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Anti-inflammatory effects of 18-nor-*ent*-pimara-9(11),15-diene-4 β -ol isolated from the roots of *Aralia continentalis* on LPS-induced in RAW264.7 cells

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Abstract

Aralia continentalis (*A. continentalis*) is a medicinal plant belonging to Araliaceae, it has been reported to exert anti-cancer, anti-bacterial, anti-inflammatory, anti-platelet and anti-oxidative activities. But the potential mechanism for the anti-inflammatory effect of compounds isolated from the roots of *A. continentalis* is still insufficient. So, we evaluated whether compounds isolated from the roots of *A. continentalis* exert anti-inflammatory effects and elucidated its potential mechanism in RAW264.7 cells. The concentrated residue was subsequently suspended in H₂O and partitioned with *n*-hexane, methylene chloride (CH₂Cl₂), ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH). The fractions were subjected to sequential column chromatography over silica-gel, RP-18, MPLC, recycling and preparative HPLC to isolated the novel compound. The novel compound was identified as 18-nor-*ent*-pimara-9(11),15-diene-4 β -ol and confirmed anti-inflammatory activity. The 18-nor-*ent*-pimara-9(11),15-diene-4 β -ol dose-dependently blocked NO production and inhibited iNOS, COX-2, TNF- α and IL-1 β expression in LPS-stimulated RAW264.7 cells. The 18-nor-*ent*-pimara-9(11),15-diene-4 β -ol inhibited LPS-stimulated degradation of I κ B- α and nuclear accumulation of p65, which resulted in the suppression of NF- κ B activation in RAW264.7 cells. Also, the 18-nor-*ent*-pimara-9(11),15-diene-4 β -ol attenuated the phosphorylation of p38 and ERK1/2 in LPS-induced RAW264.7 cells. These results suggest that the nor-*ent*-pimara-9(11),15-diene-4 β -ol isolated from the roots of *A. continentalis* may have great potential for the development of anti-inflammatory drugs.

Keywords: Anti-inflammation, *A. continentalis*, MAPK, NF- κ B, Novel compound

Introduction

In recent years, interest and expectations for medicinal resource plants have been increased, and many studies have been conducted to extract effective ingredients from natural resources and find functional materials because compounds present in medicinal resource plants have a

variety of physiological vitality and are useful for living things [1–3].

Inflammatory reactions are reported to be involved in various pathological mechanisms, such as the introduction of bacteria and viruses into the body, in which the immune cells recognize and protect the body by secreting various inflammatory intermediaries, thereby promoting the growth of cancer cells, increasing insulin resistance, and worsening arteriosclerosis. The macrophages are known to be one of the important immune cells controlling the inflammatory response, responding to the early stages of the tumor necrosis factor- α (TNF- α), cytokines and lipopolysaccharide (LPS) infection and playing a

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pivotal role in the defense of the host and in maintaining the star. In particular, they are known to be involved in inflammatory reactions such as prostaglandin E₂ (PGE₂), nitric oxide (NO), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). These substances are reported to be involved in the development of inflammatory diseases. Inhibition of inflammatory factors is very effective in preventing the onset and progression of inflammatory diseases [4–6]. The nuclear factor kappa-B (NF-κB) activation is significantly associated with inflammatory diseases and much attention is focused on developing anti-inflammatory drugs targeting NF-κB. The mitogen-activated protein kinases (MAPK) cascade is one of the important signaling pathways in immune responses, and several recent studies reported that inhibition of MAPKs in mast cells can be a suitable target for pharmacological treatment of inflammatory diseases [7, 8].

Aralia continentalis (*A. continentalis*) is a medicinal plant belonging to Araliaceae, it has been reported to exert anti-cancer, anti-bacterial, anti-inflammatory, anti-platelet and anti-oxidative activities. The root of *A. continentalis* is known to contain essential oil (α -pinene, β -pinene and sabinene) 1–2%, stearic acid 0.07%, resin, salicylic acid and diterpenic acid I, II, copper, manganese and nickel [9, 10]. We isolated 18-nor-ent-pimara-9(11),15-diene-4 β -ol from the roots of *A. continentalis* in previous study [11]. In this study, we investigated anti-inflammatory activity of 18-nor-ent-pimara-9(11),15-diene-4 β -ol isolated from the ethanol extract of the roots *A. continentalis*.

Materials and methods

Chemical reagents

Lipopolysaccharide (LPS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against phospho-I κ B- α , I κ B- α , phospho-extracellular signal-regulated kinase1/2 (p-ERK1/2), ERK1/2, phospho-p38 (p-p38), p38, p65 and β -actin were purchased from Cell Signaling technology (Beverly, MA, USA).

Isolation and confirmation of 18-nor-ent-pimara-9(11),15-diene-4 β -ol from roots of *A. continentalis*

The roots of *A. continentalis* (5 kg) were extracted with 70% ethanol for 2 days. The ethanol-soluble fraction was filtered and concentrated using a vacuum evaporator at 40 °C. This extract was suspended in distilled water and successively partitioned with n-hexane, dichloromethane (CH₂Cl₂), ethyl acetate (EtOAc) and n-butanol (n-BuOH). Repeated chromatography of the n-hexane fraction over a SiO₂ column with an n-hexane:EtOAc

solvent system (50:1 to 1:1 gradient), resulted in 14 sub-fractions. The novel compound [18-nor-ent-pimara-9(11),15-diene-4 β -ol] was isolated and identified by spectroscopic methods, including ¹H (700 MHz) and ¹³C NMR (175 MHz). Preparative HPLC was performed on a Shimadzu system (LC-8A pump and SPD-20A UV/VIS detector, Kyoto, Japan) using a YMC-Pack ODS A column (I.D. 250 × 20 mm), and a mixed solvent system of acetonitrile:H₂O (50:50 to 90:10 gradient, 50 min.) at a flow rate of 12 ml/min.

Cell culture

Mouse macrophage cell line, RAW264.7 cells were purchased American Type Culture Collection (ATCC, Virginia, USA) and grown in Dulbecco's Modified Eagle (Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin. The cells were cultured in an incubator containing 5% CO₂ at 37 °C. Trypsin–EDTA (Sigma, St. Louis, MO, USA) was used to detach the cells from a T75 flask. The novel compound was dissolved in dimethyl sulfoxide (DMSO) and treated to cells. DMSO was used as a vehicle and the final DMSO concentration did not exceed 0.1% (v/v).

Cell viability

Cell viability was performed by MTT assay. Briefly, cells were plated at a density of 1 × 10⁶ cells/well in 12-well plate and incubated for 24 h. The cells were treated with 18-nor-ent-pimara-9(11),15-diene-4 β -ol at the indicated concentrations for 24 h. Then, the cells were incubated with 200 μ l of MTT solution (1 mg/ml) for an additional 2 h. The resulting crystals were dissolved in DMSO. The formation of formazan was measured by reading absorbance at a wavelength of 570 nm using UV/Visible (Perkin Elmer, Nowolk, CT, USA).

Nitric oxide generation assay

RAW264.7 cells were incubated 12-well plate for overnight. The cells were pretreated with 18-nor-ent-pimara-9(11),15-diene-4 β -ol at the indicated concentrations for 6 h and then co-treated with LPS (1 μ g/ml) for 18 h. NO levels were evaluated by Griess assay. Briefly, 50 μ l of the cell culture supernatants were mixed with 50 μ l of Griess reagent (Sigma Aldrich, St. Louis, MO, USA) and followed by reaction for 10 min at the room temperature. After 10 min, absorbance values were determined using a UV spectrophotometer (Perkin Elmer, Nowolk, CT, USA) at 540 nm.

Isolation of cytosol and nucleus fraction

Nucleus fractions from RAW264.7 cells after treatment of 18-nor-ent-pimara-9(11),15-diene-4 β -ol and LPS

were prepared using a nuclear extract kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocols. Briefly, we harvested RAW264.7 cells with cold 1Xhypotonic buffer and reacted at 4 °C for 15 min. After adding detergent and vortexing for 10 s, the cells were centrifuged at 15,000 rpm for 10 min at 4 °C and the supernatants (cytoplasmic fraction) were collected and stored at - 80 °C for further analysis. The cell pellets were used for nuclear fraction collection. Cell pellets were re-suspended with lysis buffer by pipetting up and down, and incubated at 4 °C for 30 min under shaking. After 30 min, nuclear suspensions were centrifuged at 15,000 rpm for 10 min at 4 °C, and the supernatants (nuclear fraction) were stored at - 80 °C for further analysis.

SDS-PAGE and western blot analysis

After treatment with 18-nor-*ent*-pimara-9(11),15-diene-4β-ol and LPS, cells were washed twice with cold 1 × phosphate-buffered saline (1XPBS), and lysed in radio immune precipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA, USA) supplemented with protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA) and phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA), and centrifuged at 15,000 rpm for 10 min at 4 °C. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as the standard.

We separated the equal proteins on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred them to nitrocellulose membrane. The nitrocellulose membranes were blocked with 5% non-fat dry milk in tris-buffered saline containing 0.05% tween 20 (TBS-T) by stirring at room temperature for 1 h and then incubated with specific primary antibodies in 5% skim dry milk in 0.05% TBS-T at 4 °C for overnight. After three washes with TBS-T, the blots were incubated with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h at room temperature and chemiluminescence was detected with ECL western blotting substrate and visualized in Chemi Doc MP Imaging system (Bio-rad, CA, USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

After treatment of 18-nor-*ent*-pimara-9(11),15-diene-4β-ol and LPS, total RNA was extracted from RAW264.7 cells using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and total RNA (1 μg) was synthesized using a Verso cDNA Kit (Thermo Scientific, Pittsburgh, PA, USA) according to the manufacturer's protocol. PCR was performed using PCR Master Mix

Kit (Promega, Madison, WI, USA) and mouse primers for iNOS, COX-2, IL-1β and GAPDH (Table 1).

Statistical analysis

All the data are shown as mean ± SD (standard deviation). Statistical analysis was determined by Student's t-test. Differences with *P or #P < 0.05 were considered statistically significant.

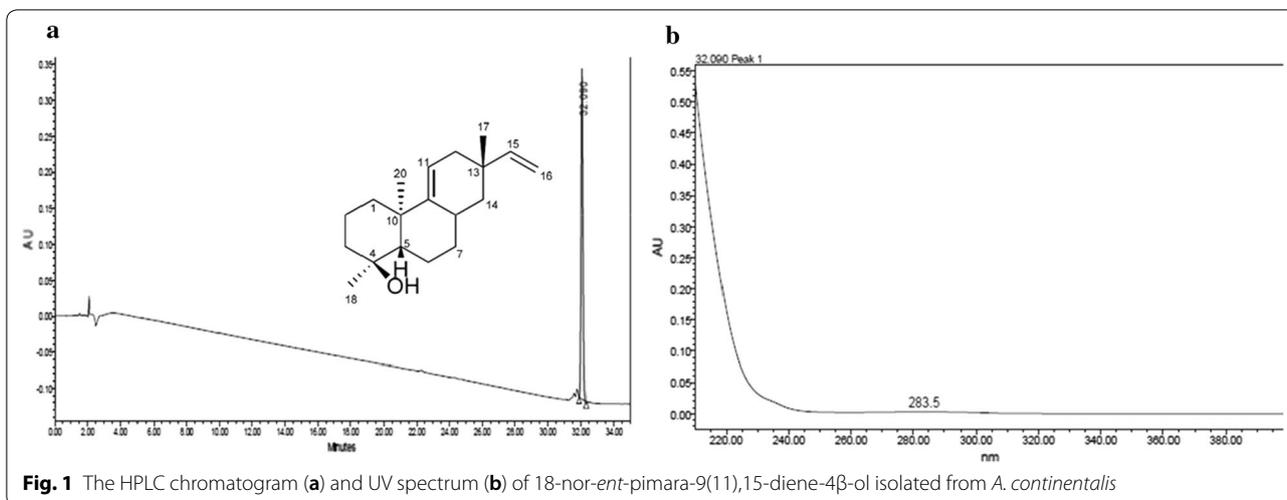
Results and discussion

Confirmation of 18-nor-*ent*-pimara-9(11),15-diene-4β-ol from *A. continentalis*

The chemical structure of 18-nor-*ent*-pimara-9(11),15-diene-4β-ol was confirmed by comparing the UV, ¹H-NMR, ¹³C-NMR and 2D-NMR spectral data to published spectra. 18-nor-*ent*-pimara-9(11),15-diene-4β-ol appeared as white amorphous powder [11]; ¹H-NMR (700 MHz, CDCl₃) δ 5.82 (1H, dd, J = 17.5, 10.5 Hz, H-15), 5.38 (1H, qui, J = 2.1 Hz, H-11), 4.93 (1H, dd, J = 17.5, 2.1 Hz, Ha-16), 4.87 (1H, dd, J = 10.5, 2.1 Hz, Hb-16), 2.36 (1H, m, H-8), 2.03 (1H, m, Ha-12), 1.99 (1H, m, Ha-6), 1.82 (1H, m, Ha-1), 1.79 (1H, m, Ha-3), 1.77 (1H, m, Hb-12), 1.74 (1H, m, H-5), 1.73 (1H, m, Ha-7), 1.60 (1H, dt, J = 7.0, 3.5 Hz, Ha-2), 1.56 (1H, m, Hb-6), 1.47 (1H, dt, J = 14.0, 3.5 Hz, Hb-2), 1.41 (1H, dq, J = 12.6, 2.1 Hz, Ha-14), 1.36 (1H, m, Ha-3), 1.26 (1H, m, Hb-7), 1.24 (1H, m, Hb-1), 1.21 (3H, s, H-19), 1.04 (1H, m, Hb-14), 1.03 (3H, s, H-20), 0.97 (3H, s, H-17); ¹³C-NMR (175 MHz, CDCl₃) δ 150.5 (C-9), 150.3 (C-15), 116.3 (C-11), 109.1 (C-16), 73.2 (C-4), 47.3 (C-5), 43.1 (C-3), 41.7 (C-14), 40.3 (C-1), 38.7 (C-10), 37.6 (C-12), 34.9 (C-13), 29.4 (C-8), 26.5 (C-7), 24.3 (C-20), 23.4 (C-19), 22.4 (C-17), 20.9 (C-2), 17.5 (C-6); ESI-MS m/z 297 [M + Na]⁺. The HPLC chromatogram and UV spectrum of the 18-nor-*ent*-pimara-9(11),15-diene-4β-ol was shown in Fig. 1.

Table 1 Sequence of oligonucleotide primers used for RT-PCR

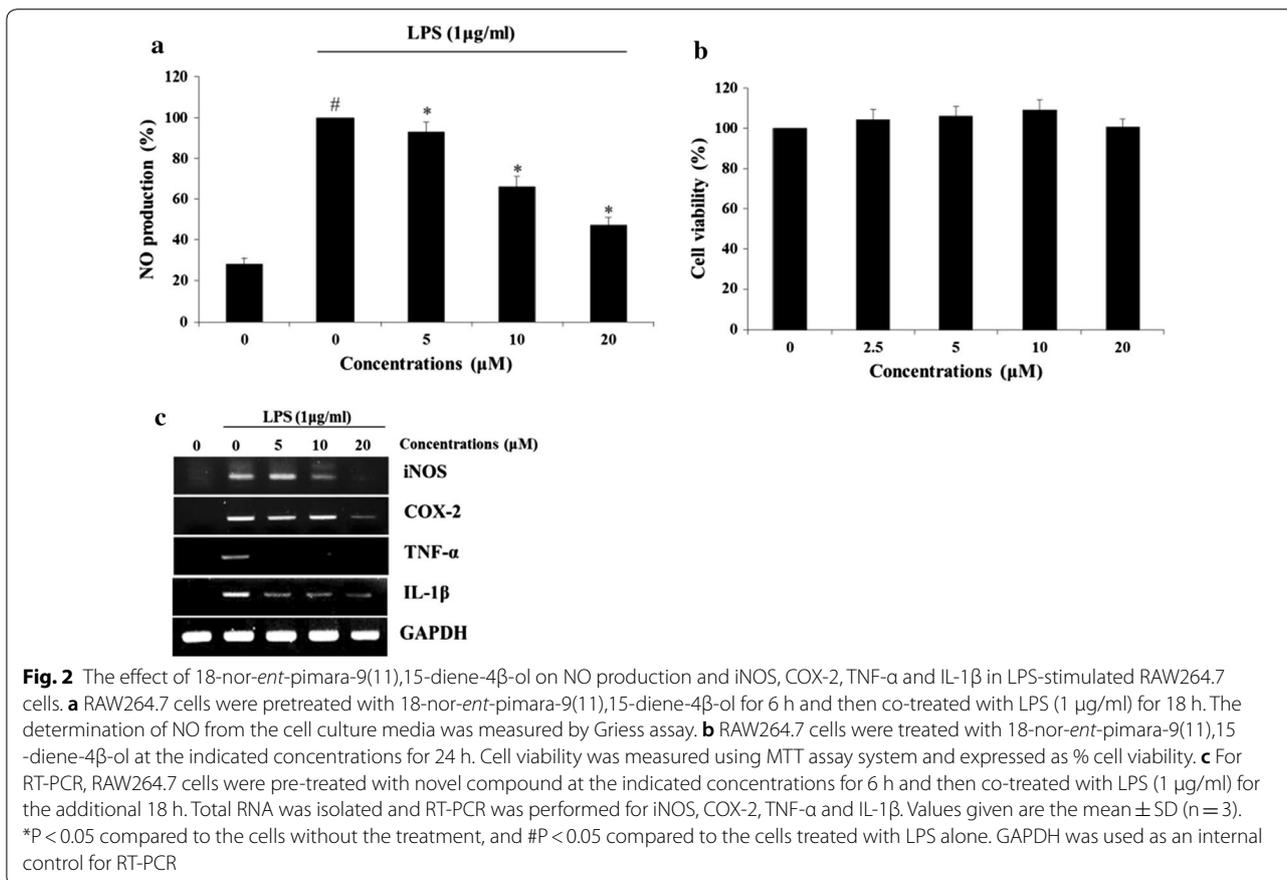
Gene name	Sequence
iNOS	Forward 5'-GTGCTGCCTCTGGTCTTGCAAGC-3' Reverse 5'-AGGGGCAGGCTGGAATTTCG-3'
COX-2	Forward 5'-GGAGAGACTATCAAGATAGTGATC-3' Reverse 5'-ATGGTCAGTAGACTTTTACAGCTC-3'
TNF-α	Forward 5'-TACTGAACTTCGGGGTGATTGGTCC-3' Reverse 5'-CAGCCTTGTCCCTTGAAGAGAACC-3'
IL-1β	Forward 5'-GAAGCTGTGGCAGCTACCTATGTCT-3' Reverse 5'-CTCTGCTTGTGAGGTGCTGATGTAC-3'
GAPDH	Forward 5'-CAGGAGCGAGACCCCACTAATCAT-3' Reverse 5'-GTCAGATCCACGACGGACACATT-3'



The effect of 18-nor-ent-pimara-9(11),15-diene-4β-ol on NO production and iNOS, COX-2, TNF-α and IL-β expression in LPS-stimulated RAW264.7 cells

The nitric oxide (NO) has been known as one of the most multifaceted players in the immune system. It is included

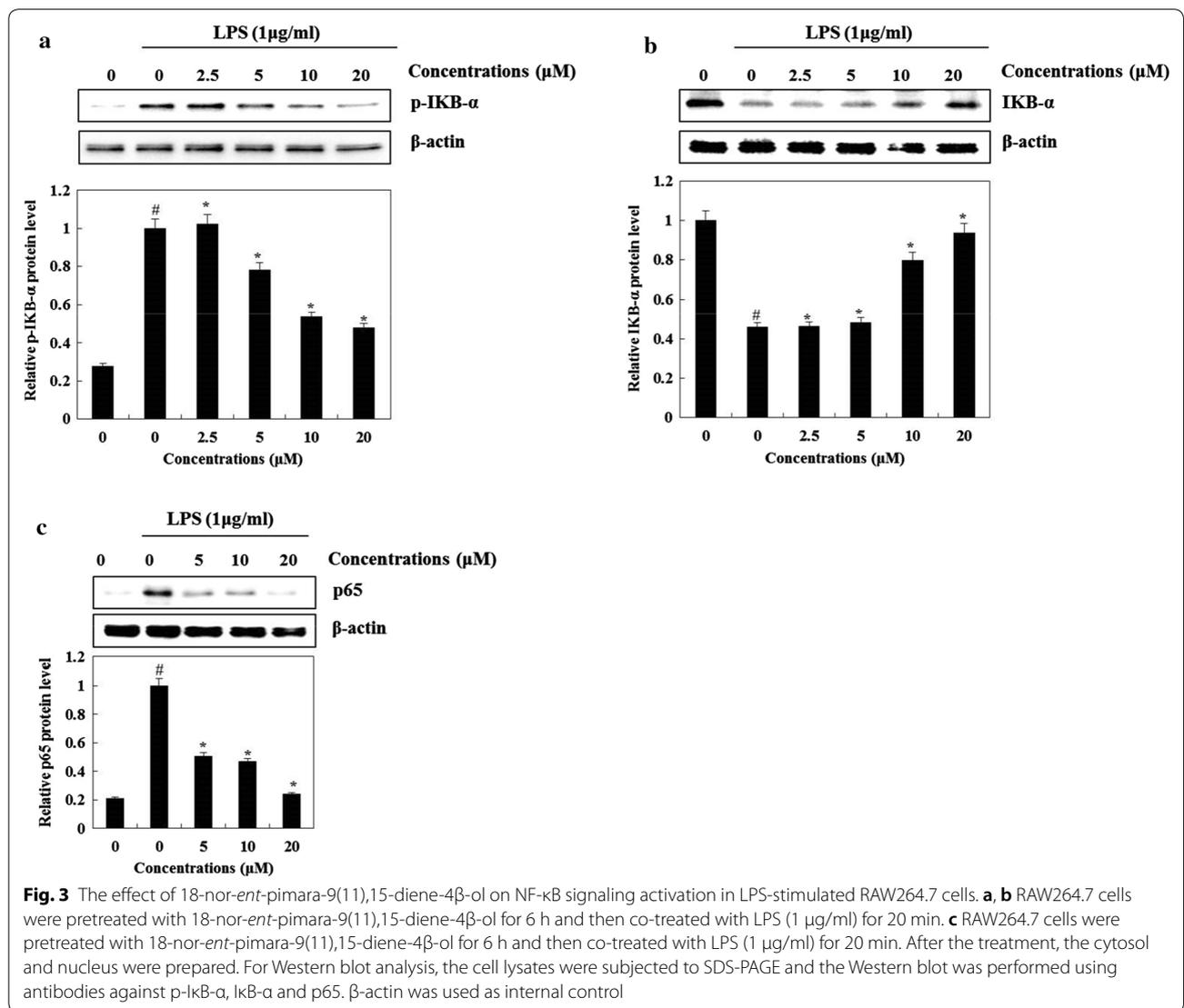
in the pathogenesis and control of tumors, infectious diseases, chronic degenerative diseases and autoimmune processes [12, 13]. NO is synthesized in mammalian cells by a family of three NO synthesis (NOS). The neuronal NOS (nNOS, Type I NOS) and endothelial NOS (eNOS,



type III NOS) have been classified as constitutive NOS because these are continuously present in cells, while an inducible NOS (iNOS, type II NOS), is revealed only after exposure to specific stimulants such as cytokines and bacterial endotoxic lipopolysaccharide (LPS) in macrophages and hepatocytes [14–19].

So, in the study, to determine if the 18-nor-ent-pimara-9(11),15-diene-4β-ol could reduce NO generation by LPS, RAW264.7 cells were pretreated with the 18-nor-ent-pimara-9(11),15-diene-4β-ol (0, 5, 10, 20 μM) for 6 h and then co-treated with LPS (1 μg/ml) for the additional 18 h. As shown in Fig. 2a, 18-nor-ent-pimara-9(11),15-diene-4β-ol significantly decreased LPS-mediated overproduction of NO in RAW264.7 cells. To exclude the

cytotoxic effect of 18-nor-ent-pimara-9(11),15-diene-4β-ol from the inhibitory effect of 18-nor-ent-pimara-9(11),15-diene-4β-ol against NO production, we tested cell viability after the treatment of 18-nor-ent-pimara-9(11),15-diene-4β-ol using MTT assay. As shown in Fig. 2b, 18-nor-ent-pimara-9(11),15-diene-4β-ol showed no cytotoxicity. Since NO production is regulated by iNOS, COX-2, IL-1β and TNF-α expression, the effect of 18-nor-ent-pimara-9(11),15-diene-4β-ol on iNOS, COX-2, IL-1β and TNF-α expression was evaluated by RT-PCR. As shown in Fig. 2c, the treatment of 18-nor-ent-pimara-9(11),15-diene-4β-ol attenuated the mRNA expression of iNOS, COX-2, TNF-α and IL-1β. Thus, 18-nor-ent-pimara-9(11),15-diene-4β-ol attenuated the



overproduction of NO by inhibiting LPS-induced iNOS, COX-2, TNF- α and IL-1 β overexpression in RAW264.7 cells.

The effect of 18-nor-ent-pimara-9(11),15-diene-4 β -ol on NF- κ B signaling activation in LPS-induced RAW264.7 cells

The nuclear factor kappa-B (NF- κ B) serves to control the activation of inflammation. NF- κ B is not a single gene but a family of closely related transcription factors that contain five genes: NF- κ B1(p50/p105) NF- κ B2(p52/p100), RelA(p65), c-Rel, and RelB. The activities of NF- κ B are strictly regulated by interaction with inhibitory I κ B protein. As with the NF- κ B transcription factors, there are several I κ B proteins (I κ B α , I κ B β , I κ B γ and I κ B ϵ) that have different affinities for individual NF- κ B dimers. The activation of NF- κ B dimers is the result of IKK-mediated, phosphorylation-induced degradation of the I κ B inhibitor, which enables the NF- κ B dimers to enter the nucleus and activate specific target gene expression. The degradation and phosphorylation of I κ B in response to LPS lead to NF- κ B translocation to the nucleus. This event is related to the activation of a wide range of NF- κ B-responsive pro-inflammatory genes [20–25].

To elucidate the effect of 18-nor-ent-pimara-9(11),15-diene-4 β -ol on NF- κ B signaling activation, we investigated a western blot for I κ B- α degradation and phosphorylation in LPS-stimulated RAW264.7 cells. As shown in Fig. 3a, LPS induced successive I κ B- α degradation and phosphorylation at 30 min after the stimulation. However, pretreatment of 18-nor-ent-pimara-9(11),15-diene-4 β -ol blocked LPS-stimulated I κ B- α degradation and phosphorylation in a dose-dependent manner. Cytosol to nucleus p65 translocation due to I κ B- α degradation by various stimuli are essential for NF- κ B activation. Thus, we performed whether

18-nor-ent-pimara-9(11),15-diene-4 β -ol inhibit the nuclear translocation of p65. As shown in Fig. 3b, LPS raised an amount of p65 in the nucleus of RAW264.7 cells. However, pretreatment of 18-nor-ent-pimara-9(11),15-diene-4 β -ol dose-dependently blocked LPS-induced p65 translocation. Translocate p65 into the nucleus binds to the NF- κ B binding site and extends NF- κ B transcriptional activity. These data show that 18-nor-ent-pimara-9(11),15-diene-4 β -ol may suppress NF- κ B activation by inhibition of p65 translocation into the nucleus via blocking the I κ B- α degradation.

The effect of 18-nor-ent-pimara-9(11),15-diene-4 β -ol on MAPKs signaling activation in LPS-stimulated RAW264.7 cells

The mitogen-activated protein kinases (MAPKs) pathway plays important role in transmitting signals from cell surface to the nucleus and manages cellular functions such as growth, migration, differentiation, proliferation and death. In addition, MAPKs pathway is the important regulator in the activation pro-inflammatory cytokines in different cell types, including T cells, epithelial cells, dendritic cells and macrophages. Some defects in MAPKs signaling are related to carcinogenesis by increased production of pro-inflammatory cytokines, cell proliferation, growth factors and anti-cancer factors. Generally growth factors are involved in activation of extracellular signal regulated kinases 1 and 2 (ERK1/2), whereas stress stimuli activates the c-Jun N-terminal kinases (JNK) and p38 MAPKs [24, 25]. Excessive activation of ERK1/2 has been associated with tumors. Also, deregulations of JNK and p38 MAPKs pathways are associated with cancer developments [26, 27]. As MAPKs have serious roles in cancer and inflammation, it has been focused and shown by different scientists that targeting MAPKs suppression is beneficial for cancer remedy [28–33].

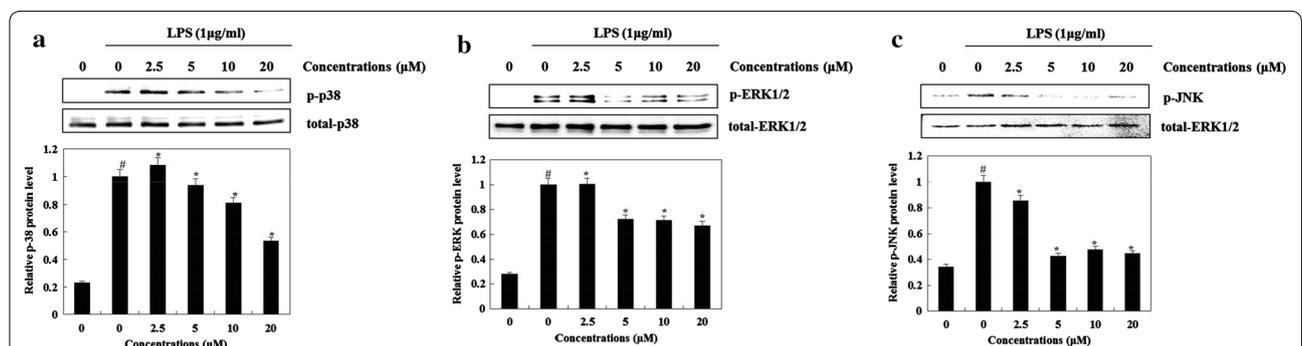


Fig. 4 The effect of 18-nor-ent-pimara-9(11),15-diene-4 β -ol on MAPK signaling activation in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pretreated with 18-nor-ent-pimara-9(11),15-diene-4 β -ol for 6 h and then co-treated with LPS (1 μ g/ml) for 20 min. For Western blot analysis, the cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibodies against p-ERK1/2, p-p38 and Total p-38 and Total ERK1/2 were used as internal control

To further examine whether decrease of MAPKs activation by 18-nor-*ent*-pimara-9 (11), 15-diene-4 β -ol treatment is associated with the regulation of p38, ERK1/2 and JNK activation, we investigated the effects of 18-nor-*ent*-pimara-9 (11), 15-diene-4 β -ol on phosphorylation of p38, ERK1/2 and JNK in LPS-stimulated RAW264.7 cells. As shown in Fig. 4, increase of p38, ERK1/2 and JNK phosphorylation was discovered in RAW264.7 cells by LPS. However, 18-nor-*ent*-pimara-9(11), 15-diene-4 β -ol suppressed phosphorylation of p38, ERK1/2 and JNK, indicating that 18-nor-*ent*-pimara-9(11), 15-diene-4 β -ol blocks the inflammatory response by inhibiting of p38, ERK1/2 and JNK activation in LPS-induced RAW264.7 cells.

Abbreviations

LPS: Lipopolysaccharide; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; RT-PCR: Reverse transcriptase-polymerase chain reaction; NO: Nitric oxide; ERK1/2: Extracellular signal-regulated kinase 1/2; NF- κ B: Nuclear factor kappa-B; MAPK: Mitogen-activated protein kinases; JNK: C-Jun N-terminal kinases.

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Authors' contributions

GHP directed, and HJE, YP and SSH designed the study. HJE, YP and SSH performed the experiments. GHP and HJE drafted manuscript. YP and SSH corrected the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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