International Immunopharmacology 15 (2013) 316–324

Contents lists available at SciVerse ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Taraxerol inhibits LPS-induced inflammatory responses through suppression of TAK1 and Akt activation

Xiangyang Yao [a,*], Guilan Li [b], Qin Bai [c], Hui Xu [a], Chaotian Lü [a]

[a] Department of Biology and Food Engineering, Bengbu College, Bengbu, PR China
[b] College of Animal Science and Veterinary Medicine, Shanxi Agricultural University, Taigu, PR China
[c] Anhui Fukang Pharmaceutical Co., Ltd., Bengbu, PR China

A R T I C L E I N F O

Article history:
Received 19 October 2012
Received in revised form 27 December 2012
Accepted 31 December 2012
Available online 15 January 2013

Keywords:
Taraxerol
TAK1
MAPK
Akt
NF-κB
RAW264.7 cells

A B S T R A C T

Taraxerol, a triterpenoid compound, has potent anti-inflammatory effects. However, the molecular mechanisms are not clear. In the study, taraxerol concentration dependently inhibited nitric-oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at the protein and mRNA levels and these inhibitions decreased the production of nitric oxide (NO), prostaglandin 2 (PGE_2), tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β induced by LPS. Furthermore, we found that taraxerol suppressed translocation of nuclear factor-κB (NF-κB), phosphorylation of IκBα, blocked the IκBα degradation as well as IKK and mitogen-activated protein kinase (MAPK) activation by inactivation of TGF-β-activated kinase-1 (TAK1) and Akt. In addition, taraxerol significantly inhibited the formation of TAK1/TAK-binding protein1 (TAB1), which was accompanied by inducing degradation of TAK1, decreasing LPS-induced polyubiquitination of TAK1 as well as TAK1 phosphorylation. Taken together, our data suggest that taraxerol downregulates the expression of proinflammatory mediators in macrophages by interfering with the activation of TAK1 and Akt, thus preventing NF-κB activation.

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1. Introduction

LPS ligation to TLR4 promotes the recruitment of a complex of proteins to the membrane, including the adaptor MyD88, IRAKs, TRAF6, and TAK1 [1]. The complex eventually results in the activation of TAK1 by the formation of a complex with the adaptor proteins TAB1 and TAB2/3, autophosphorylation and Lys^{63}-linked polyubiquitination of TAK1 [2]. TAK1 is essential for LPS-stimulated signaling pathways [3]. The TAK1 complex mediates the activation of IKK and MAPK pathways in LPS-induced cellular responses [4]. The active IKK phosphorylates IκBα, leading to sequent ubiquitination and degradation of IκBα, and translocation of NF-κB into the nucleus [5]. MAPK enhances the transcription of NF-κB-mediated pro-inflammatory genes [6]. MAPK also increases AP-1 activity through the phosphorylation of distinct substrates [7,8]. These signaling pathways in turn coordinate the expression of various proinflammatory mediators such as NO, PGE_2, IL-1β, IL-6 and TNF-α [1,5]. Thus, suppression of the signaling molecules may have great potential for preventing and treating of inflammatory diseases.

It has been demonstrated that LPS stimulation contributes to activation of the PI3K/Akt pathway [9]. Moreover, pharmacologic PI3K inhibitors prevent LPS-induced NF-κB translocation [10–12]. However, PI3K/Akt pathway plays both positive and negative roles in TLR signaling [13,14]. Therefore, disrupting the pathway may have anti-inflammatory effect, depending on specific conditions.

Taraxerol (Fig. 1) is a member of the family of triterpenoids, and has been isolated from a variety of plants including dandelion. It has been reported to exhibit anti-diabetic potential [15] and potent anti-inflammatory effects [16]. However, the molecular mechanisms are not elucidated. This study aimed to determine the anti-inflammatory effects of taraxerol on LPS-activated RAW264.7 macrophages.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies to COX-2 (C20), PARP and GAPDH were obtained from Santa Cruz Biotechnology. Monoclonal antibody to iNOS was from BD Pharmingen. NF-κBp65, phospho-IKKα/β, IKKα/β, IκBα, phospho-IκBα (Ser32), ubiquitin, phospho-Akt (Ser473), Akt, polyclonal JNK/SAPK, phospho-JNK/SAPK (Thr183/Tyr185), p38MAPK, phospho-p38MAPK (Thr180/Tyr182), p42/p44MAPK, phospho-p42/44MAPK (Thr202/Tyr204), TAB1, TAK1, and phospho-TAK1 antibodies were purchased from Cell Signaling Technology. Anti-Lys^{63}-specific ubiquitin and anti-Lys^{48}-specific ubiquitin were from Millipore. All secondary antibodies were purchased from Rockland Immunochemicals. LPS (from *Escherichia coli* 0111: B4), MTT, MG132 and LY294002 were obtained from Sigma. Taraxerol (purity 95.0%, HPLC) was purchased from Shanghai R&D Center for Standardization of Traditional Chinese Medicine (China).

* Corresponding author at: Department of Biology and Food Engineering, Bengbu College, No. 1866 Caoshan Road, Bengbu 233030, PR China. Tel./fax: +86 552 3177259.
E-mail address: leeyxy@gmail.com (X. Yao).

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http://dx.doi.org/10.1016/j.intimp.2012.12.032

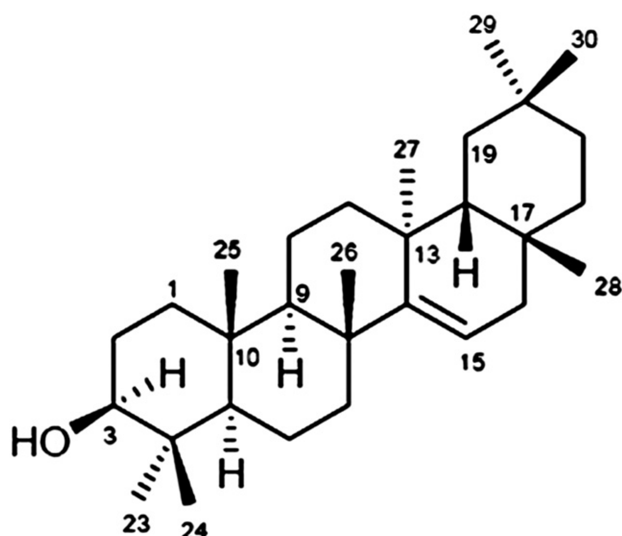

Fig. 1. Chemical structure of taraxerol.

2.2. Cell culture and transfection

RAW264.7 murine macrophage-like cells, purchased from the CBCAS (Cell Bank of the Chinese Academic of Sciences, Shanghai, PR China), were cultured in DMEM (Invitrogen) containing 10% (v/v) fetal bovine serum (HyClone) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (HyClone) at 37 °C in an atmosphere of 5% CO_2. Transient transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In all cases, the total amount of DNA was normalized by the addition of empty control plasmids.

2.3. Cell viability assay

Cell viability was determined by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW264.7 cells were seeded into 96-well plates (5×10^4 cells/well) 24 h before treatment. The cells were treated with various concentrations of taraxerol or LPS (100 ng/ml) plus taraxerol for 24 h. Then cells were incubated with 5 mg/ml of MTT working solution for 4 h at 37 °C. After being treated with 100 μl of DMSO to dissolve the crystals, the cells were detected under an ELx800 Universal Microplate Reader (Bio-Tek, Inc.) to measure the absorbance at 570 nm.

2.4. Western blotting

The cells were treated with taraxerol for 1 h before exposure to 100 ng/ml LPS for the different times. Cells were rinsed twice with ice-cold PBS, and solubilized in lysis buffer containing 20 mM Tris (pH 7.5), 135 mM NaCl, 2 mM EDTA, 2 mM DTT, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF for 30 min on ice. Lysates were centrifuged (15,000 ×g) at 4 °C for 10 min. Equal amounts of the soluble protein are denatured in SDS, electrophoresed on an 11% SDS-polyacrylamide gel, and transferred to nitrocellulose membranes. The antibody–antigen complexes were visualized by the LI-COR Odyssey Infrared Imaging System according to the manufacturer's instruction using IRDye 800 fluorophore-conjugated antibody (LI-COR Biosciences, Lincoln, NE) Quantization was directly performed on the blot using the LI-COR Odyssey Analysis software.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RAW264.7 cells were stimulated for 8 h with 100 ng/ml of LPS in the presence of various concentrations of taraxerol. Total RNA was isolated using TRIzol reagent (Gibco, USA) according to the manufacturer's instruction. RT-PCR was carried out by using an Access RT-PCR System kit (Promega, USA) with indicated primers (COX-2: sense primer 5′-tctccaacctctcctactac-3′, antisense primer 5′-gcacgtagtcttcgatcact-3′; GAPDH: sense primer 5′-tgaaggtcggtgtgaacggatttggc-3′, antisense primer 5′-tggttcacacccatcacaaacatgg-3′). PCR was performed for 30 cycles in 25 μl of reaction mixture and the products were visualized in 1.2% agarose gels stained with GoldView. GAPDH was utilized as a housekeeping gene as indicated.

2.6. Luciferase reporter assay

NF-κB-Luc and AP-1-Luc reporter vectors were generous gifts from Dr. Ze'ev Ronai (The Burnham Institute, USA). All plasmids were purified using the Wizard Plus SV Minipreps DNA Purification System Kit (Promega, USA). RAW264.7 cells cultured in 12-well plates were transiently transfected with NF-κB-Luc plasmids together with the pCMV-β-galactosidase (β-gal) control vector. Twenty-four hours after transfection, the cells were incubated with or without taraxerol for 1 h and then treated with or without LPS (100 ng/ml) for another 24 h. Cell lysates were prepared to measure luciferase activities using the Luciferase Assay System (Promega, USA) and analyzed by the Luminometer TD-20/20 (Turner, USA). Luciferase activity was normalized to β-gal activity.

2.7. Preparation of cytoplasmic and nuclear extracts

Cells were incubated with various concentrations of taraxerol for 1 h, followed by LPS stimulation (100 ng/ml) for 1 h. Cells were washed with ice-cold PBS and then lysed with hypotonic buffer (20 mM Hepes (pH 7.9), 10 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10% glycerol, 0.1 mM Na_3VO_4, 1 mM PMSF, protease inhibitor) with 0.2% NP-40 on ice for 10 min. To separate the cytoplasmic and nuclear fractions, cell pellets were processed using the NE-PER nuclear and cytoplasmic extraction kit (Pierce) according to the manufacturer's instructions. Protein concentration was determined using a Bio-Rad protein assay kit.

2.8. Determination of NO and cytokines

RAW264.7 cells were incubated with/without taraxerol for 1 h or LY294002 for 30 min before incubating with LPS (100 ng/ml) for 24 h. NO synthesis was determined by assaying the culture supernatants for nitrite using the Griess reagent (1% sulfanilic acid, 0.1% N-1-naphthyl-ethylenediamine dihydrochloride, and 5% phosphoric acid). Absorbance was measured at 550 nm and nitrite concentration was determined using sodium nitrite as a standard. PGE_2, TNF-α, IL-6 and IL-1β levels in cell culture media were quantified by using ELISA kits (R&D Systems), according to the manufacturer's instructions.

2.9. Statistics

Analysis of variance (ANOVA) was used to compare the results between two groups. Individual points were compared using a Student's *t* test and differences were considered significant for $P < 0.05$. Data are presented as means ± SD. Western blotting analysis experiments were repeated 3 times with similar trends.

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