The American Journal of Chinese Medicine, Vol. 43, No. 4, 731–742

Institute for Advanced Research in Asian Science and Medicine
DOI: 10.1142/S0192415X15500457

Alpha-Pinene Exhibits Anti-Inflammatory Activity Through the Suppression of MAPKs and the NF-κB Pathway in Mouse Peritoneal Macrophages

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Published 29 June 2015

Abstract: In this study, we found that alpha-pinene (α-pinene) exhibits anti-inflammatory activity through the suppression of mitogen-activated protein kinases (MAPKs) and the nuclear factor-kappa B (NF-κB) pathway in mouse peritoneal macrophages. α-Pinene is found in the oils of many coniferous trees and rosemary. We investigated the inhibitory effects of α-Pinene on inflammatory responses induced by lipopolysaccharide (LPS) using mouse peritoneal macrophages. α-Pinene significantly decreased the LPS-induced production of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and nitric oxide (NO).

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α-Pinene also inhibited inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions in LPS-stimulated macrophages. Additionally, the activations of MAPKs and NF-κB were attenuated by means of α-pinene treatment. These results indicate that α-pinene has an anti-inflammatory effect and that it is a potential candidate as a new drug to treat various inflammatory diseases.

Keywords: α-Pinene; Inflammation; Macrophage; Mitogen-Activated Protein Kinase; Nuclear factor-kappa B.

Introduction

Inflammation is a typical immune response which protects the human body from injury or infection. However, inappropriate immune responses cause many inflammatory diseases, such as allergies, rheumatoid arthritis, and asthma. Macrophages are considered to play an essential role in inflammatory responses. Activated macrophages stimulated by endotoxins, such as lipopolysaccharide (LPS), produce pro-inflammatory cytokines that activate other macrophages and recruit immune cells (Medzhitov and Janeway, 1997).

Inhibition of the production of pro-inflammatory cytokines and mediators serves as a key mechanism to control inflammatory responses. A number of anti-inflammatory molecules against nitric oxide (NO) and pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and prostaglandin E_2 (PGE_2) have already entered clinical trials as potential treatment for inflammatory disorders (Reinhart and Karzai, 2001). Therefore, drugs that suppress the expression of these inflammatory mediators have attracted interest as potential therapeutics for the treatment of inflammatory diseases (Park *et al.*, 2011).

Inflammatory stimuli induce the phosphorylation of p38 mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK)-1/2, and c-Jun NH2-terminal kinase (JNK), which induce the transcription of pro-inflammatory genes. One of the well-known downstream components of MAPK signaling is the nuclear factor-kappa B (NF-κB) (Ajizian *et al.*, 1999). In unstimulated cells, NF-κB is bound to the inhibitory protein I kappa B (IκB) in the cytosol. In response to stimulation, such as by LPS, IκB is rapidly phosphorylated by IκB kinase (IKK) and degraded. Consequently, the free NF-κB dimers translocate to the nucleus regulating the transcription of target genes, which include cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Li and Verma, 2002). Therefore, the regulation of MAPKs and the NF-κB pathway is a potential therapeutic strategy for inflammatory diseases.

Alpha-pinene (α-pinene) is a monoterpene compound which is found in the essential oils of coniferous trees and many other plants (Lee *et al.*, 2009). Essential oils that contain α-pinene possess anti bacterial and anti fungal effects (Hong *et al.*, 2004). However, there have been few experimental examinations of α-pinene on inflammatory responses.

In this study, we investigated α-pinene's anti-inflammatory effect and mechanisms of action in LPS-stimulated mouse peritoneal macrophages. The findings suggest that α-pinene suppresses inflammatory mediators and therefore may be potentially useful in the treatment of inflammatory diseases.

Materials and Methods

Reagents

Following chemicals were obtained from Sigma (St. Louis, MO, USA): 3-(4,5-dimethylthiazol-2-yl)-diphenyl tetrazolium bromide (MTT), bicinchoninic acid (BCA), and LPS. The anti-rabbit IgG antibody (Ab) and anti-mouse IgG Ab were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Mouse monoclonal and biotinylated monoclonal TNF-α Ab, and recombinant mouse TNF-α were purchased from R&D Systems (Minneapolis, MN, USA). Mouse monoclonal and biotinylated monoclonal IL-6, and recombinant mouse IL-6 were purchased from Pharmingen (San Diego, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM) and thioglycollate (TG) were purchased from Difco Laboratories (Detroit, MI, USA). Specific Abs against COX-2, iNOS, phospho-p38, p38, phospho-ERK, ERK, phospho-JNK, JNK, NF-κB, IκB, phospho-IκB, IKK, histone and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Animals

Male C57BL/6 mice (6 weeks old) were purchased from Da-Mool Science (Daejeon, Republic of Korea), and maintained at the College of Pharmacy, Wonkwang University. Throughout the study, mice (3 to 5 per cage) were housed in a room with laminar air flow at a temperature of $22^{\circ}C \pm 1^{\circ}C$ and a relative humidity of $55\% \pm 10\%$. The animal experimental procedures were approved by the Institutional Review Board of Wonkwang University (Approval Number WKU14-62).

Peritoneal Macrophage Culture

To isolate the peritoneal macrophages, mice were administered an intraperitoneal injection of 2.5 mL TG medium. The TG-elicited macrophages were harvested after 3-4 days, as previously reported (Narumi *et al.*, 1990). Peritoneal lavage was performed using 8 ml of Hank's balanced salt solution (HBSS) contained 10 U/mL heparin. Cells were transferred into 24-well tissue culture plates (3×10^5 cell/well) or 60 mm culture dishes (5×10^6 cell/well) containing DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and incubated for 4 hours at $37^{\circ}C$ in an atmosphere of 5% CO_2. The plates or dishes were then washed 3 times with HBSS to remove non-adherent cells and equilibrated with DMEM containing 10% FBS before treatment.

MTT Assay

Cell viability was determined by an MTT assay. Briefly, cell suspensions (500 μL) of peritoneal macrophages treated with different concentrations of α-pinene were cultured in 24-well plates (3×10^5 cell/well) for 24 hours. Fifty microliters of MTT solution (5 mg/mL) was added, and the cells were incubated for 4 hours at $37^{\circ}C$. After washing off the

supernatant, the insoluble formazan product was dissolved in dimethyl sulfoxide. The absorbance values of 96-well culture plates were measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) reader. The absorbance value of control cells was considered to represent 100% viability.

Cytokine Assay

The cytokine assays for IL-6 and TNF-α were performed by a modified ELISA method, as described previously (Kim *et al.*, 2011). In this method, 96-well plates were coated with mouse monoclonal IL-6 and TNF-α Abs overnight at 4°C. The coated plates were washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST) before proceeding with the subsequent steps of the assay. After washing with PBST, samples and standards were incubated for 2 hours at room temperature. Recombinant IL-6 and TNF-α were used as standards. Serial dilutions from 10 ng/mL solution were used to establish the standard curve. The assay plates were sequentially exposed to biotinylated mouse IL-6 and TNF-α secondary Abs, avidin peroxidase, and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate solution containing 30% distilled water. The absorbance values of the plate were measured at 405 nm.

Nitrite Assay

To measure the nitrite content, 100 μL aliquots were removed from the conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) for 10 minutes at room temperature. The absorbance was measured by a microplate reader at a wavelength of 540 nm. The NO^{2-} level was determined using sodium nitrite as a standard. The value was recorded in each experiment and subtracted from that obtained for the medium containing the peritoneal macrophages.

PGE_2 Assay

The PGE_2 concentration was measured using a PGE_2 EIA kit (Enzo Life Sciences, USA) according to the manufacturer's directions. Duplicate aliquots of supernatant were measured for each sample.

Western Blot Analysis

To determine the levels of the indicated proteins, the stimulated cells were rinsed twice with ice-cold PBS and then lysed in ice-cold lysis buffer (iNtRON Biotech, Republic of Korea). Nuclear extracts were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific, IL, USA) according to the manufacturer's protocols. Cell lysates were centrifuged at 13,000 *g* for 5 minutes, and the supernatant was mixed with an equal volume of 2 × SDS sample buffer, boiled for 5 min and separated by

electrophoresis through a 10% denaturing protein gel. After electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked by 5% skim milk for 2 hours, rinsed, and incubated overnight at 4°C with primary Abs. After washing with PBST 5 times, the membranes were incubated with horseradish peroxidase-conjugated secondary Abs for 1 hour. The membranes were washed with PBST 5 more times and the protein bands were visualized by an enhanced chemiluminescence assay according to the manufacturer's instructions.

NF-κB Binding Assay

The nuclear extract was incubated with an immobilized oligonucleotide on a 96-well plate (containing a specific NF-κB binding site) and detected by a specific p65 NF-κB subunit ELISA kit (Affymetrix, CA, USA) according to the manufacturer's instructions.

Statistical Analysis

The results shown are a summary of the data from at least three experiments and are presented as mean ± SEM. Statistical evaluation of the results were performed by Student's *t*-test. A value of $p < 0.05$ was considered significant.

Results

Effect of α-Pinene on Cell Viability and Cytokine Production

The effect of α-pinene on the cell viability was examined using an MTT assay. α-Pinene did not show a cytotoxic effect up to 20 μM (Fig. 1). To investigate the anti-inflammatory

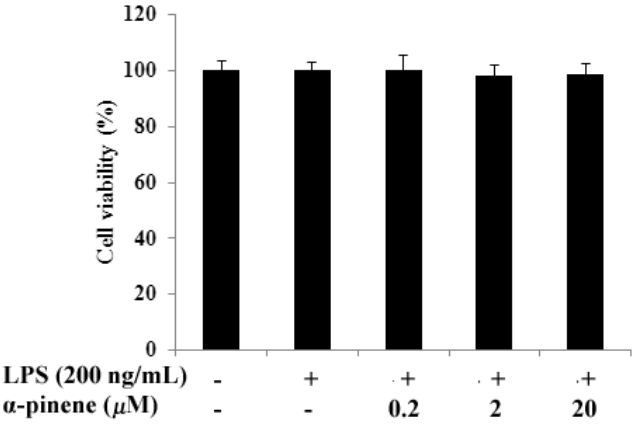


Figure 1. Cytotoxicity of α-pinene in mouse peritoneal macrophages. Cells (5×10^5 cell/well) were pretreated with various concentrations of α-pinene (0.2–20 μM) for 1 hour and then stimulated with LPS (200 ng/ml) for 24 hours, after which they were collected and assessed for viability using an MTT assay. Values are mean ± SEM of duplicate determinations from three separate experiments.

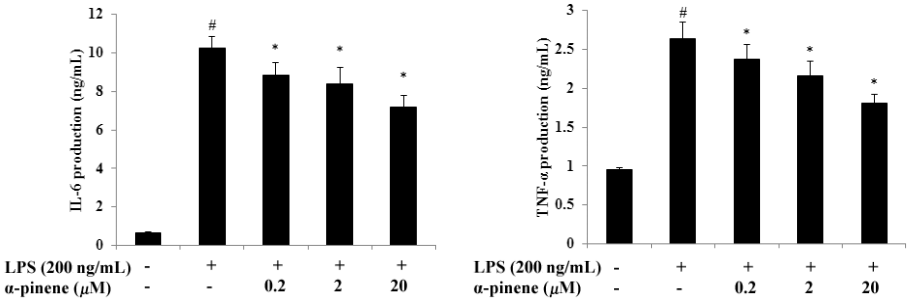


Figure 2. α-Pinene decreases IL-6 and TNF-α production in LPS-stimulated macrophages. Cells (5×10^5 cell/well) were pretreated with various concentrations of α-pinene (0.2–20 μM) for 1 hour and then stimulated with LPS (200 ng/ml) for 24 hours. IL-6 (A) and TNF-α (B) concentrations were measured in cell supernatants using the ELISA method. Values are mean ± SEM of duplicate determinations from three separate experiments ($^{\#}p < 0.05$ vs. unstimulated cells, $^{*}p < 0.05$ vs. LPS-stimulated cells).

effect of α-pinene, IL-6 and TNF-α production were measured. LPS stimulation significantly increased the production of IL-6 and TNF-α. However, α-pinene decreased the production of IL-6 and TNF-α in a dose-dependent manner in LPS-stimulated peritoneal macrophages (Fig. 2).

Effects of α-Pinene on NO, PGE_2, iNOS, and COX-2

As shown as Fig. 3A, α-pinene decreased LPS-induced NO production in a dose-dependent manner. In addition, LPS induced a high level of iNOS protein expression,

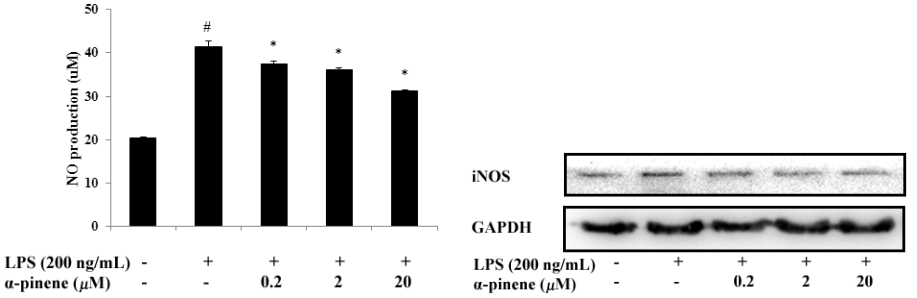
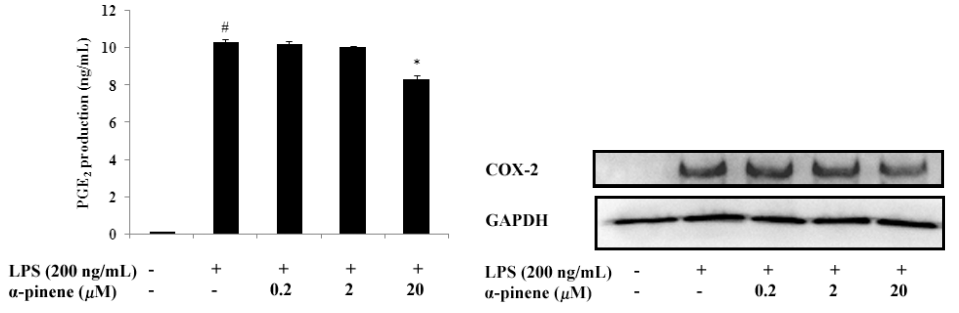
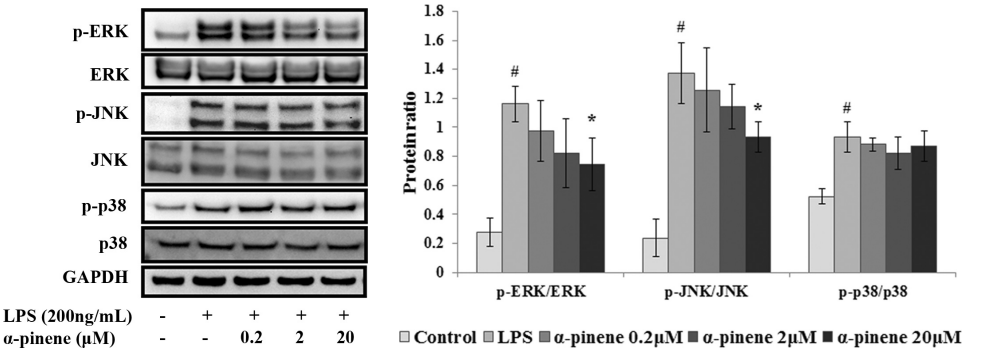


Figure 3. α-Pinene inhibits NO production and iNOS expression in LPS-stimulated macrophages. (A) To examine NO production levels, cells (5×10^5 cell/well) were pretreated with various concentrations of α-pinene (0.2–20 μM) for 1 hour and then stimulated with LPS (200 ng/ml) for 48 hours. The amount of NO production was measured via the Griess assay. (B) To examine iNOS expression, cells (5×10^6 cell/well) were treated with various concentrations of α-pinene (0.2–20 μM) for 1 hour and then stimulated with LPS (200 ng/ml) for 24 hours. iNOS expression levels were determined by a Western blot analysis, as noted in the Materials and Methods Section. Values are mean ± SEM of duplicate determinations from three separate experiments ($^{\#}p < 0.05$ vs. unstimulated cells, $^{*}p < 0.05$ vs. LPS-stimulated cells).



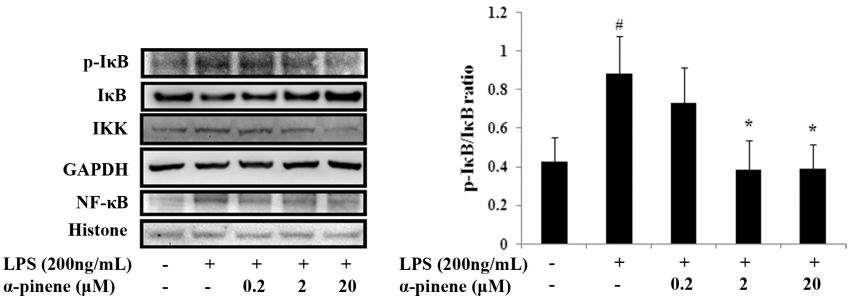
(A) (B)

Figure 4. α-Pinene inhibits PGE_2 production and COX-2 expression in LPS-stimulated macrophages. (A) To examine PGE_2 production levels, cells (5×10^5 cell/well) were pretreated with various concentrations of α-pinene (0.2–20 μM) for 1 hour and then stimulated with LPS (200 ng/ml) for 24 hours. The amount of PGE_2 production was measured with a PGE_2 assay. (B) To examine COX-2 expression levels, cells (5×10^6 cell/well) were treated with various concentrations of α-pinene (0.2–20 μM) for 1 hour and then stimulated with LPS (200 ng/ml) for 24 hours. COX-2 expression was determined by a Western blot analysis, as noted in the Materials and Methods Section. Values are the mean ± SEM of duplicate determinations from three separate experiments ($^{\#}p < 0.05$ vs. unstimulated cells, $^{*}p < 0.05$ vs. LPS-stimulated cells).

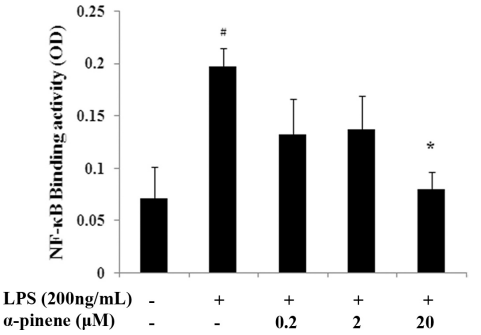


(A) (B)

Figure 5. α-Pinene inhibits MAPKs and NF-κB activation in LPS-stimulated macrophages. (A) Cells (5×10^6 cell/well) were pretreated with various concentrations of α-pinene (0.2–20 μM) for 1 hour, and then stimulated with LPS (200 ng/ml) for 30 minutes. The expressions p-ERK, p-p38, p-JNK, ERK, p38, and JNK were determined by western blot analysis as written in the Section "Materials and Methods". (B) p-ERK/ERK, p-JNK/JNK and p-p38/p38 protein ratios were calculated by densitometry. (C, D and E) Cells (5×10^6 cell/well) were pretreated with various concentrations of α-pinene (0.2–20 μM) for 1 hour, and then stimulated with LPS (200 ng/ml) for 1 hour. Nuclear extracts were prepared as described in the Section "Materials and Methods". (C) The nuclear extracts were evaluated for NF-κB expression and cytosolic extracts were examined for IκB, p-IκB and IKK expression. The expression levels of NF-κB, IκB, p-IκB, and IKK were determined by Western blot analysis. (D) p-IκB/IκB ratio was calculated by densitometry. (E) The nuclear extracts were assayed by a specific p65 NF-κB subunit ELISA kit. Values are mean ± SEM of duplicate determinations from three separate experiments ($^{\#}p < 0.05$ vs. unstimulated cells, $^{*}p < 0.05$ vs. LPS-stimulated cells).



(C) (D)



(E)

Figure 5. (*Continued*)

which is in accordance with the significant NO production in peritoneal macrophages. α-Pinene decreased the expression of iNOS protein in LPS-stimulated macrophages (Fig. 3B). Moreover, we evaluated the effect of α-pinene on PGE_2 production and COX-2 expression because COX-2 catalyzes the biosynthesis of PGE_2 in inflammatory responses. α-Pinene effectively inhibited PGE_2 production and COX-2 expression in LPS-stimulated macrophages (Fig. 4).

Effect of α-Pinene on MAPKs and the NF-κB Pathway

Activation of the MAPK/NF-κB pathway plays a key role in inflammatory reactions. Among the MAPKs, α-pinene inhibited ERK and JNK phosphorylation, but not p38 in LPS-stimulated macrophages (Figs. 5A and 5B). To investigate whether α-pinene inhibits the NF-κB pathway, we conducted a Western blot analysis and an NF-κB binding assay. α-pinene decreased the levels of cytosolic IKK levels and the levels of phosphorylated IκB and the nuclear NF-κB (Figs. 5C and 5D). To confirm the inhibitory effect of α-pinene on NF-κB activation, we performed an NF-κB binding assay. High

concentrations of α-pinene significantly inhibited NF-κB binding activity in LPS-stimulated macrophages (Fig. 5E).

Discussion

Recent studies have reported that the biological properties of α-pinene, including lipophilic, bactericidal, insecticidal, anti carcinogenesis, anti-oxidant, sedative alone or in synergy with other pinenes (Mercier *et al.*, 2009). Because α-pinene is present in essential oils, some studies on herbal essential oils containing the compound were reported to have an influence on inflammation (El-Readi *et al.*, 2013; Popović *et al.*, 2014; Kim *et al.*, 2004). In particular, α-pinene was found to modulate bone resorption and acute pancreatitis and allergic rhinitis (Mühlbauer *et al.*, 2003; Bae *et al.*, 2012; Nam *et al.*, 2014). The anti-inflammatory properties of α-pinene have been reported in different cell types. In rat peritoneal mast cells, α-pinene inhibited histamine release (Moon *et al.*, 2013), and the activation of IKK-β, NF-κB, and caspase-1 were inhibited by α-pinene in human mast cells (Nam *et al.*, 2014). It has also been reported that α-pinene inhibited NF-κB and JNK activation in chondrocytes (Rufino *et al.*, 2014). In this study, the anti-inflammatory effect of α-pinene was tested in mouse peritoneal macrophages.

Pro-inflammatory cytokines such as IL-6 and TNF-α are important in inflammatory diseases. TNF-α and IL-6 are critically involved in the inflammatory responses, which are associated with autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, asthma, and psoriasis (Takematsu *et al.*, 1991; Stokkers *et al.*, 1995; Mudter and Neurath, 2007; Park and Pillinger, 2007; Simsek, 2010; Rincon and Irvin, 2012). In the present study, we demonstrated that α-pinene treatment reduces the production of IL-6 and TNF-α in LPS-stimulated peritoneal macrophages (Fig. 2).

NO has been known as an important regulatory molecule in various physiological functions including inflammation, vasodilation, and cellular communication (MacMicking *et al.*, 1997). NO is synthesized by three different isoforms of NOS: endothelial NOS (eNOS), neuronal NOS (nNOS), and iNOS. Among them, iNOS is up-regulated in LPS-induced macrophages (Fukumoto *et al.*, 1997). COX is an enzyme that converts arachidonic acid to Prostaglandin H_2, a precursor of various mediators, such as thromboxane A_2, prostacyclin and PGE_2 (Breyer *et al.*, 2001). There are two isoforms of COX; COX-2, which is induced by many stimulants and expressed at sites of inflammation, and COX-1, which is expressed physiologically. Upon inflammatory stimulation, COX-2 is rapidly expressed in macrophages and endothelial cells and may be responsible for the edema and vasodilation associated with inflammation (Morita, 2002). The present results showed that α-pinene attenuates inflammation by reducing COX-2 and iNOS expression as well as PGE_2 and NO production in LPS-stimulated macrophages (Figs. 3 and 4).

The phosphorylation and activation of MAPKs have been known to regulate inflammatory mediators in LPS-induced macrophages. Activated p38 regulates TNF-α and iNOS production through NF-κB (Kaminska, 2005), whereas JNK activation regulates various inflammatory mediators, including the phosphorylation of c-Jun and AP-1 activation (Karin *et al.*, 1997). It has been reported that the JNK pathway is required for LPS-induced

iNOS expression in macrophages (Chan and Riches, 2001). ERK regulates IKK activation and TNF-α production (Dumitru *et al.*, 2000). Moreover, ERK is involved in iNOS expression and in the production of various cytokines in monocytes/macrophages (Chang *et al.*, 2003). In this study, the effect of α-pinene on MAPK signaling was investigated in mouse peritoneal macrophages. The α-pinene treatment attenuated LPS-induced phosphorylation of ERK and JNK, but not that of p38 (Figs. 5A and 5B). This result showed that α-pinene inhibits the expression of inflammatory mediators by blocking the phosphorylation of ERK and JNK rather than that of p38 in LPS-stimulated mouse peritoneal macrophages.

Except for the MAPK signaling, NF-κB also plays a critical role in the immune system, which controls the expression of enzymes and cytokines, including iNOS, COX-2, TNF-α, and IL-6 during inflammatory responses (Baeuerle and Baltimore, 1996; Kim *et al.*, 2007). In a resting cell, NF-κB is bound by its inhibitor, IκB, and cannot translocate to the nucleus. Once IκB is phosphorylated by IKK, IκB is degraded and NF-κB undergoes nuclear translocation, and it triggers the expression of pro-inflammatory genes. Therefore, we investigated IKK expression, IκB phosphorylation, and NF-κB translocation. Based on our results, LPS treatment induces the up-regulation of IKK, phosphorylation of IκB, and nuclear translocation of NF-κB. Addition of α-pinene reduces IKK expression, p-IκB level, and NF-κB translocation, which is also confirmed by NF-κB binding activity assay (Figs. 5C–5E). These results suggest that α-pinene exerts its anti-inflammatory effect via the NF-κB pathway.

The present study demonstrates α-pinene's anti-inflammatory activity through the down-regulation of MAPKs (ERK and JNK) phosphorylation and the NF-κB signaling pathway. Taken together, our results suggest that α-pinene is an effective natural anti-inflammatory agent.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (No. 2013R1A2A2A0106785 and No. 2011-0006220).

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