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Molecules and Cells

Inhibition of Homodimerization of Toll-like Receptor 4 by 6-Shogaol

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Toll-like receptors (TLRs) play a critical role in sensing microbial components and inducing innate immune and inflammatory responses by recognizing invading microbial pathogens. Lipopolysaccharide-induced dimerization of TLR4 is required for the activation of downstream signaling pathways including nuclear factor-kappa B (NF-κB). Therefore, TLR4 dimerization may be an early regulatory event in activating ligand-induced signaling pathways and induction of subsequent immune responses. Here, we report biochemical evidence that 6-shogaol, the most bioactive component of ginger, inhibits lipopolysaccharide-induced dimerization of TLR4 resulting in the inhibition of NF-κB activation and the expression of cyclooxygenase-2. Furthermore, we demonstrate that 6-shogaol can directly inhibit TLR-mediated signaling pathways at the receptor level. These results suggest that 6-shogaol can modulate TLR-mediated inflammatory responses, which may influence the risk of chronic inflammatory diseases.

INTRODUCTION

Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMP) and trigger the innate immune responses that are essential for host defense against invading microbial pathogens (Doyle and O'Neill, 2006; Medzhitov et al., 1997; Takeda and Akira, 2005). TLR4 has been identified as the receptor for lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria. One of the initial steps of TLR4 activation after TLR4 agonist treatment is the dimerization of the receptor (Saitoh et al., 2004). TLR2 is also capable of forming a heterodimer with TLR1 or TLR6 to recognize triacyl- or diacyl-lipopeptide, respectively (Hajjar et al., 2001; Takeuchi et al., 2001; 2002). These results suggest that agonist-induced dimerization of TLRs is required for the activation of downstream signaling pathways. Therefore, receptor dimerization in response to an agonist is considered one of the first means of regulation in activating TLR-mediated downstream signaling pathways and induction of subsequent innate and adaptive immune responses.

Dimerization of TLR4 leads to the recruitment of myeloid differential factor 88 (MyD88) or TIR domain-containing adaptor inducing interferon-β (TRIF) (Takeda and Akira, 2005). The interaction of MyD88 with the TIR domain of TLR recruits interleukin-1 receptor-associated kinase (IRAK)-4 and induces phosphorylation of IRAK-4. The phosphorylated IRAK-4 induces phosphorylation and degradation of IRAK-1, which associates with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) leading to the activation of the canonical inhibitor κB kinase (IKK) complex resulting in the activation of transcription factor NF-κB. TRIF is another adaptor molecule responsible for the activation of the MyD88-independent signaling pathway leading to the expression of type I interferon (IFN) and IFN-inducible genes (Fitzgerald et al., 2003; Sato et al., 2003). The TRIF pathway also leads to delayed NF-κB activation while MyD88-dependent NF-κB activation occurs in earlier than TRIF-dependent pathway (Sato et al., 2003). TRIF activates TNF-receptor-associated factor family member-asso-ciated NF-κB activator (TANK)-binding kinase 1 (TBK1) and IKKε leading to the activation of IRF3 and the expression of IFNβ and IFN-inducible genes (Fitzgerald et al., 2003; Toshchakov et al., 2002). The activation of TLR4 signaling pathway by lipopolysaccharide (LPS) leads to the induction of pro-inflammatory gene products such as cytokines, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS). These observations suggest that TLR4 and its signaling components could be excellent therapeutic targets for chronic inflammatory diseases.

Herbal medicine has been used for the treatment of several diseases for centuries. Ginger, which is the rhizome of the herb *Zingiber officinale* Roscoe, is widely used as a spice, food ingredient, and herbal medicine. Ginger has traditionally been used to treat a wide range of ailments including rheumatism, nervous diseases, gingivitis, toothache, asthma, stroke, constipation, diabetes, and arthritis (Afzal et al., 2001). Ginger extracts, which are a multi-component mixture of biologically active constituents, have been reported to have anti-inflammatory (Chang et al., 1995), anti-oxidant (Ippoushi et al., 2003), antithrombotic (Thomson et al., 2002) and anti-cancer effects (Surh, 2002). The major compounds accounting for ginger's anti-inflammatory properties are gingerols, shogaols, and paradols (Fig. 1). Gingerols readily undergoes dehydration to form shogaols, which are a major component of dried ginger powder. Shogaols are further converted to paradols by hydrogenation. Shogaols are found only in small quantities in fresh ginger but

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are present in large amounts in stored ginger. In particular, ginger extracts and ginger-derived components can inhibit NF-κB activation and COX-2 induced by a variety of agents (Kim et al., 2008; Kim et al., 2005; 2008; Takada et al., 2005). However, the direct molecular targets for the anti-inflammatory ginger-derived components are largely unknown. Given that the most bioactive component of ginger is 6-shogaol (Rhode et al., 2007), we presently investigated 6-shogoal-mediated modulation of TLR4 activation to elucidate the anti-inflammatory effects of 6-shogaol.

MATERIALS AND METHODS

Reagents

6-Shogaol was purchased from Wako Pure Chemical (Japan). Purified LPS was purchased from List Biological Lab (USA). All other reagents were purchased from Sigma-Aldrich (USA) unless otherwise described.

Cell culture

RAW 264.7 cells (a murine monocytic cell line) and 293T human embryonic kidney cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Invitrogen, USA), 100 units/ ml Penicillin, and 100 μg/ml Streptomycin (Invitrogen). Ba/F3, an IL-3 dependent murine pro-B cell line that expresses Flag- and GFP-TLR4, CD14, Flag-MD2, and NF-κB luciferase reporter gene were described previously (Saitoh et al., 2004). Cells were cultured in RPMI1640 medium containing recombinant murine IL-3 (70 U/ml), 10% (v/v) heat-inactivated FBS, 100 units/ml Penicillin, and 100 μg/ml Streptomycin. Cells were maintained at 37°C in an atmosphere of 5% CO_2.

Plasmids

NF-κB (2x)-luciferase reporter construct was provided by Frank Mercurio (Signal Pharmaceuticals, USA). Heat shock protein 70 (HSP70)-β-galactosidase reporter plasmid was provided by Robert Modlin (University of California, USA). All DNA constructs were prepared in large scale using EndoFree Plasmid Maxi kit (Qiagen, USA) for transfection.

Transfection and luciferase assays

The assays were performed as described previously (Youn et al., 2006a; 2006b). RAW 264.7 cells were co-transfected with NF-κB- or COX-2-luciferase plasmid and HSP70-β-galactosidase plasmid as an internal control using SuperFect transfection reagent (Qiagen, USA) according to the manufacturer's instructions. Luciferase enzyme activities were determined using the Luciferase Assay System (Promega, USA) according to the manufacturer's instructions. β-galactosidase enzyme activities were determined using the β-galactosidase enzyme system. Luciferase activity was normalized by β-galactosidase activity.

Immunoblotting

These were performed as previously described (Youn et al., 2005; 2008a). Equal amounts of protein extracts were resolved using 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 3% nonfat dry milk. The membranes were blotted with the indicated antibodies and secondary antibodies conjugated to horseradish peroxidase (Amersham, USA). The reactive bands were visualized using an enhanced chemiluminescence system (ECL; Amersham Biosciences, USA).

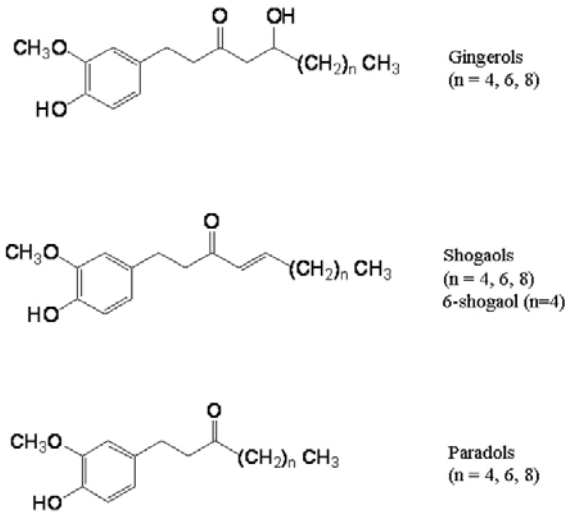

Fig. 1. The structure of gingerols, shogaols, and paradols

Immunoprecipitation

Protein extracts from Ba/F3 cells expressing TLR4 (Flag or GFP-tagged), CD14, MD2 (Flag-tagged), and NF-κB luciferase reporter gene were prepared for immunoprecipitation as described previously (Saitoh et al., 2004; Youn et al., 2006b). Briefly, the samples were immunoprecipitated by an overnight application of mouse-GFP antibody (Molecular Probes, USA). The solubilized immune complex was resolved using 8% SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was blocked with PBS containing 0.1% Tween-20 and 3% nonfat dry milk, and was blotted overnight with Flag antibodies. Thereafter, the blot was exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h and the binding of the secondary antibodies was detected via ECL Western blot detection reagents. The blot was reprobed with rabbit green fluorescent protein antibodies.

RESULTS AND DISCUSSION

6-Shogaol suppresses TLR4 ligand-induced NF-κB activation and COX-2 expression

TLRs signaling pathway can trigger the activation of NF-κB through the MyD88-dependent pathway (Takeda and Akira, 2005). NF-κB is the major transcription factor known to be activated in TLR4 signaling pathways. To investigate whether 6-shogaol could modulate the activation of TLR4, we determined the effect of 6-shogaol on LPS (TLR4 agonist)-induced NF-κB activation. 6-Shogaol suppressed LPS-induced NF-κB activation in macrophages (RAW264.7) (Fig. 2A). The inhibition of transcription factor activation by 6-shogaol resulted in the decrease of LPS-induced expression of COX-2 as determined by a COX-2 promoter luciferase reporter assay (Fig. 2B) and COX-2 immunoblotting (Fig. 2C).

6-Shogaol inhibits NF-κB activation induced by MyD88 or IKKβ, and LPS-induced degradation of IRAK-1

IKKβ is the key kinase for NF-κB activation mediated through the MyD88-dependent pathway in response to various agonists, including LPS (Youn et al., 2006b). The 6-Shogaol inhibited NF-κB activation induced by the overexpression of MyD88 or

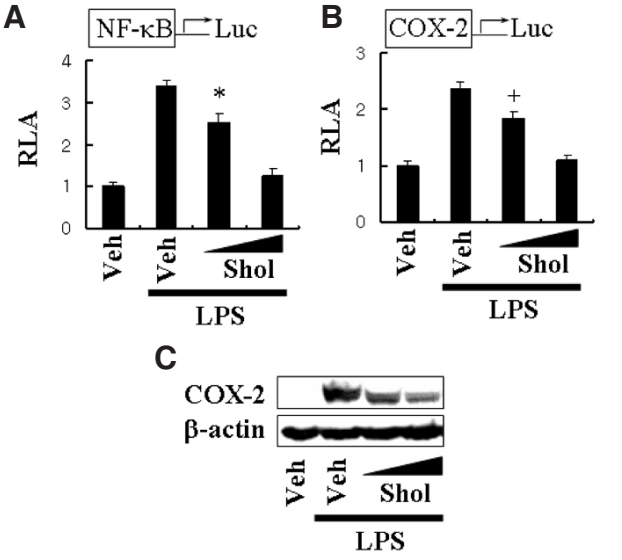

Fig. 2. The 6-Shogaol suppressed the NF-κB activation and COX-2 expression induced by LPS. (A, B) RAW264.7 cells were transfected with NF-κB (A) or COX-2 (B) luciferase reporter plasmid and pre-treated with 6-shogaol (20, 30 μM) for 1 h and then treated with LPS (5 ng/ml) for an additional 8 h. Cell lysates were prepared and luciferase and β-galactosidase enzyme activities were measured as described in "Materials and Methods". Relative luciferase activity (RLA) was normalized with β-galactosidase activity. Values are mean ± SEM (n = 3). *, Significantly different from LPS alone (A), $p < 0.05$. +, Significantly different from LPS alone (B), $p < 0.05$. (C) RAW264.7 cells were pretreated with 6-shogaol (20, 30 μM) for 1 h and then further stimulated with LPS (5 ng/ml) for 8 h. Cell lysates were analyzed for COX-2 and β-actin protein by immunoblots. Veh, vehicle; Shol, 6-shogaol.

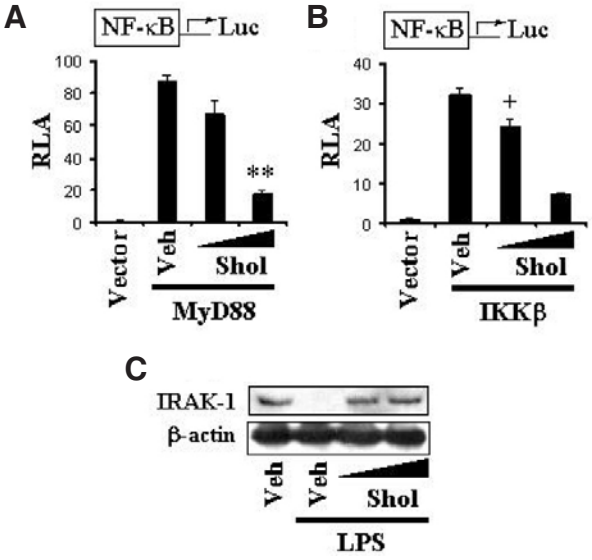

Fig. 3. The 6-Shogaol inhibited NF-κB activation induced by MyD88 or IKKβ, and LPS-induced degradation of IRAK-1. (A, B) 293T cells were co-transfected with NF-κB-luciferase reporter plasmid and an expression plasmid for MyD88 or IKKβ. pcDNA was used as a vector control for MyD88 and IKKβ. After 3 h, cells were treated with 6-shogaol (20, 30 μM) for 18 h. Relative luciferase activity (RLA) was determined by normalization with β-galacto-sidase activity. Values are mean ± SEM (n = 3). **, Significantly different from MyD88 plus vehicle (A), $p < 0.01$. +, Significantly different from IKKβ plus vehicle (B), $p < 0.05$. (C) RAW264.7 cells were pretreated with 6-shogaol (20, 30 μM) for 1 h and then stimulated with LPS (50 ng/ml) for 30 min. Cell lysates were subjected to SDS-PAGE and proved with anti-IRAK-1 (upper) or anti-β-actin (lower) antibody. Veh, vehicle; Shol, 6-shogaol.

IKKβ in 293T cells (Figs. 3A and 3B). These observations are consistent with the previous report describing IKKβ as the molecular target of 6-shogaol (Pan et al., 2008). However, 6-shogaol also inhibited LPS-induced IRAK-1 degradation (Fig. 3C). The results suggest that the target of inhibition by 6-shogaol is upstream of IRAK-1 in addition to IKKβ. The target may be TLR4 itself or events leading to TLR4 activation by agonist.

6-Shogaol suppresses LPS-induced dimerization of TLR4

TLR4 dimerization is one of the critical steps in the initiation of activation of TLR4 signaling and induction of subsequent immune responses. Appropriately, we investigated whether 6-shogaol could affect dimerization of TLR4 as determined by a co-immunoprecipitation experiment using differently tagged TLRs. For this study, we used IL-3-dependent Ba/F3 cells stably transfected with murine TLR4-Flag and TLR4-GFP, MD2, CD14, and NF-κB-luciferase reporter gene as described previously (Saitoh et al., 2004). 6-Shogaol suppressed the LPS-induced dimerization of TLR4 in Ba/F3 cells (Fig. 4A). Receptor dimerization of TLRs leads to the recruitment of MyD88. The interaction of MyD88 with the TIR domain of TLR recruits IRAK-4 and induces IRAK-4-induced phosphorylation of IRAK-1 leading to its degradation. The phosphorylated IRAK-1 associates with TRAF6 leading to the activation of IKK complex resulting in activation of NF-κB. Thus, IRAK-1 degradation and NF-κB activation were used as additional readouts for the dimerization of TLR4. 6-Shogaol inhibited the degradation of IRAK-1 (Fig. 4B) and NF-κB activation (Fig. 4C) induced by LPS in a dose dependent manner.

The results from our previous reports have shown that curcumin, helenalin, and cinnamaldehyde with the α, β-unsaturated carbonyl group structural motif that confers Michael addition inhibit TLR4 dimerization (Youn et al., 2006b; 2008b). It is well documented that molecules with such a structural motif are highly reactive with the sulfhydryl group of cysteine by Michael addition. We have also previously demonstrated that a gold compound having thiol binding affinity inhibits the dimerization of TLR4 induced by LPS (Youn et al., 2006c). Therefore, cysteine residues have been implicated as potential targets for 6-shogaol, since 6-shogaol also has an α,β-unsaturated carbonyl moiety to react with the sulfhydryl group of cysteine. TLRs have several cysteine residues in extracellular and cytoplasmic domains that may be involved in disulfide bond formation for receptor dimerization (Tao et al., 2002). Therefore, it is conceivable that 6-shogaol may interact with the cysteine residue in TLR4 leading to the inhibition of TLR4 dimerization.

TLRs play a critical role in the regulation of the immune and inflammatory responses, of which dysregulation would be one of the key etiological factors for the development of chronic diseases. Therefore, it is important to find the modulating mechanism of TLR activity to prevent or treat chronic disease. TLR dimerization may be one of the first regulatory points in the activation TLR-mediated signaling pathways and in the induction of subsequent immune and inflammatory responses. Therefore, disruption of TLR4 dimerization would deregulate TLR activation. Our results show that TLR activity can be regu-

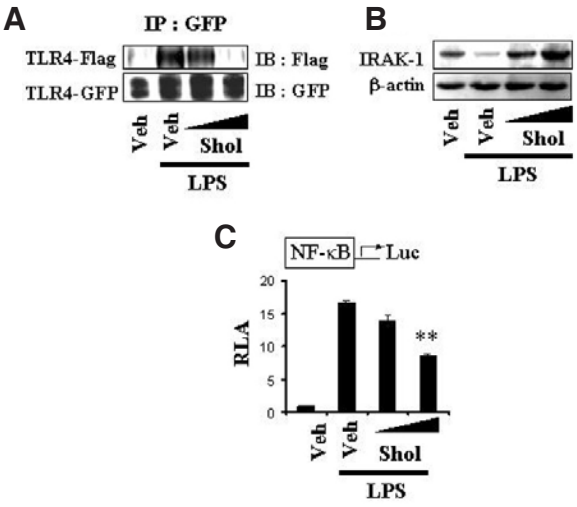

Fig. 4. The 6-Shogaol inhibited LPS-induced TLR4 dimerization. (A) Ba/F3 cells expressing TLR4-Flag (TLR4F), TLR4-GFP (TLR4G), MD2-Flag (MD2F), CD14, and NF-κB luciferase were pre-treated with 6-shogaol (20, 30 μM) for 1 h and then treated with LPS (50 ng/ml) for 20 min. Cells were then subjected to immunoprecipitation with anti-mouse GFP antibody and immunoblotted with anti-Flag (upper) or anti-rabbit GFP (lower) antibody. (B) The same Ba/F3 cells in Fig. 4A were pre-treated with 6-shogaol (20, 30 μM) for 1 h and then treated with LPS (50 ng/ml) for 30 min. Cell lysate was subjected to SDS-PAGE and probed with anti-IRAK-1 (upper) or β-actin (lower) antibody. (C) The same Ba/F3 cells in Fig. 4A were pre-treated with 6-shogaol (20, 30 μM) for 1 h and then treated with LPS (1 ng/ml) for an additional 8 h. Cell lysates were prepared and luciferase enzyme activities were measured as described in the legend of Fig. 1. Values are mean ± SEM (n = 3). **, Significantly different from LPS alone, $p < 0.01$. Veh, vehicle; Shol, 6-shogaol.

lated by 6-shogaol mediated through the modulation of receptor dimerization leading to decreased inflammatory gene expression.

Our studies demonstrate for the first time that the 6-shogal phytochemical from ginger can modulate inflammatory responses by suppressing TLR activation. 6-Shogaol inhibits the LPS-induced TLR4 dimerization, leading to the inhibition of NF-κB activation and COX-2 expression. These results provide a new paradigm in identifying specific molecular targets of anticancer and anti-inflammatory agents. Furthermore, these results suggest that TLRs themselves may be important molecular targets in the prevention of many chronic diseases.

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