Chemico-Biological Interactions 244 (2016) 16–26

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

Inhibitory effects of alternaramide on inflammatory mediator expression through TLR4-MyD88-mediated inhibition of NF-κB and MAPK pathway signaling in lipopolysaccharide-stimulated RAW264.7 and BV2 cells

Wonmin Ko [a], Jae Hak Sohn [b], Jae-Hyuk Jang [c], Jong Seog Ahn [c], Dae Gill Kang [d], Ho Sub Lee [d], Jong-Su Kim [a], Youn-Chul Kim [a, **], Hyuncheol Oh [a, *]

[a] College of Pharmacy, Wonkwang University, Iksan 570-749, Republic of Korea
[b] College of Medical and Life Sciences, Silla University, Busan 617-736, Republic of Korea
[c] Chemical Biology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongwon, Republic of Korea
[d] Hanbang Body-Fluid Research Center, Wonkwang University, Iksan 570-749, Republic of Korea

ARTICLE INFO

Article history:
Received 15 August 2015
Received in revised form
11 November 2015
Accepted 20 November 2015
Available online 24 November 2015

Keywords:
Alternaramide
Marine-derived fungus
Anti-inflammation
Toll-like receptor 4
Nuclear factor-kappa B
MAPK

ABSTRACT

Alternaramide (**1**), a novel lipophilic depsipeptide, has been isolated from the extract of the marine-derived fungus *Alternaria* sp. SF-5016. In the course of extensive biological evaluation of **1**, its anti-inflammatory effects in lipopolysaccharide (LPS)-stimulated RAW264.7 and BV2 cells were observed. In our initial study of the anti-inflammatory effects of **1**, the compound suppressed production of nitric oxide (NO) and prostaglandin E2 (PGE_2) in LPS-stimulated RAW264.7 and BV2 cells. Suppression of NO and PGE_2 production was correlated with the inhibitory effect of **1** on expression of LPS-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at the protein level in RAW264.7 and BV2 cells. In addition, **1** reduced production of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and IL-12 in LPS-stimulated RAW264.7 and BV2 cells. In the evaluation of the molecular mechanisms underlying the anti-inflammatory effects of **1**, the compound was found to suppress the nuclear factor-kappa B (NF-κB) signaling pathway in RAW264.7 and BV2 cells stimulated with LPS. This suppression was mediated by disruption of phosphorylation and degradation of IκBα, an inhibitor of NF-κB, in the cytoplasm, and blocking of nuclear translocation of the NF-κB p50–p65 heterodimer. Furthermore, **1** inhibited phosphorylation of c-Jun N-terminal kinases (JNKs) and p38 mitogen-activated protein kinase (MAPK), demonstrating its capacity to inhibit MAPK signaling. Finally, **1** markedly reduced expression of Toll-like receptor 4 (TLR4) and myeloid differentiation primary response gene 88 (MyD88) at the mRNA and protein levels in LPS-stimulated RAW264.7 and BV2 cells. Taken together, the results of the present study suggest that **1** modulates several TLR4-mediated inflammatory pathways, demonstrating its potential in the treatment of inflammatory and neuroinflammatory conditions.

1. Introduction

Immune cells, such as macrophages and microglia, play a central role in inflammatory reactions and recognize pathogen-associated molecule patterns (PAMPs) [1]. Upon infection, PAMPs, such as the lipopolysaccharide (LPS) of Gram-negative bacteria and the peptidoglycan (PGN) of Gram-positive bacteria, activate immune cells through Toll-like receptors (TLRs) expressed on the cell surface [2]. TLRs were the first pattern-recognition receptors (PRRs) to be identified and have been well characterized. TLR activation triggers recruitment of adapter molecules, such as myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) [3]. TLR4 activates downstream signaling pathways, including nuclear factor kappa B (NF-κB)-mediated signaling, which plays a key role in LPS-induced expression of pro-

* Corresponding author.
** Corresponding author.
E-mail addresses: yckim@wku.ac.kr (Y.-C. Kim), hoh@wku.ac.kr (H. Oh).

http://dx.doi.org/10.1016/j.cbi.2015.11.024
0009-2797/

inflammatory genes and cytokines [4]. Activation of the NF-κB pathway is regulated by IκBα, which is retained in the cytoplasm. LPS induces degradation of IκBα through the ubiquitin-proteasome system, while phosphorylation of IκBα leads to nuclear translocation of NF-κB p50/p65 heterodimers [5]. In the nucleus, NF-κB induces transcription of inflammation-associated genes such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) [6–9].

There are three major mitogen-activated protein kinase (MAPK) signaling pathways, which are mediated by extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2), c-Jun N-terminal kinases (JNKs), and p38 MAPK. Following phosphorylation and activation of MAPKs, MAPK signaling mediates cell growth, differentiation, and death via regulation of the expression of numerous genes [10,11]. Recent studies have shown that MAPK signaling mediates LPS-stimulated expression of inflammatory mediators, including iNOS, COX-2, and inflammatory cytokines. Several studies have shown that MAPKs are major downstream mediators of the effects of TLR activation [12,13]. Moreover, inhibition of MAPK phosphorylation by specific molecules was shown to reduce inflammation and relieve inflammatory conditions [14–16].

Marine-derived fungi are of significant interest to the drug discovery research communities because they produce a wide variety of chemical compounds with diverse structures and bioactivities [17–21]. In the course of chemical investigation of the extract of the marine-derived fungus *Alternaria* sp. SF-5016, a cyclic pentadepsipeptide, alternaramide (**1**), was identified and shown to possess weak antimicrobial activity and protein tyrosine phosphatase 1B (PTP1B) inhibitory activity [22]. Because **1** was identified as a novel peptide-type metabolite with unusual hydrophobic D-amino acid residues, further evaluation of its biological effects was conducted, leading to observation of its anti-inflammatory activity. Murine macrophage-like cell lines, such as RAW264.7 cells, and immortalized murine brain macrophages, such as BV2 cells, are suitable models for evaluating inflammatory responses characterized by production of NO, PGE_2, TNF-α, and ILs, which can be triggered by LPS [23,24]. Thus, the aim of the present study was to describe the anti-inflammatory and anti-neuroinflammatory effects of **1** in LPS-stimulated RAW264.7 and BV2 cells.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL Co. (Grand Island, NY, USA). All chemicals were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Primary antibodies, including anti-COX-2, anti-iNOS, anti-IκBα, anti-p-IκBα, anti-p50, anti-p65, anti-TLR4, anti-MyD88, anti-beta-actin, and anti-proliferating cell nuclear antigen (PCNA) antibodies, as well as anti-mouse, anti-goat, and anti-rabbit secondary antibodies, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p-ERK, anti-ERK, anti-p-JNK, anti-JNK, anti-p-p38, and anti-p38 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for PGE_2, IL-1β, IL-6, IL-12, and TNF-α were purchased from R & D Systems, Inc. (Minneapolis, MN, USA). The isolation and structure determination of **1** have been described elsewhere [22].

2.2. Cell culture and viability assays

RAW264.7 and BV2 cells were maintained at 5×10^5 cell/mL in DMEM medium supplemented with 10% heat-inactivated FBS, penicillin G (100 units/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The effects of the various experimental treatments on cell viability were evaluated by determining mitochondrial reductase function with an assay based on the reduction of the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into formazan crystals [25]. Following exposure of living cells to MTT, formazan formation is proportional to the number of functional mitochondria. For the determination of cell viability, 50 mg/mL of MTT was added to 1 mL of each cell suspension (1×10^5 cell/mL in 96-well plates) for 4 h. The resulting formazan was dissolved in acidic 2-propanol, after which the optical density of the solution was measured at 540 nm. The optical density of the formazan solution from the control (untreated) cells was considered to indicate 100% viability.

2.3. Determination of nitrite as an indicator of NO production

Production of nitrite, a stable end-product of NO oxidation, was used as a measurement of iNOS activity. The concentration of nitrite in the conditioned media was determined by a method based on the Griess reaction [26]. An aliquot of each supernatant (100 μL) was mixed with the same volume of Griess reagent (0.1% (w/v) N-(1-naphathyl)-ethylenediamine and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid) for 10 min at room temperature. The absorbance of the final product was measured spectrophotometrically at 540 nm using an ELISA plate reader. The nitrite concentration in the samples was determined from a standard curve of sodium nitrite prepared in phenol red-free DMEM.

2.4. PGE_2, IL-1β, IL-6, IL-12, and TNF-α assays

The culture medium was collected and the level of PGE_2, IL-1β, IL-6, IL-12, and TNF-α present in each sample was determined using a commercially available kit from R & D Systems, Inc. The assays were performed according to the manufacturer's instructions.

2.5. Western blot analysis

Western blot analysis was performed by lysing the cells in 20 mM Tris–HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM PMSF, 5 mg/mL aprotinin, 5 mg/mL pepstatin A, and 1 mg/mL chymostatin). The protein concentration was determined using a Lowry protein assay kit (P5626; Sigma–Aldrich, St. Louis, MO, USA). An equal amount of protein for each sample was resolved by performing 7.5% and 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrophoretically transferred onto Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), which were blocked with 5% skim milk and sequentially incubated with particular primary antibodies (Santa Cruz Biotechnology) and horseradish peroxidase (HRP)-conjugated secondary antibodies, followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.6. Preparation of cytosolic and nuclear fractions

RAW264.7 and BV2 cells were homogenized (1:20, w:v) in PER-Mammalian Protein Extraction buffer (Pierce Biotechnology, Rockford, IL, USA) containing freshly added protease inhibitor cocktail I (EMD Biosciences, San Diego, CA, USA) and 1 mM