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Inhibition of LPS binding to MD-2 co-receptor for suppressing TLR4-mediated expression of inflammatory cytokine by 1-dehydro-10-gingerdione from dietary ginger

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ABSTRACT

Myeloid differentiation protein 2 (MD-2) is a co-receptor of toll-like receptor 4 (TLR4) for innate immunity. Here, we delineated a new mechanism of 1-dehydro-10-gingerdione (1D10G), one of pungent isolates from ginger (*Zingiber officinale*), in the suppression of lipopolysaccharide (LPS)-induced gene expression of inflammatory cytokines. 1D10G inhibited LPS binding to MD-2 with higher affinity than gingerol and shogaol from dietary ginger. Moreover, 1D10G down-regulated TLR4-mediated expression of nuclear factor-κB (NF-κB) or activating protein 1 (AP1)-target genes such as tumor necrosis factor α (TNF-α) and interleukin-1β, as well as those of interferon (IFN) regulatory factor 3 (IRF3)-target IFN-β gene and IFN-γ inducible protein 10 (IP-10) in LPS-activated macrophages. Taken together, MD-2 is a molecular target in the anti-inflammatory action of 1D10G.

1. Introduction

Lipopolysaccharide (LPS) in Gram-negative bacteria consists of a lipid A moiety and polysaccharide units of the inner and outer cores [1]. Bacterial LPS displays endotoxic activity after binding with its specific receptor complex called myeloid differentiation protein 2 (MD-2)/toll-like receptor 4 (TLR4) in the mammalian host [2]. MD-2 plays a pivotal role in the recognition of bacterial LPS, and is requisite in the TLR4-mediated inflammatory cascade across the plasma membrane. Upon binding with LPS, the receptor components are activated to a multimer that consists of two copies of LPS, MD-2 and TLR4 [3,4]. The intracellular domain of TLR4 within the activated receptor multimer recruits adaptor molecules such as the myeloid differentiation factor 88 (MyD88) and the toll/interleukin (IL)-1 receptor-containing adaptor inducing interferon (IFN)-β (TRIF) [5]. TLR4 signaling then diverges into MyD88- and TRIF-dependent pathways. The MyD88-dependent pathway activates the transcription factors, nuclear factor-κB (NF-κB) and activating protein 1 (AP1), which induce inflammatory cytokines such as tumor necrosis factor α (TNF-α) and IL-1β [6,7]. On the other hand, the TRIF-dependent pathway activates another transcription factor called IFN regulatory factor 3 (IRF3) to up-regulate IFN-β gene and IFN-γ inducible protein 10 (IP-10) [6,7].

Although commonly used as a spice or food supplement, ginger (*Zingiber officinale*) has been reported to exert anti-inflammatory activity [8–10]. Pungent constituents of ginger inhibit both cyclooxygenase 2 (COX-2) and 5-lipoxygenase in the metabolism of arachidonic acid, and also down-regulate the gene expression in chronic inflammation conditions [10,11]. However, molecular basis for the latter is only partially defined. 6-Gingerol, a major pungent constituent of ginger, decreases the expression of inducible nitric oxide (NO) synthase (iNOS) or TNF-α by suppressing the phosphorylation of inhibitory κB (IκB) and nuclear activation of NF-κB in LPS-activated macrophages [12]. 6-Shogaol, another pungent constituent of ginger, suppresses LPS-induced expression of iNOS or COX-2 in macrophages by inhibiting phosphatidylinositol-3 kinase, IκB kinase, and mitogen-activated protein kinase for the activation of NF-κB or AP1 [13]. 6-Shogaol also inhibits not only MyD88-dependent NF-κB activation but also TRIF-dependent IRF3 activation in LPS-activated macrophages, and it disrupts the receptor multimer of MD-2/TLR4 in LPS-activated Ba/F3 cells expressing MD-2 and TLR4 [14,15]. 1-Dehydro-10-gingerdione (1D10G, Fig. 1A) from ginger exerts more effective inhibition than

Abbreviations: 1D10G, 1-dehydro-10-gingerdione; AP1, activating protein 1; Bis-ANS, 1,1′-bis(anilino)-4,4′-bis(naphthalene)-8,8′-disulfonate; COX-2, cyclooxygenase 2; IFN, interferon; IκB, inhibitory κB; IL, interleukin; iNOS, inducible nitric oxide synthase; IP-10, IFN-γ inducible protein 10; IRF3, IFN regulatory factor 3; LPS, lipopolysaccharide; LBP, LPS-binding protein; MD-2, myeloid differentiation protein 2; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor α; TRIF, toll/IL-1 receptor-containing adaptor inducing IFN-β.

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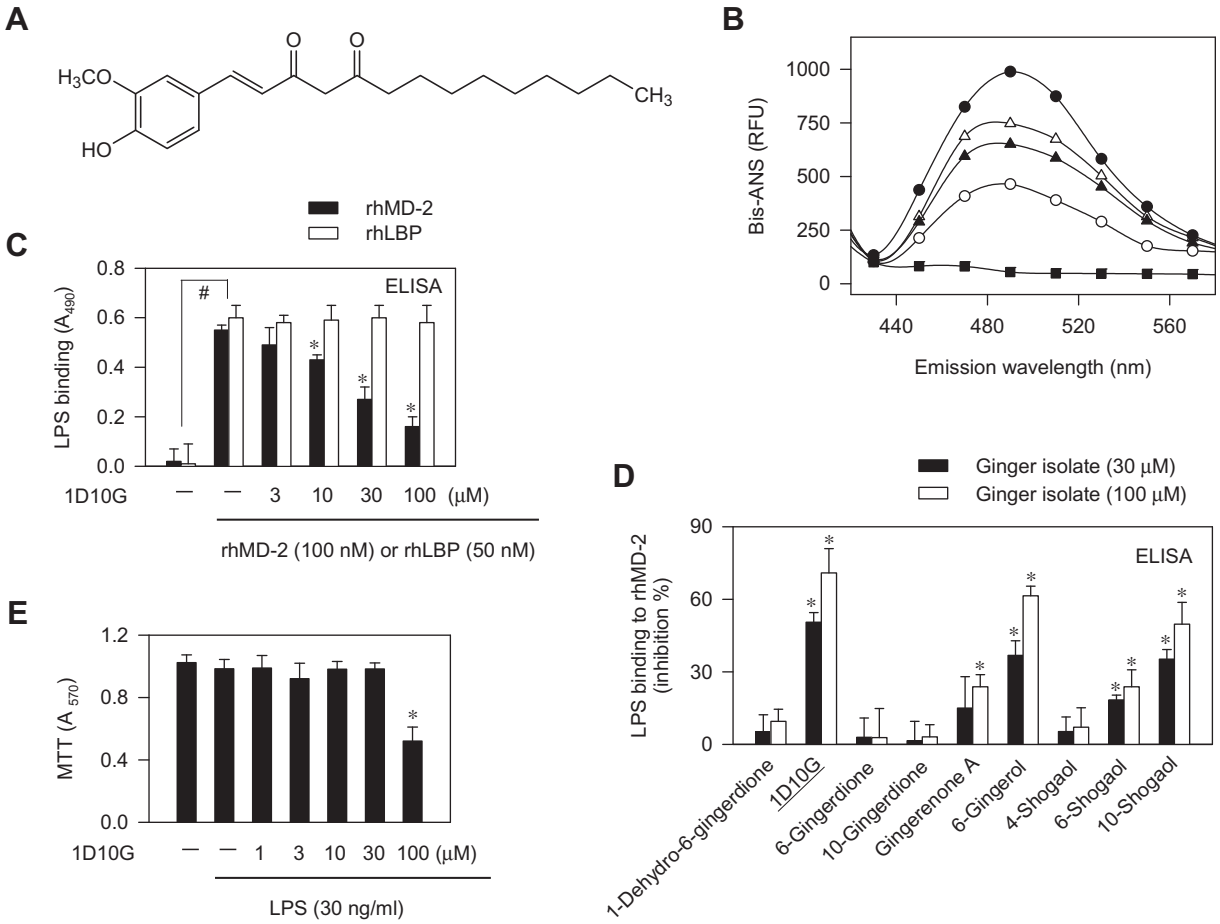

Fig. 1. Effect of 1D10G on LPS binding to MD-2. (A) Chemical structure of 1D10G. (B) Bis-ANS (1 μM) was pre-incubated with rhMD-2 (200 nM) to reach stable fluorescence values and then treated with nonfluorescent 1D10G for 2 h. Emission spectra are represented as relative fluorescence units (RFUs). Symbols denote Bis-ANS + rhMD-2 group (●), Bis-ANS + rhMD-2 + 1D10G (10 μM) group (△), Bis-ANS + rhMD-2 + 1D10G (30 μM) group (▲), Bis-ANS + rhMD-2 + 1D10G (100 μM) group (○), and Bis-ANS alone (■). LPS (15 μg/ml) was immobilized and then incubated with either rhMD-2 or rhLBP for 2 h in the presence of 1D10G (C) or other pungent isolates from ginger (D). LPS binding to rhMD-2 or rhLBP was determined by ELISA and is represented as absorbance values at 490 nm (C) or inhibition% compared with rhMD-2 alone-added group (D). (E) RAW 264.7 cells were incubated with 1D10G for 24 h in the presence of LPS. Cell viability was analyzed by MTT assay and is represented as absorbance values at 570 nm. Data are mean ± S.D. of three to five separate experiments. $^{\#}P < 0.05$ vs. vehicle alone-added group. $^{*}P < 0.05$ vs. rhMD-2 alone-added group (C, D) or LPS alone-treated group (E).

6-shogaol and other pungent constituents against LPS-induced NO production in macrophages [16].

In the present study, we decided to elucidate a primary target of 1D10G in the down-regulation of inflammatory cytokines in LPS-activated macrophages. 1D10G inhibited LPS binding to MD-2 for the TLR4 activity, thus suppressing both MyD88- and TRIF-dependent pathways in the gene expression of TNF-α, IL-1β, IFN-β or IP-10.

2. Materials and methods

2.1. Materials

1D10G and other pungent compounds were isolated from dietary ginger (*Zingiber officinale*) as described previously [16]. MD-2 protein and another LPS-binding protein (LBP) were purchased from R&D Systems (Minneapolis, MN). Primary and secondary antibodies used in this study were purchased from Santa Cruz Biotech (Santa Cruz, CA) or Cell Signaling Tech (Danver, MA). Luciferase reporter plasmids of NF-κB-Luc, AP1-Luc and IRF3-Luc were purchased from Millipore (Billerica, MA), and those of TNF-α (−1260/+60)-Luc and IP-10 (−875/+97)-Luc from Dr. P.F. Johnson (National Cancer Institute, Frederick, MD) and Dr. R.M. Ransohoff (Lerner Research Institute, Cleveland, OH). TLR9 agonist CpG DNA (5′-TCCACGTTCCTGACGTT-3′) was purchased from Invitrogen (Carlsbad, CA). All other chemicals including TLR4 agonist LPS (*E. coli* 055:B5) were otherwise purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Competition displacement assay

1,1′-Bis(anilino)-4,4′-bis(naphthalene)-8,8′-disulfonate (Bis-ANS, 1 μM) and rhMD-2 (200 nM) were incubated to reach stable fluorescence values under excitation at 385 nm. Nonfluorescent 1D10G was then treated for 2 h, and followed by measuring relative fluorescence units (RFUs) emitted at 420–580 nm. LPS binding to either rhMD-2 or rhLBP was determined by ELISA. In brief, LPS (15 μg/ml) was immobilized to microplates overnight and then incubated with rhMD-2 (100 nM) or rhLBP (50 nM) for 2 h in the presence of 1D10G. After rinsing, microplates were added with anti-MD-2-antibody followed by horseradish peroxidase-conjugated secondary antibody. These immune complexes were reacted with *o*-phenylenediamine (2 mg/ml) containing 0.2% H_2O_2 for 30 min, and stopped with 1 N H_2SO_4. Absorbance values were then measured at 490 nm.

2.3. Western blot analysis

Cell extracts were resolved on SDS-acrylamide gels by electrophoresis, and transferred to a polyvinylidene difluoride membrane. Either 5% non-fat milk in PBS with Tween 20 or 5%