Effect of α-pinene on nuclear translocation of NF-κB in THP-1 cells

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ABSTRACT

AIM: To study the effects of α-pinene on nuclear translocation of nuclear factor-κB (NF-κB) and the expression of the inhibitor of NF-κB (IκBα) in human monocyte THP-1 cell line. **METHODS:** THP-1 cells were incubated with α-pinene (1, 10, and 100 mg/L, for 30 min) before being stimulated with lipopolysaccharide (LPS, 1 mg/L, 30 min). The location of NF-κB p65 subunit (NF-κB/p65) in THP-1 cells was detected by immunofluorescence and laser scanning confocal microscope (LSCM). The expression of NF-κB/p65 in nuclei and that of IκBα in cytoplasm were measured by Western-blot analysis. **RESULTS:** The majority of FITC-labelled NF-κB/p65 was located in the nuclei being stimulated with LPS. Whereas, no such fluorescence was seen in the nuclei of the groups pretreated with α-pinene or control cells. α-Pinene pretreatment decreased the NF-κB/p65 nuclear translocation in LPS-stimulated THP-1 cells, and this effect was dose-dependent, but there was no reaction in LPS-unstimulated THP-1 cells. α-Pinene pretreatment increased IκBα protein level in cytoplasm, compared with that in LPS-stimulated THP-1 cells. **CONCLUSION:** In a dose-related fashion, α-pinene inhibits the nuclear translocation of NF-κB induced by LPS in THP-1 cells, and this effect is partly due to the upregulation of IκBα expression.

INTRODUCTION

Terpenes are widely used in the treatment of upper and lower airway diseases, such as chronic sinusitis and bronchitis[1]. α-Pinene is a principal constituent of most *Eucalyptus* oils, *Camphor*, *Bupleurum fruticescens*, *Psidium* and many other essential oils. Martin *et al* has investigated the anti-inflammatory activity of α-pinene in against rat hindpaw edema induced by carrageenin or by PGE_1[2]. However, the molecular mechanism of the anti-inflammatory activity of α-pinene is still not well understood.

Nuclear factor-κB (NF-κB) is an important pluripotent transcription factor involved in the regulation of many proinflammatory responses. Its gene products include cytokine, chemokines, cell adhesion molecules, and immunoreceptors. Upon activation, NF-κB is released from IκBα, the major inhibitor of NF-κB, then translocates into the nucleus where it binds specific DNA motifs in the promoter/enhancer regions of target genes and activates transcription[3,4].

To elucidate the anti-inflammatory mechanism of α-pinene, in the present study, we investigated the effects of α-pinene on subcellular localization of NF-κB

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p65 subunit (NF-κB/p65), expression of NF-κB/p65 in nucleus, and IκBα in cytoplasm in human THP-1 cells.

MATERIALS AND METHODS

Drugs and reagents α-Pinene was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing). Lipopolysaccharide (LPS from *Escherichia coli* 026:B6), *N*-α-tosyl-*L*-lysine chloromethyl ketone (TLCK) protease inhibitors, phorbol 12-myristate 13-acetate (PMA), and propidium iodide (PI) were purchased from Sigma (USA). Rabbit polyclonal anti-IκBα and anti-p65 antibody (Ab) were obtained from Santa Cruz (USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was obtained from Jackson Immuno Research (USA). Gel shift assay systems were from Bio-Rad (USA).

Cell culture and treatments THP-1 cells were obtained from American Type Culture Collection (ATCC, No TIB-202). Cells were cultured and treated with RPMI-1640 (Gibco, USA), supplemented with 10 % heat-inactivated fetal bovine serum, glutamine 2 mmol/L, benzylpenicillin 100 kU/L, streptomycin 100 kU/L at 37 °C under 5 % CO_2. Before incubation with α-pinene, which was emulsified with Tween 80/phosphate-buffered saline (PBS), THP-1 cells were divided into two groups: with or without LPS treatment respectively. In normal culture condition group, cells were incubated with α-pinene 1, 10, and 100 mg/L for 30 min; while in the stimulated group, cells were incubated with LPS 1 mg/L for 30 min after an α-pinene treatment. Vehicle control was incubated with 0.001 % Tween 80/PBS as the same volume. Then the subcellular localization of NF-κB/p65 and the expression of NF-κB/p65 in nucleus and IκBα in cytoplasm in THP-1 cells were examined.

Immunofluorescent staining and images capture[6]. The cells, which adhered on coverslips induced by PMA 20 μg/L, were fixed with freshly prepared 3 % paraformaldehyde for 15 min and permeabilized with 0.5 % Triton X-100 for 15 min. After a 1-h incubation with 10 % normal goat serum/PBS, cells were incubated with anti-p65 Ab diluted at 1:50 in PBS for 2 h, washed and then incubated with FITC-conjugated IgG, diluted at 1:100 in PBS, for 1 h. In order to identify the nuclei, the FITC-labeled samples were counterstained with PI 25 mg/L for 2 min.

To acquire dual-color images, a 510 confocal laser scan confocal microscope (Zeiss, Germany) was used, which equipped with a Zeiss inverted research biological microscope, with a 100×oil immersion objective (NA 1.30). The samples labeled with both FITC and PI were excited at 488 nm, and the fluorescence emissions were captured through 510 to 550 nm (530 nm in center) and 590 to 620 nm (605 nm in center) bandpass with spectral grating, respectively.

Preparation of cytoplasmic and nuclear extracts The cytoplasmic and nuclear protein extracts were prepared according to the protocol of Schreiber *et al*[5] with some modifications. Briefly, after culture the cells were collected and washed twice with cold PBS, lysed in 400 μL of cold buffer A (HEPES 10 mmol/L pH 7.9, KCl 10 mmol/L, edetic acid 0.1 mmol/L, egtazic acid 0.1 mmol/L, phenylmethanesulphonylfluoride (PMSF) 1 mmol/L, dithiothreitol (DTT) 1 mmol/L, aprotinin 1 mg/L, leupeptin 1 mg/L, and pepstatin A 1 mg/L). After 15-min incubation on ice, 0.1 % NP-40 was added to the homogenates and the tubes were vigorously rocked for 1 min. Then the homogenates were centrifuged (20 800×*g*, 5 min) in a microcentrifuge at 4 ºC. The supernatant fluid (cytoplasmic extracts) was collected and stored in aliquots at -70 ºC. The nuclear pellets were washed once with cold buffer A, then suspended in 50 μL of cold buffer B (HEPES 20 mmol/L, pH 7.9, NaCl 420 mmol/L, edetic acid 0.1 mmol/L, egtazic acid 0.1 mmol/L, PMSF 1 mmol/L, DTT 1 mmol/L, aprotinin 1 mg/L, leupeptin 1 mg/L, and pepstatin A 1 mg/L), and vigorously rocked at maximum speed at 4 ºC for 30 min. The solution was clarified by centrifugation at 20 800×*g* for 5 min, and the supernatant fluid (nuclear extract) was stored in aliquots at -70 ºC. The protein concentration was determined by the Folin method.

Western-blot analysis Nuclear or cytoplasmic extracts (30 μg total protein/lane) were separated by 8 % SDS-polyacrylamide gel electrophoresis and blotted to polyvinylidenedifluoride membranes using a semi-dry blotting apparatus. Membranes were blocked (1 h, room temperature) in 5 % non-fat dried milk plus Tris-buffered saline (TBS) plus 0.05 % Tween 20 (blocking buffer), incubated with primary antibody (1 h, room temperature) in blocking buffer, and washed 5 times with TBS/0.05 % Tween 20 before incubation (1 h, room temperature) with secondary HRP-conjugated antibody in blocking buffer. After successive washes, the membranes were developed with an enhanced chemiluminescence kit. Anti-p65 Ab, anti-IκBα Ab, and HRP-conjugated IgG were applied at a dilution of

1:2000. Semi-quantitative analysis of immunoreactivity was measured by Lab Works image acquisition and analysis software (UVP GDS 8000, USA), and the results were expressed as OD (optical density).

Statistical analysis of data The data are presented as means±SD and compared with ANOVA and least significant difference test using SPSS statistical program. The level of the statistical significance was set at $P<0.05$.

RESULTS

Effect of α-pinene on the subcellular localization of NF-κB/p65 in THP-1 cells The dual-color images of FITC-labeled NF-κB/p65 and PI-labeled nuclei in each groups were detected by indirect immunofluorescence and confocal microscopy. Samples were immunocytochemically labeled with FITC for NF-κB/p65 protein in green, followed by incubation with the nuclear stain PI in red (Fig 1). Normal THP-1 cells were labeled in the absence of primary antibody to NF-κB/p65 to identify autofluorescence and nonspecific labeling, a faint or invisible signal of FITC-labelled green, but only PI label red was observed in these cell sheets (Fig 1.1). There was a strong nuclear staining of NF-κB/p65 after stimulation with LPS 1 mg/L for 30 min in THP-1 cells (Fig 1.3) compared with the cytoplasmic distribution in unstimulated cells (Fig 1.2). This nuclear translocation of NF-κB/p65 by LPS was blocked by α-pinene 100 mg/L pretreatment as demonstrated by the cytoplasmic staining pattern (Fig 1.4). This change suggested α-pinene inhibited the LPS-mediated NF-κB/p65 internalization.

Effect of α-pinene on NF-κB/p65 nuclear translocation In the unstimulated group, THP-1 cells were only incubated with α-pinene for 30 min. Treatment with α-pinene for 30 min respectively in 1, 10, and 100 mg/L did not significantly affect the NF-κB nuclear translocation in normal THP-1 cells (Fig 2).

In the stimulated group, THP-1 cells were pretreated with α-pinene for 30 min before addition of LPS 1 mg/L for 30 min. In the presence of α-pinene, the LPS-induced NF-κB nuclear translocation was markedly inhibited. This inhibitory effect of α-pinene was in a dose-dependent manner (Fig 3).

The α-pinene concentration (1, 10, and 100 mg/L for 30 min) was determined from our previous work showing significant effects in THP-1 cells without toxicity. The cell viability evaluated by MTT assay did not change in both cells at all doses used (data not shown).

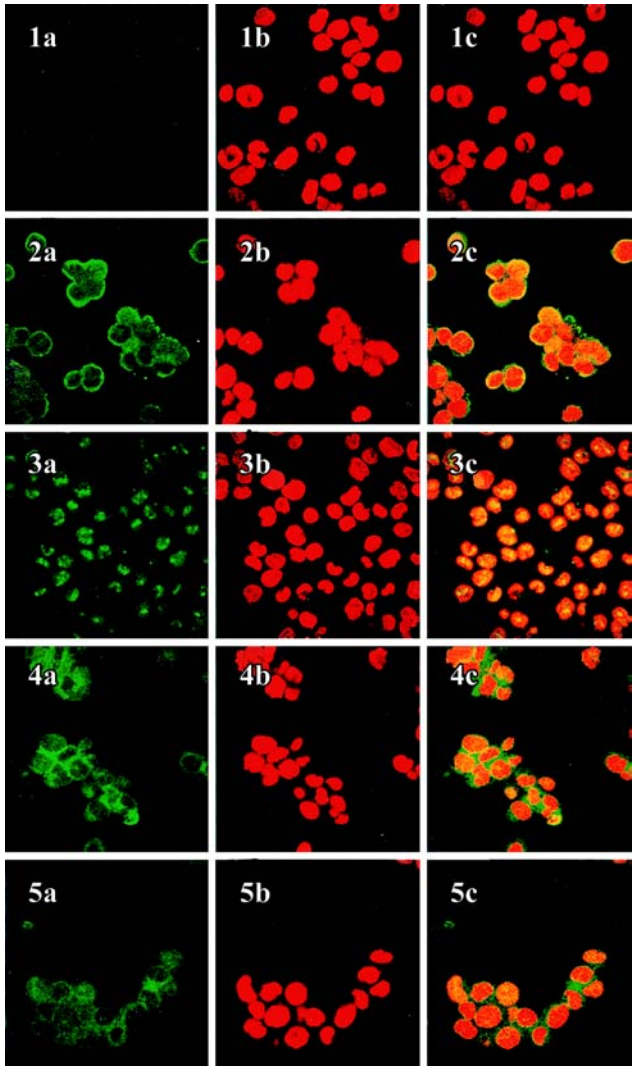

Fig 1. Indirect immunofluorescence and confocal microscopy analysis demonstrating the effect of α-pinene on LPS-induced subcellular localization of NF-κB/p65 in THP-1 cells. (1) The normal THP-1 cells labeled in the absence of primary antibody to NF-κB/p65; (2) control; (3) LPS 1 mg/L for 30 min; (4,5): α-pinene 100 mg/L or TLCK 50 μmol/L for 30 min before the addition of LPS for 30 min, respectively. a, b, c were green channel, red channel and the combining fig. Results are representatives of three independent experiments.

Effect of α-pinene on LPS-mediated degradation of IκBα in THP-1 cells The IκBα protein level in LPS-stimulated THP-1 cells was markedly decreased at 30 min. The LPS-induced degradation of IκBα was blocked by α-pinene (Fig 4).

DISCUSSION

NF-κB is made up of two subunits. The most

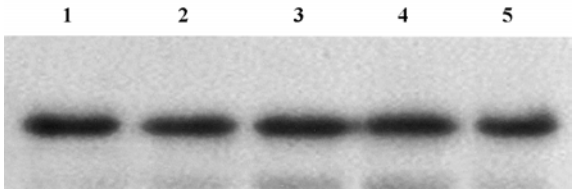

Fig 2. Representative Western-blot analysis of nuclear proteins demonstrating the effect of α-pinene on nuclear translocation of the NF-κB/p65 in normal THP-1 cells. Lane 1: control; Lane 2-5: α-pinene 1, 10, and 100 mg/L or TLCK 50 μmol/L for 30 min, respectively. Results are representatives of three independent experiments.

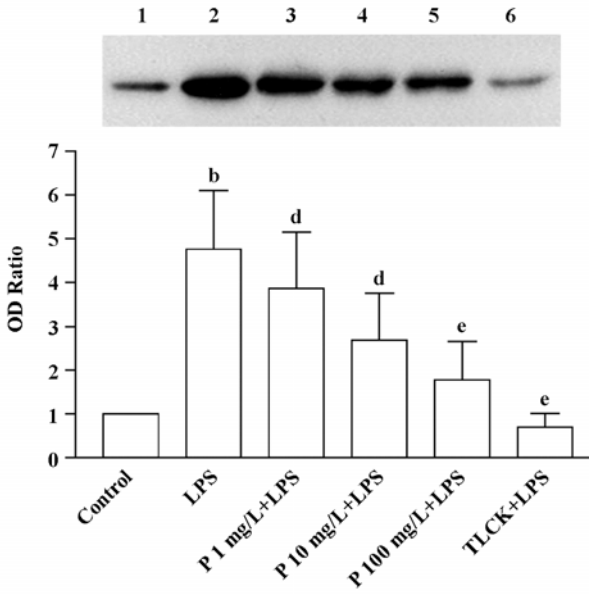

Fig 3. Representative Western-blot analysis of nuclear proteins demonstrating the effect of α-pinene on LPS-mediated nuclear translocation of the NF-κB/p65 in THP-1 cells. Lane 1: control; Lane 2: LPS 1 mg/L for 30 min; Lane 3-6: α-pinene 1, 10, and 100 mg/L or TLCK 50 μmol/L for 30 min before the addition of LPS for 30 min, respectively. *n*=3. Mean±SD. $^{b}P<0.05$ *vs* control. $^{d}P>0.05$, $^{e}P<0.05$ *vs* LPS.

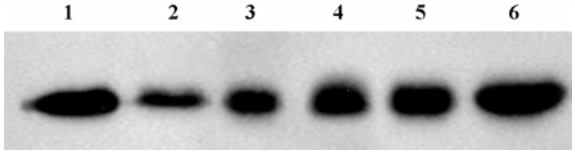

Fig 4. Representative Western-blot analysis of cytoplasmic proteins demonstrating the effect of α-pinene on LPS-mediated degradation of IκBα in THP-1 cells. Lane 1: control; 2: LPS 1 mg/L for 30 min; Lane 3-6: α-pinene 1, 10, and 100 mg/L or TLCK 50 μmol/L for 30 min before the addition of LPS for 30 min, respectively. Results are representatives of three independent experiments.

usual form of NF-κB is a heterodimer of p65 and p50, p65 contain a transactivation domain. The heterodimer normally exists in the cytoplasm in a dormant form bound to an inhibitory protein, IκB, of which several forms exist. IκBα is a mainly form related to inflammatory responses. In response to a variety of proinflammatory signals, such as LPS, TNF-α, viruses, and oxidants, IκBα is rapidly degraded by a phosphorylation-dependent and ubiquitination-dependent mechanism. Rapid degradation of IκBα allows for nuclear translocation of NF-κB where it directs the expression of target genes[3,4].

NF-κB plays a key role in immune and inflammatory responses. NF-κB activation can lead to enhanced expression of proinflammatory cytokines, chemokines, inflammaroty enzymes such as inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), adhension molecules and inflammatory receptors[4,7,8]. Therefore, modulation of NF-κB activation may provide a direct way of inhibiting inflammatory mediators[9]. Directing drug discovery efforts towards NF-κB activation rather than towards any one of its many target genes could produce a much greater therapeutic benefit by inhibiting expression of the constellation of NF-κB-induced pro-inflammatory genes[10].

TLCK is a serine protease inhibitor. It antagonizes the translocation of NF-κB indirectly by blocking the signalling pathway for stimuli-induced phosphorylation of IκBα and thus preventing its degradation[11].

Eucalyptus oil shows particular anti-inflammation and anti-oxidation properties[12]. As the active agent and main component of eucalyptus oil, α-pinene is an ethereal oil with a camphor-like smell. Martin has investigated the anti-inflammation activity of α-pinene against rat hindpaw edema induced by carrageenin or by PGE_1[2]. α-Pinene at dose of 150 or 300 mg/kg, being administered orally 1 h before the injection of the inflammatory agent, showed anti-inflammatory activity against carrageenin-induced edema, which was greater at 5 h after administration of inflammatory agent (the maximum percentages of anti-inflammatory activity were 31.5 % and 45.7 %, respectively). At the same dose, α-pinene also markedly inhibited the inflammation induced by PGE_1, which maintained its activity from 15 min after injection of PGE_1 as long as the experiment lasted[2].

In this study, we investigated the effects of α-pinene on NF-κB nuclear translocation in THP-1 cells. Our studies showed that in a dose-dependent fashion,

α-pinene inhibited the NF-κB nuclear translocation induced by LPS in THP-1 cells (Fig 1.4, 3), but did not significantly affect the nuclear translocation of NF-κB in normal THP-1 cells (Fig 2). α-Pinene is an inhibitor of NF-κB nuclear translocation in THP-1 and this effect is partly due to the upregulation of IκBα expression (Fig 4). The effects of TLCK (Fig 1.5, 3, 4) on NF-κB and IκBα in LPS-induced THP-1 cells were similar with α-pinene. These data indicated that stabilization prevents IκBα degradation and NF-κB nuclear translocation, thus potentially blocking the gene regulatory effects of NF-κB, which represents one of the anti-inflammatory mechanisms of α-pinene.

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