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Nepetaefuran and leonotinin isolated from *Leonotis nepetaefolia* R. Br. potently inhibit the LPS signaling pathway by suppressing the transactivation of NF-κB

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ABSTRACT

Leonotis nepetaefolia R. Br., also known as Klip Dagga or Lion's Ear, has traditionally been used as a folk medicine to treat inflammatory diseases such as rheumatism, bronchitis, and asthma; however, the components that exhibit its anti-inflammatory activity have not yet been identified. In the present study, we investigated the effects of three types of diterpenoids, nepetaefuran, leonotinin, and leonotin, which were isolated from *L. nepetaefolia* R. Br., on the LPS signaling pathway in order to elucidate the anti-inflammatory mechanism involved. Nepetaefuran more potently inhibited the LPS-induced production of NO and CCL2 than leonotinin by suppressing the expression of iNOS mRNA and CCL2 mRNA. On the other hand, leonotin failed to inhibit the production of NO and CCL2 induced by LPS. Although nepetaefuran and leonotinin had no effect on the LPS-induced degradation of IκBα or nuclear translocation of NF-κB p65, they markedly inhibited the transcriptional activity of NF-κB. Nepetaefuran and leonotinin also inhibited the transcriptional activity of the GAL4-NF-κB p65 fusion protein. On the other hand, nepetaefuran, leonotinin and leonotin did not affect the LPS-induced activation of MAP kinase family members such as ERK, p38, and JNK. In addition, inhibitory effect of nepetaefuran and leonotinin on NF-κB activation is well correlated with their ability to induce activation of Nrf2 and ER stress. Taken together, these results demonstrated that nepetaefuran and leonotinin could be the components responsible for the anti-inflammatory activity of *L. nepetaefolia* R. Br. by specifically inhibiting the LPS-induced activation of NF-κB.

1. Introduction

Inflammation is important for host defense against bacterial or viral infections and macrophages play a central role in defense against invading pathogens [1,2]. However, excessive inflammatory responses by aberrantly activated macrophages, which produce an excess amount of inflammatory mediators, may disrupt immune homeostasis and result in immunopathological conditions such as sepsis. Sepsis is the systemic inflammatory response to infection and has a high rate of mortality [3,4]. Endotoxins such as lipopolysaccharide (LPS) from Gram-negative bacteria are known to potently stimulate macrophages in inflammatory responses. LPS has also been shown to induce the production of inflammatory mediators such as nitric oxide (NO) as well as cytokines in order to promote inflammatory responses in macrophages [5–7]. Among host factors, NO is the foremost key mediator responsible for sepsis syndrome, which is generated by inducible NO synthase (iNOS). Previous studies reported that large amounts of NO were generated during sepsis [8,9]. Furthermore, decreased responses to bacterial infections and endotoxic shock were detected in mice lacking iNOS [10]. Activated macrophages produce proinflammatory cytokines and chemokines, such as interleukin 1 (IL-1), tumor necrosis factor α (TNFα), and CCL-2/MCP-1 [11–13], and, as a result, various biological responses, including tissue injury, shock, and apoptosis, are rapidly induced in vivo. Therefore, novel compounds that suppress the LPS-induced production of NO, cytokines, and chemokines may potentially be effective anti-inflammatory drugs [14–16].

LPS has been shown to induce the production of pro-inflammatory mediators through its receptor Toll-line receptor 4 (TLR4) [17]. The binding of LPS to TLR4 causes the recruitment of a set of adaptor proteins and serine/threonine kinase, such as myeloid differentiation factor 88 (MyD88), TNF receptor-associated factor 6 (TRAF6), and IL-1 receptor-associated kinase 1 (IRAK-1) to TLR4 [18–21]. These interactions trigger downstream signaling cascades that activate the transcription factor,

Abbreviations: CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; FBS, fetal bovine serum; HO-1, heme-oxygenase-1; IκB, inhibitory of NF-κB; IKK, IκB kinase; iNOS, inducible NO synthase; IL, interleukin; IRAK-1, IL-1R-associated kinase-1; Keap1, Kelch-like ECH-associated protein 1; LPS, lipopolysaccharide; MMC, mitomycin C MyD88, myeloid differentiation factor 88; NQO1, NADPH quinone oxidoreductase; NO, nitric oxide; Nrf2, nuclear factor erythroid 2-related factor 2; NF-κB, nuclear factor-kappa B; ROS, reactive oxygen species; TLR4, Toll-line receptor 4; TNFα, tumor necrosis factor alpha; TRAF6, TNF receptor-associated factor 6; WST-1, water-soluble tetrazolium-1.

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nuclear factor kappaB (NF-κB) through IκB kinases (IKKs). In mammals, the NF-κB family has 5 members: RelA/p65, RelB, c-Rel, NF-κB1/p50, and NF-κB2/p52, and the p65/p50 heterodimer is a typical member [22–24]. In unstimulated cells, NF-κB remains inactive in the cytoplasm through an association with inhibitor proteins of the IκB family, such as IκBα. Activated IKKs phosphorylate IκBα, leading to their ubiquitination and proteasomal degradation [25,26]. These events release NF-κB dimers in the cytosol, allowing them to translocate into the nucleus in which they enhance the transcription of target genes, including iNOS and inflammatory cytokines and chemokines such as CCL2 [27–30].

Leonotis nepetaefolia R. Br. (family: Lamiaceae, syn. Labiatae), also known as Klip Dagga or Lion's Ear, is widely distributed throughout tropical Africa, southern India, and the tropical regions of America. The leaf decoctions of *L. nepetaefolia* R. Br. have traditionally been used as a folk medicine to treat a wide array of human diseases such as coughs, fever, stomachache, skin infections, rheumatism, bronchitis, and asthma [31,32]. Previous studies demonstrated that the crude extract of *L. nepetaefolia* R. Br. exhibited anti-bacterial activity and anti-fungal activities [33,34]; however, their effective components and other effects such as anti-inflammatory activity have not yet been elucidated.

In the present study, we focused on three kinds of diterpenoids: nepetaefuran, leonotinin, and leonotin, which were isolated from *L. nepetaefolia* R. Br. and investigated their effects on the LPS signaling pathway in order to elucidate the mechanism underlying the anti-inflammatory activity of *L. nepetaefolia* R. Br.

2. Materials and methods

2.1. Extraction and isolation of nepetaefuran, leonotinin, and leonotin

The aerial parts of *L. nepetaefolia* R.Br. were collected from the Medicinal Plant Garden of Keio University (Saitama, Japan) in 2009. Dried and powdered aerial parts of *L. nepetaefolia* R.Br. (530 g) were extracted two times with acetone (each 5 L) for 24 h at room temperature. The extracts were filtered and concentrated under reduced pressure to give a residue (37.7 g), which was then dissolved in acetone and passed through a column of activated charcoal to give a de-pigmented fraction (19.3 g). This fraction was chromatographed on silica gel column with hexane/AcOEt (9:1, 5:1, 3:1, 2:1, 1:1 and 1:2) to give eight fractions (fr. a-1–a-8). Fraction a-6 (679 mg) was subjected to column chromatography with Lobar RP-18 (size A, 60–80% MeCN) to give nepetaefuran (32.5 mg) and leonotinin (62.7 mg). Fraction a-5 (1.37 g) was rechromatographed on a COSMOSIL $^{75}C_{18}$-OPN column using 50–100% MeCN to afford two fractions (fr. b-1–b-2). The crystallization of b-1 from chloroform yielded leonotin (113 mg) as a colorless plate. The proton NMR of nepetaefuran, leonotinin and leonotin was shown in Supplement Fig. 1.

2.2. Reagents

LPS (*Escherichia coli* 055:B5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). An anti-NF-κB p65 antibody, anti-IκBα antibody, anti-Lamin B antibody, anti-iNOS antibody, anti-p38 antibody, and anti-β-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An anti-phospho ERK1/2 antibody (T202/Y204), anti-ERK1/2 antibody, anti-phospho p38 antibody (T180/Y182), anti-phospho JNK antibody (T183/Y185), anti-JNK antibody, anti-phospho NF-κB p65 (S536) and anti-Nrf2 antibody were purchased from Cell Signaling Technology (West Lafayette, St. Indiana, USA). Peroxidase-conjugated rabbit anti-mouse, rabbit anti-goat, and goat anti-rabbit secondary antibodies were purchased from Dako-Japan (Tokyo, Japan).

2.3. Cell culture

The murine macrophage cell line, RAW264.7 and HEK293T cells were cultured at 37 °C under 5% CO_2/95% air in DMEM (Nacalai Tesque, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies, CA, USA) and 1% Penicillin–Streptomycin Mixed Solution (Nacalai Tesque).

2.4. Measurement of NO_2^-

RAW264.7 cells (2×10^5 cells) were cultured on a 24-well plate and preincubated with various concentrations of nepetaefuran, leonotinin, leonotin, or 0.1% DMSO (Wako, Osaka, Japan) for 1 h at 37 °C prior to the stimulation with LPS (1 μg/mL) for 24 h. Nitrate concentrations were measured in culture supernatants using Griess reagent (1% sulfanilamide, 0.1% *N*-naphthylethylenediamine, and 2.5% H_3PO_4) as previously described [35].

2.5. Water-soluble tetrazolium-1 (WST-1) assay

The cell proliferation reagent water-soluble tetrazolium-1 (WST-1) (Roche Applied Science, Indianapolis, IN, USA) was used to detect the metabolic activities of cells. RAW264.7 cells (5×10^4 cells/100 μL) were cultured on a 96-well plate and preincubated with nepetaefuran, leonotinin, leonotin, or 0.1% DMSO for 1 h prior to the stimulation with LPS (1 μg/mL). After a 24-h incubation, 10 μL WST-1 was added to the culture media and the cells were incubated for 2 h at 37 °C. Absorbance at 450/690 nm was measured by the microplate reader Infinite M1000 (Tecan Group Ltd., Tokyo, Japan).

2.6. Immunoblotting

RAW264.7 cells (5×10^6 cells) were preincubated with nepetaefuran, leonotinin, leonotin, or 0.1% DMSO for 1 h at 37 °C prior to the stimulation with LPS (1 μg/mL). Cells were then lysed with Nonidet P-40 lysis buffer (50 mM Tris–HCl pH 8.0, 120 mM NaCl, 1 mM EDTA pH 8.0, 0.5% Nonidet P-40, 10 mM β-glycerophosphate, 2.5 mM NaF, and 0.1 mM Na_3VO_4) supplemented with protease inhibitors. To prepare nuclear extracts, cells were lysed in buffer A (10 mM HEPES-KOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 2 μg/ml leupeptin). Nuclei were then isolated as a precipitate by centrifugation at 5000 rpm for 2 min. Isolated nuclei were lysed in Nonidet P-40 lysis buffer and homogenized using the ultrasonic homogenizer VP-50 (TAITEC, Japan). Nuclear extracts were then centrifuged at 15,000 rpm for 15 min at 4 °C and the supernatant was mixed with Laemmli's sample buffer. Denatured samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were probed using the designated antibodies and visualized with the ECL detection system (GE Healthcare, Little Chalfont, UK) as described previously [35]. The band intensity was measured with an image analysis software (ImageJ, NIH, Bethesda, MD).

2.7. Enzyme-linked immunosorbent assay (ELISA)

RAW264.7 cells (2×10^5 cells) were cultured on a 24-well plate and preincubated with various concentrations of nepetaefuran, leonotinin, leonotin, or 0.1% DMSO for 1 h at 37 °C prior to the stimulation with LPS (1 μg/mL) for 24 h. The culture supernatants were collected, and the amounts of CCL2 in the supernatants were then determined by using the Immunoassay kit (eBioscience, San Diego, CA, USA).

2.8. RT-PCR (reverse transcription-polymerase chain reaction)

RAW264.7 cells (5×10^6 cells) were preincubated with nepetaefuran, leonotinin, leonotin, or 0.1% DMSO for 1 h at 37 °C prior to the stimulation with LPS (1 μg/mL). Total RNA was extracted using TRIzol (Life Technologies, Waltham, MA, USA). The synthesis of single strand cDNA was performed using an oligo $(dT)_{20}$ primer and 1 μg