithelial cells were incubated in the presence or absence of various concentrations of alpinetin which

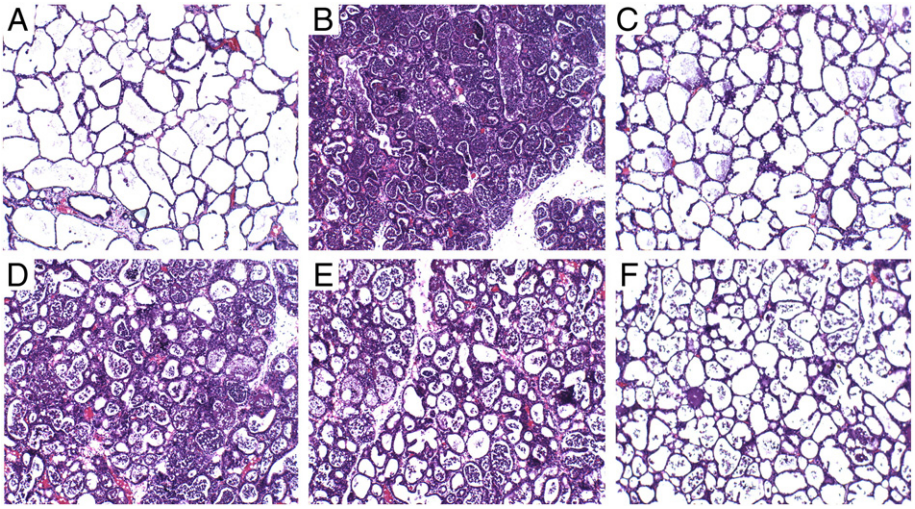

Fig. 3. Effects of alpinetin on histopathological changes in mammary gland tissues in LPS-induced mastitis in mice. Mice were given an intraperitoneal injection of alpinetin (10, 25 and 50 mg/kg) 1 h before and 12 h after LPS instillation respectively. Mammary gland (n = 4-6) from each experimental group were processed for histological evaluation at 24 h after LPS challenge. Representative histological changes of mammary gland obtained from mice of different groups. A: Control group, B: LPS group, C: LPS + DEX group, D: LPS + alpinetin (10 mg/kg) group, E: LPS + alpinetin (25 mg/kg) group F: LPS + alpinetin (50 mg/kg) group (Hematoxylin and eosin staining, magnification 100×).