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Molecules and Cells

Suppression of the TRIF-Dependent Signaling Pathway of Toll-Like Receptors by Isoliquiritigenin in RAW264.7 Macrophages

Se-Jeong Park[1], Ho-Yeon Song[2,*], and Hyung-Sun Youn[1,3,*]

Toll-like receptors (TLRs) play an important role in host defense by sensing invading microbial pathogens and initiating innate immune responses. The stimulation of TLRs by microbial components triggers the activation of myeloid differential factor 88 (MyD88)- and toll-interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF)-dependent downstream signaling pathways. Isoliquiritigenin (ILG), an active ingredient of Licorice, has been used for centuries to treat many chronic diseases. ILG inhibits the MyD88-dependent pathway by inhibiting the activity of inhibitor-κB kinase. However, it is not known whether ILG inhibits the TRIF-dependent pathway. To evaluate the therapeutic potential of ILG, we examined its effect on signal transduction via the TRIF-dependent pathway of TLRs induced by several agonists. ILG inhibited nuclear factor-κB and interferon regulatory factor 3 activation induced by lipopolysaccharide or polyinosinic-polycytidylic acid. ILG inhibited the lipopolysaccharide-induced phosphorylation of interferon regulatory factor 3 as well as interferon-inducible genes such as interferon inducible protein-10, and regulated activation of normal T-cell expressed and secreted (RANTES). These results suggest that ILG can modulate TRIF-dependent signaling pathways of TLRs, leading to decreased inflammatory gene expression.

INTRODUCTION

Toll-like receptors (TLRs) recognize diverse molecular products derived from all the major classes of microbes including bacteria, viruses, yeast, and fungi. TLRs induce innate immune responses that are essential for host defense against invading microbial pathogens (Bjorkbacka et al., 2004a; Medzhitov et al., 1997). TLRs are type I transmembrane receptors, consisting of leucine-rich repeats (LRR) in the extracellular domain and a Toll/interleukin (IL)-1R (TIR) homologous region in the cytoplasmic domain (Uematsu and Akira, 2007). The activation of these receptors by their respective ligands recruits one or more TIR domain-containing adapter molecules, such as myeloid differentiation protein-88 (MyD88) or toll-interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF).

MyD88, the first TIR-domain adapter molecule to be described, is an essential molecule for signaling by all mammalian TLRs except for TLR3 (Takeda and Akira, 2005). MyD88 recruits IL-1 receptor-associated kinase (IRAK) to TLRs. Two members of the IRAK family, IRAK-1 and IRAK-4, are activated by phosphorylation and associate with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) leading to the activation of the canonical I kappa B kinase (IKK) complex followed by the activation of the nuclear factor-κB (NF-κB) transcription factor. This signaling pathway, the MyD88-dependent pathway, induces the expression of inflammatory cytokine genes including TNF-α, IL-6, IL-12, and IL-1β (Akira et al., 2006).

In addition to the proinflammatory signals, TLR3 and TLR4 have the ability to induce type I interferons (IFNs) in a MyD88-independent pathway (Akira et al., 2006). TRIF is an essential adapter molecule for the TLR3- and TLR4-mediated MyD88-independent pathway. TRIF activates two noncanonical IKKs, inducible IKK (IKK*i*)/IKKε and TRAF family member associated NF-κB activator-binding kinase1 (TBK1)/NF-κB-activating kinase (NAK)/TRAF2-associated kinase (T2K), leading to the activation of interferon regulatory factor 3 (IRF3) (Fitzgerald et al., 2003). The representative target genes regulated through TRIF-dependent signaling pathway of TLRs include IFN-β and IFN-inducible genes such as inducible nitric oxide synthase (iNOS), interferon inducible protein-10 (IP-10), and regulated on activation normal T-cell expressed and secreted (RANTES) (Bjorkbacka et al., 2004a; Gao et al., 1998; Kawai et al., 2001). The activation of the TRIF pathway also leads to the delayed activation of NF-κB mediated through the association of TRIF with receptor-interacting protein-1 (RIP1) (Takeda and Akira, 2005).

Licorice (*Glycyrrhiza uralensis*) has been used for centuries as a flavoring agent in foods, beverages, and tobacco, and to treat several diseases. Studies have revealed that licorice-derived compounds that include glycyrrhizin, isoliquiritigenin (ILG), licochalcone, and glabridin have a variety of pharmaceutical effects. Of these, ILG, a flavonoid with chalcone structure (4,2′,4′-trihydroxychalcone) (Fig. 1), has been evaluated for its

[1]Departments of Medical Science, College of Medical Sciences, Soonchunhyang University, Asan 336-745, Korea, [2]Department of Microbiology, School of Medicine, Soonchunhyang University, Cheonan 330-090, Korea, [3]Department of Biomedical Laboratory Science, College of Medical Sciences, Soonchunhyang University, Asan 336-745, Korea
*Correspondence: songmic@sch.ac.kr (HS); hyoun@sch.ac.kr (HY)

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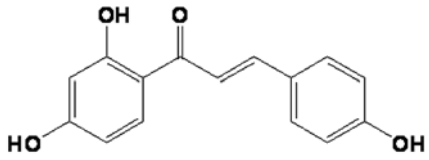

Fig. 1. The structure of isoliquiritigenin

various biological activities including anti-inflammatory and antioxidant activity (Kakegawa et al., 1992; Vaya et al., 1997). In particular, ILG was found to suppress lipopolysaccharide (LPS)-induced cyclooxygenase-2 (COX-2) and iNOS expression in RAW264.7 cells (Takahashi et al., 2004).

The activation of TLRs by agonists induces inflammatory responses that are key etiological conditions for the development of many chronic inflammatory diseases. It is already known that ILG inhibits MyD88-dependent signaling pathway of TLRs by the inhibition of IKKβ (Kumar et al., 2007). However, it is not known whether ILG inhibits the TRIF-dependent signaling pathway of TLRs. The present study was undertaken to investigate this.

MATERIALS AND METHODS

Reagents

Isoliquiritigenin was purchased from Sigma-Aldrich (USA). It was dissolved in dimethyl sulfoxide prior to use. Purified LPS was purchased from List Biologicals (USA) and dissolved in endotoxin-free water. Polyinosinic-polycytidylic acid (poly[I:C]) was purchased from Amersham Biosciences (USA). All other reagents were purchased from Sigma-Aldrich unless otherwise described.

Cell culture

RAW 264.7 cells (a murine monocytic cell line) purchased from the American Type Culture Collection (USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/ml Penicillin and 100 μg/ml Streptomycin (Invitrogen, USA). Cells were maintained at 37°C in a 5% CO_2/air environment.

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 from Robert Modlin (University of California, Los Angeles, Worcester, USA). IFNβ PRDIII-I luciferase plasmid was kind gift from Katherine A. Fitzgerald (University of Massachusetts Medical School, Worcester, USA). IP-10- and RANTES-luciferase reporter constructs were from Dr. Daniel Hwang (University of California, Davis, USA). All DNA constructs were prepared in large scale using EndoFree Plasmid Maxi kit (Qiagen, USA) for transfection.

Transfection and luciferase assay

The assays were performed as described previously (Ahn et al., 2009; Youn et al., 2009). Briefly, RAW264.7 cells were co-transfected with a luciferase plasmid and HSP70-β-galactosidase plasmid as an internal control using SuperFect transfection reagent (Qiagen, USA) according to the manufacturer's instructions. Luciferase and β-galactosidase enzyme activities were determined using commercial luciferase assay and β-galactosidase enzyme systems (Promega, USA) according to the manufacturer's instructions. Luciferase activity was normalized by β-galactosidase activity. Data were obtained from triplicate experiments. Values are expressed as mean ± standard error of the mean (SEM).

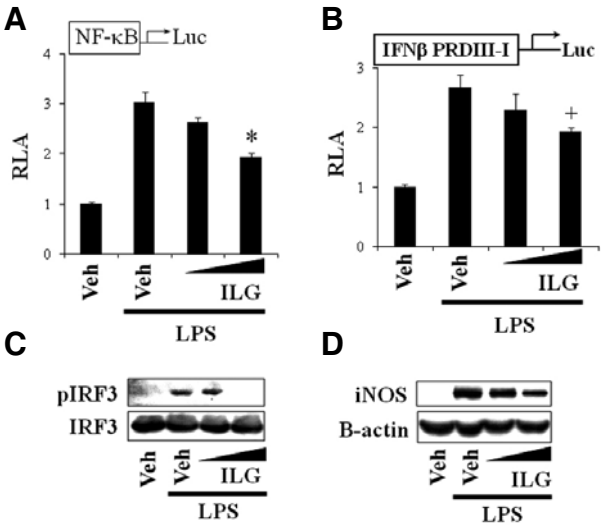

Fig. 2. Isoliquiritigenin inhibits LPS-induced IRF3 activation and phosphorylation. (A, B) RAW264.7 cells were transfected with NF-κB (A) or IRF3 binding site (IFNβ PRDIII-I) (B) luciferase reporter plasmid and pre-treated with isoliquiritigenin (20, 50 μM) for 1 h and then treated with LPS (10 ng/ml) 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 isoliquiritigenin (20, 50 μM) for 1 h and then further stimulated with LPS (10 ng/ml) for 2 h. Cell lysates were analyzed for phospho-IRF3 (S396) and IRF3 proteins by Western blotting. (D) RAW264.7 cells were pretreated with isoliquiritigenin (20, 50 μM) for 1 h and then further stimulated with LPS (10 ng/ml) for 8 h. Cell lysates were analyzed for iNOS and actin proteins by Western blotting. Veh, vehicle; ILG, isoliquiritigenin.

Western blotting

These were performed the same as previously described (Youn et al., 2006). Equal amounts of extracts were resolved on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and separated proteins were electrotransferred to a polyvinylidene difluoride membrane. The membrane was blocked with phosphate-buffered saline containing 0.1% Tween 20 and 3% nonfat dry milk and were blotted with the indicated antibodies and secondary antibodies conjugated to horseradish peroxidase (Amersham, USA). The reactive bands were visualized with an enhanced chemiluminescence system (Amersham Biosciences, USA). To reprobe with different antibodies, the membrane was stripped in 0.2 N of NaOH at room temperature for 10 min. IRF3 and phospho-IRF3 antibodies were purchased from Zymed Laboratories (USA) and Cell Signaling (USA), respectively.

ELISA

For the measurement of IP-10 concentration, cells were pretreated with ILG (20.50 μM) for 1 h and then treated with LPS (10 ng/ml) or poly[I:C] (10 μg/ml) for an additional 8 h. The levels of IP-10 were determined with culture medium by using IP-10 ELISA kit according to the manufacturer's instruction (R&D

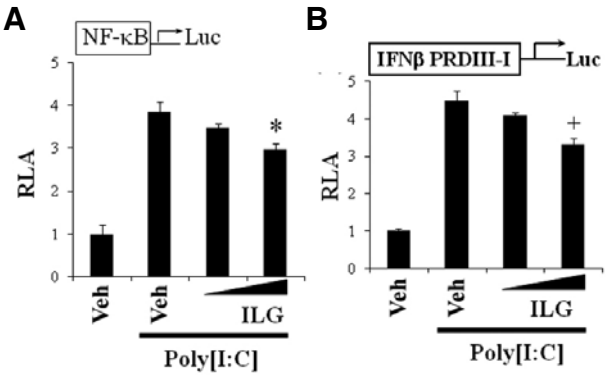

Fig. 3. Isoliquiritigenin inhibits poly[I:C]-induced NF-κB and IRF3 activation. (A, B) RAW264.7 cells were transfected with NF-κB (A) or IRF3 binding site (IFNβ PRDIII-I) (B) luciferase reporter plasmid and pre-treated with isoliquiritigenin (20, 50 μM) for 1 h and then treated with poly[I:C] (10 μg/ml) 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 poly[I:C] alone (A), $p < 0.05$. +, Significantly different from poly[I:C] alone (B), $p < 0.05$. Veh, vehicle; ILG, isoliquiritigenin.

Systems, USA).

Cell viability test

Cell viability was assessed by using MTS [3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] based colorimetric assay. Assays are performed by adding a small amount of the CellTiter 96 AQ_{ueous} One Solution Reagent (Promega, USA) directly to culture wells, incubating for 4 h and then recording the absorbance at 490 nm with a 96-well plate reader.

RESULTS AND DISCUSSION

ILG suppresses LPS-induced NF-κB and IRF3 activation

TLR signaling pathways trigger the activation of NF-κB mediated through both MyD88- and TRIF-dependent pathways. LPS (a TLR4 agonist) triggers both MyD88- and TRIF-dependent pathways leading to the activation of NF-κB. Therefore, the activation of NF-κB was used as the readout for LPS-induced TLR4 activation. ILG inhibited LPS-induced NF-κB activation in RAW264.7 cells as determined by a luciferase reporter gene assay (Fig. 2A).

Next, we determined whether ILG could inhibit TRIF-dependent signaling pathway of TLR4. This pathway induces the activation of downstream kinases such as TBK1 and IKKi, and then phosphorylates IRF3 leading to the activation of IRF3 (Fitzgerald et al., 2003). Therefore, IRF3 activation was used as the readout for TRIF-dependent pathway. ILG inhibited LPS-induced IRF3 activation as determined by a reporter gene assay using the IFNβ promoter domain containing the IRF3 binding site (IFNβ PRDIII-I) (Fig. 2B). ILG also inhibited the phosphorylation of IRF3 (Fig. 2C) and the expression of iNOS (Fig. 2D) as determined by Western blotting. These results suggest that ILG inhibits TRIF-dependent signaling pathway derived from TLR4 activation.

ILG suppresses poly[I:C]-induced NF-κB and IRF3 activation

Although the TLR4 signaling pathway can trigger NF-κB and IRF3 activation mediated through both MyD88- and TRIF-dependent pathways, TLR3 triggers NF-κB and IRF3 activation only through the TRIF-dependent pathway. Therefore,

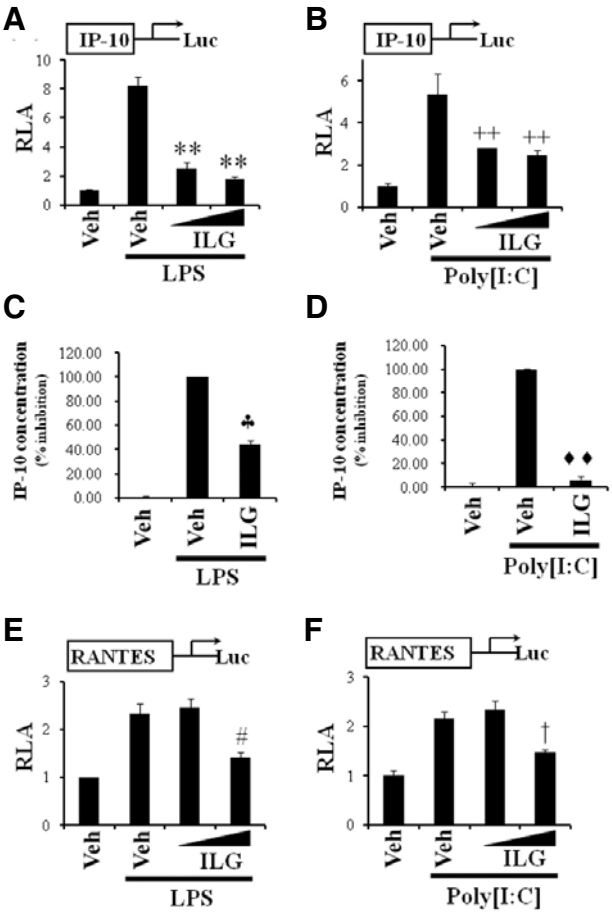

Fig. 4. Isoliquiritigenin inhibits LPS or poly[I:C]-induced IP-10 and RANTES expression. (A, B) RAW264.7 cells were transfected with IP-10-luciferase reporter plasmid and pre-treated with isoliquiritigenin (20, 50 μM) for 1 h and then treated with LPS (10 ng/ml) (A) or poly[I:C] (10 μg/ml) (B) for an 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), $p < 0.01$. ++, Significantly different from poly[I:C] alone (B), $p < 0.01$. (C, D) RAW264.7 cells were pre-treated with isoliquiritigenin (50 μM) for 1 h and then treated with LPS (10 ng/ml) (C) or poly[I:C] (10 μg/ml) (D) for an additional 8 h. Protein concentrations from cell culture supernatants were determined by ELISA as described in "Materials and Methods". Values are mean ± SEM (n = 2). ♣, Significantly different from LPS alone (C), $p < 0.05$. ♦♦, Significantly different from poly[I:C] alone (D), $p < 0.01$. (E, F) RAW264.7 cells were transfected with RANTES-luciferase reporter plasmid and pre-treated with isoliquiritigenin (20, 50 μM) for 1 h and then treated with LPS (10 ng/ml) (E) or poly[I:C] (10 μg/ml) (F) 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 (E), $p < 0.05$. †, Significantly different from poly[I:C] alone (F), $p < 0.05$. Veh, vehicle; ILG, isoliquiritigenin.

induction of NF-κB and IRF3 activation by poly[I:C] (a TLR3 agonist) can be used as the readout for the TRIF-dependent pathway. ILG inhibited poly[I:C]-induced NF-κB and IRF3 activation as determined by luciferase reporter gene assay (Figs. 3A and 3B).

ILG suppresses LPS or poly[I:C]-induced IP-10 and RANTES expression

To further identify the inhibition of TRIF-dependent pathway by ILG, the expression of genes associated with the TRIF-dependent pathways such as IP-10 and RANTES were measured by a luciferase reporter gene assay and ELISA. ILG inhibited LPS or poly[I:C]-induced IP-10 and RANTES expression (Fig. 4). The results are consistent with ILG inhibition of the TRIF-dependent signaling pathway of TLR3 and TLR4 for the inhibition of NF-κB and IRF3.

Recently, it was demonstrated that ILG effectively inhibits LPS-induced iNOS and COX-2 expression and iNOS, COX-2, TNFα, and IL-6 transcriptions by inhibiting the degradation and phosphorylation of IκBα in RAW264.7 macrophages (Kim et al., 2008). The anti-inflammatory effects of ILG are also accomplished by reducing NF-κB activation via IKK signaling pathway on human endothelial cells and human lung epithelial cells (Kumar et al., 2007). Therefore, the NF-κB inhibition by ILG is not a cell type specific. ILG inhibited TNFα-induced NF-κB activation but not AP-1 and Oct-1 transcription factors (Kumar et al., 2007). Thus, the inhibitory activity of ILG is specific to NF-κB.

All TLR signaling pathways culminate in the activation of NF-κB, which induces cytokine gene expression leading to pro-inflammatory responses. In addition to NF-κB activation, TLR3 and TLR4 lead to IRF3 activation that is mediated through the TRIF-dependent signaling pathway (Lin et al., 1998; Navarro and David, 1999). The activation of TLR3 and TLR4 recruits TRIF that activates the downstream kinases TBK1 and IKKε. Their activation 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 IFN-stimulated response elements (ISRE) found in the promoter regions of genes such as those encoding IFNβ, IP-10, and RANTES (Bjorkbacka et al., 2004a; Lin et al., 1999; Schafer et al., 1998). Since more than 70% of LPS-inducible genes are regulated through TRIF-dependent pathway (Bjorkbacka et al., 2004b), the modulation of the TRIF-dependent pathway of TLRs might be useful and novel anti-inflammatory strategy. Our results showed that ILG suppressed IFNβ production induced by TLR3 or TLR4 agonists and the transcriptional activation of IRF3 by inhibiting TRIF-dependent signaling pathway.

In the present study, we demonstrate that ILG suppresses TRIF-dependent pathways of TLR3 and TLR4. The suppression of TRIF pathway of TLR3 and TLR4 by ILG is accompanied by the down-regulation of the activation of NF-κB and IRF3, and of their target genes including IFNβ, IP-10, and RANTES. Our results further the understanding of the mechanism of ILG for its anti-viral, anti-bacterial, and anti-inflammatory activities.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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