TBK1-Targeted Suppression of TRIF-Dependent Signaling Pathway of Toll-Like Receptors by 6-Shogaol, an Active Component of Ginger

Se-Jeong Park, Mi-Young Lee, Bu-Soon Son, and Hyung-Sun Youn

Department of Medical Science, College of Medical Sciences, Soochunhyang University, Asan-Si, Chungnam 336-745, Korea
Department of Medical Biotechnology, College of Medical Sciences, Soochunhyang University, Asan-Si, Chungnam 336-745, Korea
Department of Environmental Health Science, College of Natural Sciences, Soochunhyang University, Asan-Si, Chungnam 336-745, Korea
Department of Biomedical Laboratory Science, College of Medical Sciences, Soochunhyang University, Asan-Si, Chungnam 336-745, Korea

Received October 15, 2008; Accepted December 27, 2008; Online Publication, July 7, 2009 [doi:10.1271/bbb.80738]

Toll-like receptors (TLRs) are primary sensors that detect a wide variety of microbial components involving induction of innate immune responses. After recognition of microbial components, TLRs trigger the activation of myeloid differential factor 88 (MyD88) and Toll-interleukin-1 (IL-1) receptor domain-containing adapter inducing interferon-β (TRIF)-dependent downstream signaling pathways. 6-Shoagol, an active ingredient of ginger, inhibits the MyD88-dependent signaling pathway by inhibiting inhibitor-κB kinase activity. Inhibitor-κB kinase is a key kinase in nuclear factor κB (NF-κB) activation. However, it is not known whether 6-shogaol inhibits the TRIF-dependent signaling pathway. Our goal was to identify the molecular target of 6-shogaol in the TRIF-dependent pathway of TLRs. 6-Shogaol inhibited the activation of interferon-regulatory factor 3 (IRF3) induced by lipopolysaccharide (LPS) and by polyriboinosinic polyribocytidylic acid (poly[I:C]), overexpression of TRIF, TANK-binding kinase1 (TBK1), and IRF3. Furthermore, 6-shogaol inhibited TBK1 activity in vitro. Together, these results suggest that 6-shogaol inhibits the TRIF-dependent signaling pathway of TLRs by targeting TBK1 and, they imply that 6-shogaol can modulate TLR-derived immune/inflammatory target gene expression induced by microbial infection.

Key words: Toll-like receptors; 6-shogaol; lipopolysaccharide; Toll-interleukin-1 (IL-1) receptor domain-containing adapter inducing interferon-β; TANK-binding kinase1

Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns and trigger the innate immune responses that are essential for host defense against invading microbial pathogens.1,2 Molecular events triggered by TLRs rely on two signal transducers, myeloid differential factor 88 (MyD88) and Toll-interleukin-1 (IL-1) receptor domain-containing adapter inducing interferon-β (TRIF), which activate signaling cascades, ultimately leading to activation of transcription factor nuclear factor-κB (NF-κB) and to the initiation of innate immune responses.3,4 MyD88 is the immediate adaptor molecule for all mammalian TLRs, except for TLR3.5 It recruits IL-1 receptor-associated kinases (IRAK4 and IRAK1) and TNF receptor-associated factor 6 (TRAF6) leading to activation of the canonical inhibitor-κB kinase (IKK) complex followed by activation of NF-κB. Activation of NF-κB induces gene expression of cytokine, leading to pro-inflammatory responses.4

Recognition of viral double-stranded RNA (dsRNA) by TLR3 or bacterial lipopolysaccharide (LPS) by TLR4 leads to transcription factor NF-κB and interferon-regulatory factor 3 (IRF3) activation.5,6 Activation of TLR3 and TLR4 recruits TRIF which activates the downstream kinases, TANK-binding kinase1 (TBK1) and IKKε leading to IRF3 activation7 and receptor interacting protein-1 (RIP1), leading to delayed activation of NF-κB.8,9 TBK1 and IKKε are two serine/threonine kinases that lie upstream of IRF3. Activated TBK1 phosphorylates IRF3, resulting in IRF3 dimerization and subsequent translocation to the nucleus. IRF3 translocated into the nucleus binds to consensus DNA sequences known as the interferon (IFN)-stimulated response element (ISRE), found in the promotor regions of genes such as encoding IFNβ and Regulated on Activation Normal T-cell Expressed and Secreted (RANTES).9,10 These IRF3-regulated genes play an important role in anti-viral and anti-bacterial innate immune responses.11

Ginger, the powered rhizome of the herb Zingiber officinale Roscoe, is widely used as a spice, food, and herbal medicine. In Ayurvedic medicine, ginger has...
traditionally been used as a treatment for rheumatism, nervous diseases, gingivitis, toothache, asthma, stroke, constipation, diabetes, and arthritis. Ginger extracts, a multicomponent mixture of biologically active constituents, have anti-inflammatory, anti-oxidant, and anti-cancer effects. The major ginger-derived components that account for ginger’s anti-inflammatory properties are gingerols and the dehydration products of gingerols (shogaols) (Fig. 1). In particular, gingerols and a number of gingerol derivatives are effective inhibitors of prostaglandin and leukotriene synthesis enzyme extracts. Ginger-derived components can also inhibit NF-κB activation and cyclooxygenase-2 (COX-2) induced by a variety of agents. The anti-inflammatory effects of ginger-derived components depend on the lengths, of the side chains. These components inhibit the activation of NF-κB by inhibiting IκBα, which lies downstream of the MyD88-dependent pathway of TLRs, but it is not known whether ginger-derived components inhibit the TRIF-dependent signaling pathway of TLRs. More than 70% of LPS-induced genes are regulated through the TRIF-dependent pathway. Since the most bioactive component of ginger is 6-shogaol, we attempted to identify the molecular target of 6-shogaol in the TRIF-dependent signaling pathways of TLRs.

Materials and Methods

Reagents. 6-Shogaol was purchased from Wako Pure Chemical (Osaka, Japan). Purified LPS was from List Biological Lab (San Jose, CA). Poly[I:C] was from Amersham Biosciences (Piscataway, NJ). All other reagents were from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

Cell culture. RAW 264.7 cells (murine monocytic cell line, ATCC TIB-71) and 293T cells (human embryonic kidney cells) were cultured in Dulbecco’s Modified Eagle’s Medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml, penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). The cells were maintained at 37°C in a 5% CO₂/air environment.

Plasmids. NF-κB (2×)-luciferase reporter construct was provided by Dr. Frank Mercurio (Signal Pharmaceuticals, San Diego, CA). Heat shock protein 70 (HSP70)-β-galactosidase reporter plasmid was provided by Dr. Robert Modlin (University of California, Los Angeles, CA). All DNA constructs were prepared on a large scale using an EndoFree Plasmid Maxi kit (Quiagen, Chatsworth, CA) for transfection.

Transfection and luciferase assay. Both procedures were performed as described previously. RAW 264.7 or 293T cells were co-transfected with NF-κB-luciferase plasmid and HSP70-β-galactosidase plasmid as an internal control using SuperFect transfection reagent (Qiagen) according to the manufacturer’s instructions. Various expression plasmids or the corresponding empty vector plasmids for signaling components were co-transfected. Luciferase enzyme activities were determined using a commercial luciferase assay system (Promega, Madison, WI) 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.

Statistic analysis. Statistic analysis was determined by standard error of the mean (SEM). All the data were from more than three independent experiments.

Immunoblotting. Immunoblotting was performed as described previously. Equal amounts of protein extracts were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a polyvinylidene difluoride membrane. The membranes were blocked with phosphate-buffered saline containing 0.1% Tween-20 and 3% nonfat dry milk. They were then blotted with the indicated antibodies and secondary antibodies conjugated to horseradish peroxidase (HRP; Amersham, Arlington Heights, IL). The reactive bands were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

In vitro TBK1 kinase assay. TBK1 kinase activity was conducted with recombinant human TBK1 (Upstate Biotechnology, Lake Placid, NY) and a chemiluminescence detection myelin basic protein (MBP) assay kit (Upstate Biotechnology) according to the manufacturer’s instructions. The assay kit is designed to measure the phosphotransferase activity of numerous kinases, including TBK1, c-Jun kinase, and mitochondrion-activated kinase based on phosphorylation of MBP using a non-radioactive magnesium/ATP cocktail as phosphate donor. A mixture of reagents, magnesium/ATP and recombinant TBK1 was added to MBP-coated wells. After incubation of the wells for 30 min at 30°C, they were blocked with blocking buffer. HRP-conjugated anti-phospho-MBP was added to them. After washing with washing buffer, luminescent intensity was determined by the LumiGLO™ chemiluminescent system (Upstate Biotechnology).

Results and Discussion

6-Shogaol suppressed the activation of NF-κB induced by TLR3 and by TLR4 agonists

TLR signaling pathways trigger the activation of NF-κB mediated through MyD88- and TRIF-dependent pathways. TLR4 activates both MyD88- and TRIF-dependent signaling pathways, leading to NF-κB activation and expression of target genes such as COX-2. Hence, activation of NF-κB and expression of COX-2 were used as the readout for LPS-induced TLRs activation. 6-Shogaol inhibited the activation of NF-κB induced by LPS in RAW264.7 cells, as determined by luciferase reporter gene assay (Fig. 2A) (Supplemental Fig. 1; see Biosci. Biotechnol. Biochem. Web site). 6-Shogaol also inhibited LPS-induced COX-2 expression, as determined by COX-2 immunoblotting (Fig. 2B).

Although the TLR4 signaling pathway can trigger activation of NF-κB through both MyD88- and TRIF-dependent pathways, TLR3 triggers activation of NF-κB only through the TRIF-dependent signaling pathway. Therefore, the activation of NF-κB induced by poly[I:C] (TLR3 agonist) can be used as the readout for the TRIF-dependent pathway. 6-Shogaol inhibited NF-κB activation induced by poly[I:C], as determined by luciferase reporter gene assay in RAW 264.7 (Fig. 2C). Poly[I:C]-
assay using an IFN-β/C12 reporter gene assay (Fig. 2D).

...shogaol, as determined by COX-2 immunoblotting induced COX-2 expression was also inhibited by 6-shogaol, as determined by COX-2 immunoblotting (Fig. 2D).

6-Shogaol suppressed the TRIF-dependent signaling pathways of TLR3 and TLR4.

The TRIF-dependent signaling pathways of TLR3 and TLR4 lead to the activation of IFN-β transcription factor mediated through TRIF. Activation of IFN-β was used as the readout for the TRIF-dependent pathways. Activation of IFN-β was determined by reporter gene assay using an IFN-β promoter domain containing a IFN-β binding site (IFN-β PRDIII-I). 6-Shogaol suppressed the expression of IFN-β induced by poly[I:C] and by LPS (Fig. 3A and B). These results confirm that 6-shogaol suppresses TRIF-dependent signaling pathways derived from TLR3 and TLR4 activation.

To further identify the molecular targets of 6-shogaol for inhibition of the TRIF-dependent pathways, downstream components (TRIF, TBK1, and IRF3) of the pathway were transfected into 293T cells. The TRIF-dependent pathways of TLRs lead to activation of the IFN-β transcription factor mediated through TRIF and TBK1. 6-Shogaol inhibited TRIF-, TBK1- and constitutively active IFN-β-induced IFN-β activation, as determined by IFN-β binding site (IFN-β PRDIII-I) reporter gene assay (Fig. 4A–C). Together, these results indicate that 6-shogaol inhibits the TRIF-dependent signaling pathway of TLRs.
We next sought to determine which signaling molecules in the TRIF-dependent pathway of TLRs are the targets of 6-shogaol. We investigated to determine whether 6-shogaol would affect TBK1 kinase activity in vitro using a MBP chemiluminescence detection assay kit. The results showed that 6-shogaol suppressed TBK1 kinase activity in a dose-dependent manner (Fig. 4D) suggesting that TBK1 is the molecular target of 6-shogaol which mediates anti-inflammatory activity through TLRs.

Both the NF-κB and IRF transcription factor families are simultaneously activated in response to viral and bacterial infection, but the target genes induced by these signaling pathways are distinct. Indeed, whereas NF-κB activation induces proinflammatory cytokines, IRF activation induces type I IFN genes.11,27,28 IRFs are involved in the regulation of the cell cycle, apoptosis, and tumor suppression.11,29

NF-κB activation induced by viral or bacterial infections involves signal-induced phosphorylation and subsequent degradative polyubiquitination of the inhibitory-κB (IκB) protein through the canonical IKK complex the composed of two catalytic kinase subunits, IκKα and IκKβ, and a nonenzymatic regulatory subunit, NF-κB essential modulator (NEMO) or IκKγ.2,3,30-33 In contrast to NF-κB activation, IRF3 activation in the cytoplasm occurs directly through C-terminal phosphorylation by an additional IKK: TBK1, also referred to as NF-κB activating kinase (NAK),34 or TNF-receptor-associated factor 2 (TRAF2)-interacting kinase (T2K)35 and IκKε (also referred to as IκKι).36,37 These modifications induce homodimerization of IRF3 and subsequent translocation of IRF3 into the nucleus.37 In spite of similar homology and functional similarities between TBK1 and IκKε, the expression patterns of these two kinases are distinct. While TBK1 expression is ubiquitous and constitutive in a wide variety of cells, IκKε expression is relegated to cells of the immune compartment, but is inducible in non-hematopoietic cells by stimulation with activating agents such as TNF, phorbol myristate acetate (PMA), and LPS, and by virus infection.36,38-40 Analysis of mouse embryonic fibroblasts derived from TBK1 knockout mice indicated that TBK1 is principally involved in downstream signaling to IRF3 activation and IFNβ induction after stimulation with TLR3 and TLR4 ligands,40-42 but mice lacking IκKε show no obvious changes with respect to IRF3 activation and IFNβ production.40 These results suggest that TBK1 has a crucial role in IRF3 activation and IFN gene induction.

The contributions of TBK1 and IκKε to NF-κB activation mediated through TLRs have been investigated using TBK1 and IκKε double knockout mice.40 In those mice, IκBα degradation and NF-κB DNA binding showed no changes after stimulation with TLR3 and TLR4 agonists. Thus the production of inflammatory cytokines is normal in these mice. These results indicate that TBK1 and IκKε are not required for TLR-mediated NF-κB activation.

Activation of TLR4 by LPS leads to both early and late activation of NF-κB, through the MyD88- and TRIF-dependent signaling pathways respectively. Activation of IRF3 mediated through TRIF and TBK1 is responsible for the expression of IFNβ- and IFN- inducible genes.7 More than 70% of LPS-inducible genes are regulated through the TRIF-dependent pathway.22 These facts suggest that suppression of the TRIF-dependent signaling pathway and consequent down-regulation of IFR3 and NF-κB activation by 6-shogaol can significantly suppress the target gene expression of TRL3 and TLR4.

In the present study, we found for the first time that 6-shogaol suppressed the TRIF-dependent pathways of TLR3 and TLR4. TBK1 is the molecular target of the inhibitory effect of 6-shogaol. Suppression of the TRIF pathway of TLR3 and TLR4 by 6-shogaol is accompanied by down-regulation of the activation of NF-κB and IRF3 and of their target genes, including COX-2 and IFNβ.22 Our results perhaps provide new insight the mode of action of 6-shogaol as to its anti-bacterial, anti-viral, and anti-inflammatory activities.

Acknowledgments

This study was supported by the Ministry of Environment of the Republic of Korea the Eco-Technopia 21 Project.

References