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Zerumbone suppresses the activation of inflammatory mediators in LPS-stimulated U937 macrophages through MyD88-dependent NF-κB/MAPK/PI3K-Akt signaling pathways

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ABSTRACT

Zerumbone (ZER), isolated mainly from the *Zingiber zerumbet* (*Z. zerumbet*) rhizomes was found to be effective against numerous inflammatory and immune disorders, however, the molecular and biochemical mechanisms underlying its anti-inflammatory and immunosuppressive properties have not been well studied. This study was carried out to examine the profound effects of ZER on inflammatory mediated MyD88-dependent NF-κB/MAPK/PI3K-Akt signaling pathways in LPS-stimulated U937 human macrophages. ZER significantly suppressed the up-regulation pro-inflammatory mediators, TNF-α, IL-1β, PGE_2, and COX-2 protein in LPS-induced human macrophages. Moreover, ZER significantly downregulated the phosphorylation of NF-κB (p65), IκBα, and IKKα/β as well as restored the degradation of IκBα. ZER correspondingly showed remarkable attenuation of the expression of Akt, JNK, ERK, and p38 MAPKs phosphorylation in a concentration-dependent manner. ZER also diminished the expression of upstream signaling molecules TLR4 and MyD88, which are prerequisite for the NF-κB, MAPK and PI3K-Akt activation. Additionally, quantification of relative gene expression of TNF-α, IL-1β, and COX-2 indicated that, at a higher dose (50 μM), ZER significantly downregulated the elevated mRNA transcription levels of the stated pro-inflammatory markers in LPS-stimulated U937 macrophages. The strong suppressive effects of ZER on the activation of inflammatory markers in the macrophages via MyD88-dependent NF-κB/MAPK/PI3K-Akt signaling pathways suggest that ZER can be a preventive and potent therapeutic candidate for the management of various inflammatory-mediated immune disorders.

1. Introduction

Immunity is the host defense mechanism against various pathogenic conditions and is categorized into innate and adaptive immune responses. Any imbalance of both immune responses may lead to abnormal immune states like inflammation, inflammatory bowel diseases, cancers, rheumatoid arthritis, and autoimmune diseases. Conversely, these immune responses are involved with various immune cells including macrophages to trigger various cellular processes. Macrophages are mainly differentiated blood monocytes and considered as the first effector cells of innate immune response; found mainly in tissues all over the body [1,2]. These differentiated monocytes are the vital source of cytokines, chemokines, and inflammatory markers like tumor necrosis factor (TNF-α), prostaglandins, and nitric oxide (NO) that play crucial role during the origination and propagation of various immune responses, hematopoiesis, and inflammation [1,3,4]. During the inflammatory progression, any endogenous factors, microbes or microbial products like lipopolysaccharides (LPS) selectively bind with the upstream signaling molecules like Toll-like receptors (TLR4), which further triggers the activation of another upstream adaptor protein molecule known as myeloid differentiation primary response gene 88 (MyD88) in macrophages. Subsequently, MyD88 triggers the interleukin 1 receptor-associated kinases (IRAK4 and IRAK1) those involving the activation of several inflammatory mediated signaling events, specifically, nuclear factor-kappaB (NF-κB), mitogen-activated protein kinases (MAPKs) and PI3K-Akt signaling pathways, and contribute to the release of numerous inflammatory mediators like prostaglandin E2 (PGE_2) cyclooxygenase 2 (COX-2), TNF-α, and interleukin-1 beta (IL-1β) [5–7].

NF-κB, a transcription factor, plays a principal role in the propagation of both innate and adaptive immune responses [8,9]. Diverse downstream and upstream signaling events are affiliated to this pathway. This imperative pathway particularly plays key role in inducing major pro-inflammatory mediators [10]. In general, the activities of NF-κB are strictly regulated by several inhibitors of NF-κB namely, IκB proteins (e.g., IκBα). These IκB proteins are regulated discretely by

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phosphorylation and proteolysis. Nonetheless, NF-κB is found as inactive, dormant, and as IκB bound complex in the cytoplasm. The activation of NF-κB occurs mainly via activation of the IκB kinase (IKK) complex that consists of catalytic subunits like IKK-α and/or β as well as a sensing protein namely, NF-κB essential modulator (NEMO). The phosphorylation of IκB by IKK and NEMO leads to degradation of IκB and hence, following this degradation, NF-κB particles e.g., p65 are released. Then the NF-κB dimers translocate into the nucleus and thus, bind the deoxyribonucleic acid (DNA) to activate the targeted gene expressions followed by the transcription process [11,12]. Analogously, PI3K-Akt is the threonine/serine kinase and plays crucial roles in numerous cellular and molecular processes including cell cycle regulation, cell migration, cell survival, and NF-κB activation [13]. Phosphorylation of Akt interferes and promotes the activation of NF-κB pathways during inflammatory processes. Nevertheless, malfunctioning of these signaling molecules may lead to chronic inflammatory ailments along with neurodegenerative dysfunctions and cancers [14]. MAPKs signaling pathway also mediates fundamental role during various cellular and biological processes related to immune response. This pathway comprises of three major classes namely, the extracellular signal-regulated kinase (ERK1/2) and two stress-activated protein kinases (SAPKs), c-Jun N-terminal kinase (JNK1/2), and p38. Activation of ERK1/2 is generated by the MAP kinase kinase (MKK) and MKK2. Similarly, JNK is activated by MKK4 as well as MKK7 and p38 is activated by the MKK3, MKK4, and MKK6 [15,16]. Activation of this pathway further activates the NF-κB pathway. Likewise, NF-κB and PI3K-Akt, the impaired and uncontrolled regulation of the MAPK pathway may also cause diverse immunological disorders including inflammation and cancers. Accordingly, considering these signaling pathways can be a prospective therapeutic approach in treating immune disorders and inflammation.

Zingiber zerumbet (*Z. zerumbet*) or shampoo ginger is widely used as ethnomedicine and the rhizomes of this plant have been consumed traditionally for the management of numerous inflammatory disorders including allergies, fever, asthma, severe sprains, torment, toothache, stomachache, and wounds. Zerumbone (ZER), chemically known as 2,6,9,9-tetramethyl-[2E,6E,10E]-cycloundeca-2,6,10-trien-1-one, is a cyclic eleven-membered sesquiterpene and isolated from the rhizomes of *Z. zerumbet*. It has been described to be active against different types of cancers and possessed potent immunosuppressive and anti-inflammatory effects on several immune cells [17,18]. The biological activities of this potent metabolite were thought to be due to existing α,β-unsaturated carbonyl-based group in its chemical structure. Apart from the prevailing research on anti-inflammatory and immunosuppressive properties of ZER, there are still research gaps specifically, in terms of suggested mechanistic investigation targeting prospective signaling pathways specifically, NF-κB, MAPKs, and PI3K-Akt in human macrophages. Hence, the current investigation was aimed to explore the potential effects of the bioactive metabolite ZER on the MyD88-dependent NF-κB/MAPK/PI3K-Akt signaling pathways in LPS-primed U937 macrophages.

2. Materials and methods

2.1. Chemicals and reagents

Roswell Park Memorial Institute-1640 (RPMI) medium, Phosphate-buffered saline (PBS), fetal bovine serum (FBS), and penicillin-streptomycin (Pen-Strep) were procured from Gibco (Grand Island, NY, USA). Phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS) (*Escherichia coli*), Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Radioimmunoprecipitation assay (RIPA) buffer were acquired from Sigma Chemical Co. (St. Louis, MO, USA). 1 × Halt Protease and Phosphatase Inhibitor Cocktail were obtained from Pierce (Rockford, IL, USA). Human TNF-α, IL-1β, and PGE_2 ELISA kits were procured from R&D Systems (Minneapolis, MN, USA). Primary antibodies specific to COX-2, p-IκBα, IκBα, p-IKKα/β, IKKα/β, p-p65, p65, p-JNK1/2, JNK1/2, p-ERK1/2, ERK1/2, p-p38, p38, p-Akt, Akt, TLR4, MyD88 as well as β-actin, and anti-rabbit secondary antibody conjugated to horseradish peroxidase were purchased from Cell Signaling Technology (Beverly, MA). SB202190 (p38 inhibitor), U0126 (ERK inhibitor), SP600125 (JNK inhibitor), LY294002 (Akt inhibitor), and BAY 11-7082 (NF-κB inhibitor) were purchased from Tocris Biosciences (Bristol, UK). Dexamethasone (DEX) was obtained from CCM Duopharma Biotech Bhd (Selangor, Malaysia). All the chemicals and reagents including biologics and synthetics used in this study are endotoxin free.

2.2. Collection of plant materials

The rhizomes of *Z. zerumbet* were collected from Landang Kizaherbs, Kampong Batu Gading, Kuala Karu, Temerloh, Pahang, Malaysia. Identification of the specimen was carried out by Dr. Abdul Latif Mohamad, Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM). A voucher specimen (No. HF137) was deposited for further reference at the herbarium of UKM, Bangi, Malaysia.

2.3. Extraction of the plant materials and isolation of ZER

The rhizomes of *Z. zerumbet* were air-dried under shade at 26 ± 2 °C and ground into powder. Extraction was carried out using 80% EtOH. Briefly, 1750 g of powder was macerated in 80% EtOH at the ratio of 1:6 (sample: solvent) (w/v) for 72 h at room temperature and filtered with Whatman no. 1 filter paper (Whatman plc, Maidstone, UK). The filtrates obtained were evaporated in vacuo to obtain 248 g of gummy crude extract (14.17%). The crude extract (10 g) was subjected to repeated chromatography on silica gel column (40–63 μm, 3 × 60 cm) with a gradient elution of *n*-hexane: EtOAc (10:0–7:3 ratios; v/v). The eluates were evaporated and re-crystallization from *n*-hexane-EtOAC yielded 87.4 mg of ZER (0.87%). The identity and purity (> 98%) of ZER (Fig. 1) was confirmed based on its physicochemical property, NMR data, and ESI-MS. Since endotoxin is commonly exist in many plant materials like isolated metabolites, the possible endotoxin contamination of ZER was evaluated by Limulus Amebocyte Lysate (LAL) assay kit (Cambrex BioScience, Walkersville, MD) following the manufacturer's recommended instructions. ZER did not found to contain significant amounts of endotoxin which would potentially interfere with our investigations at the concentrations used. Essential precautions had also been taken to avoid any endotoxin contamination throughout the study by using endotoxin free reagents, sterile water, and buffers.

2.4. Cell culture and differentiation induction

U937 monocytes were obtained from American Type Culture Collection, Manassas, USA (ATCC® CRL1593.2™). Cells were grown in

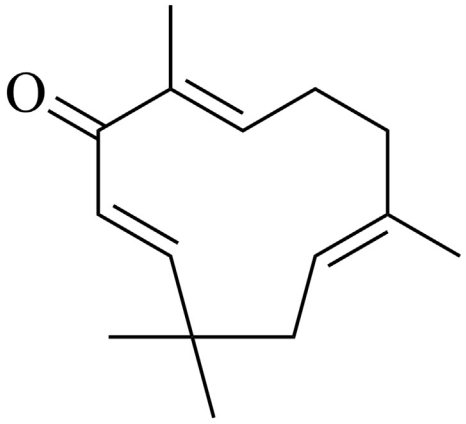

Fig. 1. The chemical structure of ZER.