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Methylene chloride fraction of the leaves of *Thuja orientalis* inhibits *in vitro* inflammatory biomarkers by blocking NF-κB and p38 MAPK signaling and protects mice from lethal endotoxemia

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ABSTRACT

Aim of the study: Thuja orientalis (TO) has been a recognized herbal medicine across Northeast Asian countries for thousands of years and used for the treatment of various inflammatory diseases through as yet undefined mechanisms. In this study, we set out to determine whether the anti-inflammatory effects of this plant are mediated to suppress mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) activation in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.

Materials and methods: RAW 264.7 cells were pretreated with the methylene chloride fraction of TO (MTO) and stimulated with LPS. Nitric oxide (NO) release was determined by the accumulation of nitrite in the culture supernatants and tumor necrosis factor-α (TNF-α) and IL-6 secretion were determined by immunoenzymatic assay. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression were evaluated via RT-PCR and Western blotting. NF-κB activation was also evaluated by reporter gene assay and electrophoretic mobility shift assay (EMSA). In addition, the protective effect of MTO was evaluated by use of the LPS-induced endotoxin shock model in mice.

Results: We found that MTO significantly suppressed LPS-stimulated NO and IL-6 production without affecting cell viability. MTO inhibited the expression of LPS-induced iNOS and COX-2 protein and their mRNA expression. Also, TNF-α and IL-6 secretion were decreased by MTO in both PMA and ionomycin-stimulated splenocytes. As a result, MTO inhibited pro-inflammatory cytokines such as TNF-α and IL-6, which is hypothesized as being due to the suppression of LPS-induced p38 MAPK and NF-κB activation. Moreover, MTO improved the survival rate during lethal endotoxemia by inhibiting the production of TNF-α in an animal model and our LC–MS analysis showed that a major component of MTO was pinusolide.

Conclusions: We demonstrate here the evidence that the methylene chloride fraction of *Thuja orientalis* (MTO) potentially inhibits the biomarkers related to inflammation *in vitro* and *in vivo*, and might be provided as a potential candidate for the treatment of inflammatory diseases.

1. Introduction

Thuja orientalis L. (also known as *Platycladus orientalis* L.) is a large, dense and compact shrub tree belonging to the Cupressaceae family. It has traditionally been used in the treatment of various inflammatory diseases (Tang et al., 1999). The plant exhibits a broad variety of biological activities: anti-plasmodial (Asili et al., 2004), platelet activating factor (PAF) receptor binding antagonistic (Yang et al., 1995), improvements for impaired memory acquisition (Nishiyama et al., 1995), hypouricemic effect (Zhu et al., 2004), fungitoxic (Guleria et al., 2008), molluscicidal (Singh and Singh, 2009), and anti-neurotoxic activities (Ju et al., 2010). In addition, Choi et al. showed that 15-methoxypinusolidic acid from the plant exhibits anti-inflammatory activity in microglial cells (Choi et al., 2008) and Morishige et al. reported that juniperonic acid, a polymethylene-interrupted (PMI) fatty acid which occurs in the plant, has an anti-proliferative activity on the bombesin-induced proliferation of Swiss 3T3 cells (Morishige et al., 2008). However, exactly how the plant mediates these anti-inflammatory effects is not yet completely understood.

NF-κB is the most pro-inflammatory transcription factor, consisting of p50, p65, and IκBα, that resides in the cytoplasm; and its activation is involved in iNOS, COX-2, and TNF-α expression

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because of the containment of the NF-κB consensus site in the upstream promoter region of the iNOS, COX-2, and TNF-α gene (Ahn and Aggarwal, 2005; Ahn et al., 2007). These gene products are essential components of the inflammatory response and are implicated in the pathogenesis of several inflammatory diseases (Barnes and Karin, 1997). Recent studies also demonstrate that the MAPKs signaling pathway, especially p38, can lead to NF-κB activation (Jijon et al., 2004; Olson et al., 2007), and this interaction (MAPKs and NF-κB) can collaborate synergistically to induce pro-inflammatory cytokine gene products and release (Craig et al., 2000). Thus, NF-κB activation and MAPKs pathways have been used as tools for the screening of anti-inflammatory activity.

In addition, septic shock is a syndrome in which a potentially lethal drop in blood pressure occurs as a result of an overwhelming bacterial infection. Indeed, the administration of the endotoxin LPS to experimental animals leads to pathophysiologic changes similar to human septic shock syndrome, and lethal endotoxemia has been extensively used as an experimental model of Gram-negative septic shock (Danner et al., 1991). The septic shock syndrome is characterized by a hyperactive and out-of-balance network of endogenous pro-inflammatory cytokines, including TNF-α, IL-12, and IL-6 (Netea et al., 2003). The overproduction of inflammatory cytokines mediates the systemic activation, which affects vascular permeability and cardiac function and induces metabolic changes that can lead to tissue necrosis and eventually to multiple-organ failure and death. Therefore, novel therapeutic approaches for the treatment of human septic shock are being designed to down-regulate the exacerbated inflammatory responses typical of endotoxemia (Sessler and Shepherd, 2002; Bochud and Calandra, 2003).

Thuja orientalis L. has anti-inflammatory properties through unknown mechanisms**.** Thus, we attempted to elucidate the anti-inflammatory potential of the plant by investigating the effect of the methylene chloride fraction of *Thuja orientalis* L. (MTO) induced by LPS. Here, we assessed the inhibitory effect of MTO on inflammatory biomarkers such as NO production and expressions of iNOS, COX-2; pro-inflammatory cytokines in RAW 264.7 cells and PMA/ionomycin-stimulated secretion of TNF-α and IL-6 in splenocytes. To investigate the underlying mechanisms, the involvement of MAPKs and NF-κB was examined. Moreover, we investigated the effect of MTO on the production of the pro-inflammatory mediator and its therapeutic action in a mouse model of endotoxemia.

2. Materials and methods

2.1. Cell culture

The RAW264.7 macrophage cells came from the Korean Cell Line Bank (KCLB, Seoul National University, College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-744, Korea). These cells were maintained at subconfluence in a 95% air, 5% CO_2 humidified atmosphere at 37 °C. The medium used for routine subcultivation was Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY) that was supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). To check NF-κB activity, transfectant RAW264.7 macrophage cells were cultured with 500 μg/ml of geneticin (100 μg/ml) for the selection and maintenance of stable transformants (Ahn et al., 2003). Cells were counted with a hemocytometer and the number of viable cells was determined through trypan blue dye exclusion.

2.2. Materials

LPS (Escherichia coli 055:B5) and fetal bovine serum (FBS), antibiotic-antimycotic were purchased from GIBCO-BRL. Antibodies against β-actin (sc-47778) and PARP (sc-7150) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-COX-2 and anti-iNOS antibodies were obtained from BD Biosciences (San Diego, CA). Phospho-specific anti-p38 (Thr180/Tyr180), anti-p38, phospho-specific anti-ERK (Thr202/Tyr204), anti-ERK, phospho-specific anti-JNK (Thr183/Tyr185), anti-JNK, phospho-specific anti-p65 (Ser536), and phospho-specific anti-p65 (Ser276) antibodies were purchased from Cell Signaling (Beverly, MA).

2.3. Plant materials

The leaves of *Thuja orientalis* were purchased in the Kyungdong market, Korea and the voucher specimen (D-602) was deposited at the Herbarium of KIST Gangneung Institute, Korea.

2.4. Preparation of various fractions from Thuja orientalis

The leaves *Thuja orientalis* (1.5 kg) was extracted four times with hot EtOH (7.0 l) for 4 h. This residue was evaporated *in vacuo* to yield the total extract (320.0 g). This extract which was vacuum-dried was then suspended in distilled water and sequentially subjected to partitioning with *n*-hexane (49.7 g), methylene chloride (21.0 g), ethyl acetate (31.2 g), and *n*-butyl alcohol (29.4 g).

2.5. Measurement of cell viability

The cytotoxicity of TO was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW264.7 cells were plated at a density of 1×10^4 cells per well in a 96-well plate, and incubated at 37 °C. The cells were treated with a 50 μg/ml concentration of various TO fractions. After 24 h incubation, 20 μl of MTT solution (5 mg/ml) was added to each well and incubated at 37 °C. The change in color was monitored with an ELISA reader (Model 680, Bio-Rad) at 570 nm.

2.6. Nitrite assay

The RAW264.7 macrophage cells were plated at a density of 2×10^5 cells per well in a 24-well plate. The cells were pre-treated with the indicated concentrations of MTO for 2 h, and then induced with a 1 μg/ml concentration of LPS for an additional 22 h. Nitrite accumulation in the culture was measured colorimetrically by the Griess reaction using a Griess reagent (Sigma, St. Louis, MO). For the assay, equal volumes of the cultured medium and Griess reagent were mixed, and the absorption coefficient was calibrated using a sodium nitrite solution standard (Sigma, St. Louis, MO). The absorbance of each sample after the Griess reaction was determined by an ELISA plate reader at 540 nm.

2.7. Enzyme-linked immunosorbent assay (ELISA) for IL-6

The amount of IL-6 produced by the mouse macrophages was indicated by the amount of substance measured in the RAW264.7 cell culture supernatant. RAW264.7 cells were plated at a density of 5×10^5 cells in a 24-well cell culture plate with 500 μl of culture medium and incubated for 12 h. They were then pretreated with combinations of various times and doses of MTO in the presence and absence of LPS (1 μg/ml). The amount of IL-6 production was determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

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

Total RNA from RAW264.7 cells was prepared using a TRIzol reagent (Gibco, Grand Island, NY). The concentration and