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Molecules and Cells

Parthenolide Inhibits TRIF-Dependent Signaling Pathway of Toll-like Receptors in RAW264.7 Macrophages

Se-Jeong Park[1], Hwa-Jeong Shin[2], and Hyung-Sun Youn[1,2,*]

Toll-like receptors (TLRs) play an important role in induction of innate immune responses for host defense against invading microbial pathogens. Microbial component engagement of TLRs can trigger the activation of myeloid differential factor 88 (MyD88)- and toll-interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF)-dependent downstream signaling pathways. Parthenolide, an active ingredient of feverfew (*Tanacetum parthenium*), has been used for centuries to treat many chronic diseases. Parthenolide inhibits the MyD88-dependent pathway by inhibiting the activity of inhibitor-κB kinase. However, it is not known whether parthenolide inhibits the TRIF-dependent pathway. To evaluate the therapeutic potential of parthenolide, its effect on signal transduction via the TRIF-dependent pathway of TLRs induced by lipopolysaccharide (LPS) or polyinosinic-polycytidylic acid (poly [I:C]) was examined. Parthenolide inhibited nuclear factor-κB and interferon regulatory factor 3 activation induced by LPS or poly[I:C], and the LPS-induced phosphorylation of interferon regulatory factor 3 as well as interferon-inducible genes such as interferon inducible protein-10. These results suggest that parthenolide can modulate TRIF-dependent signaling pathways of TLRs, and may be the basis of effective therapeutics for chronic inflammatory diseases.

INTRODUCTION

Parthenolide (Fig.1), one of the major sesquiterpene lactones found in the medicinal plant feverfew (*Tanacetum parthenium*), has shown many antitumor effects against human acute myeloid leukemia (Guzman et al., 2005), acute and chronic lymphocytic leukemia (Steele et al., 2006), and such solid tumors as breast and pancreatic cancer (Nakshatri et al., 2004; Yip-Schneider et al., 2005). The anti-inflammatory effects of parthenolide are accomplished by reducing NF-κB activation (Hehner et al., 1999). Parthenolide inhibits lipopolysaccharide (LPS)-induced cyclooxygenase-2 (COX-2) expression by inhibiting the degradation and phosphorylation of nuclear factor-κB (NF-κB) inhibitor IκBα (Hehner et al., 1998). Parthenolide inhibited TNF- or IL-1β-induced IL-8 secretion and prevented NF-κB activation, IκBα degradation, and IκB kinase complex activity (Saadane et al., 2007).

Toll-like receptors (TLRs) play an important role for host defense by sensing invading microbial pathogens and initiate immune responses. The activation of TLRs triggers inflammatory responses that are essential for host defense against invading pathogens (Bjorkbacka et al., 2004; Medzhitov et al., 1997). The activation of these receptors 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 is an essential molecule for signaling by all mammalian TLRs except for TLR3 (Takeda and Akira, 2005). MyD88 is a common downstream adaptor molecule 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. 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 trigger the TRIF-dependent signaling pathway to induce inflammatory cytokines and type I interferons (IFNs) (Akira et al., 2006). 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 (IRF3) (Fitzgerald et al., 2003), which results in expression of 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., 2004; Gao et al., 1998; Kawai et al., 2001). The activation of the TRIF pathway also leads to delayed NF-κB activation mediated through the association of TRIF with receptor-interacting protein-1 (RIP1) (Takeda and Akira, 2005).

Parthenolide inhibits MyD88-dependent signaling pathway of TLRs by the inhibition of IKKβ (Saadane et al., 2007). However, it is not known whether parthenolide inhibits the TRIF-depen-

[1]Department of Medical Science, College of Medical Sciences, Soonchunhyang University, Asan 336-745, Korea, [2]Department of Biomedical Laboratory Science, College of Medical Sciences, Soonchunhyang University, Asan 336-745, Korea
*Correspondence: hyoun@sch.ac.kr

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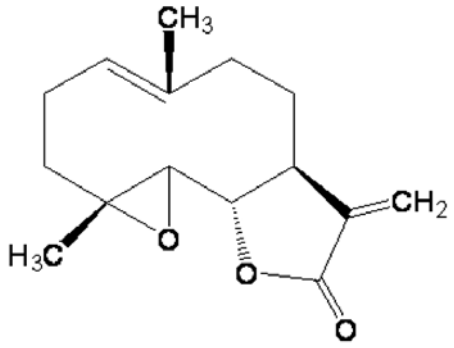

Fig. 1. The structure of parthenolide

dent signaling pathway of TLRs. More than 70% of LPS-induced genes are regulated through TRIF-dependent signaling pathway of TLRs (Bjorkbacka et al., 2004), indicating that the modulation of TRIF-dependent signaling pathway might be a useful anti-inflammatory strategy. Appropriately, the present study investigated whether parthenolide can modulate the TRIF-dependent signaling pathway of TLRs.

MATERIALS AND METHODS

Reagents

Parthenolide purchased from Sigma-Aldrich (USA) was dissolved in dimethyl sulfoxide prior to use. Purified LPS purchased from List Biologicals (USA) was 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

RAW264.7 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 (Hyclone, USA), 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 and heat shock protein 70 (HSP70)-β-galactosidase reporter construct were provided by Frank Mercurio (Signal Pharmaceuticals, USA) and Robert Modlin (University of California, USA), respectively. IFNβ PRDIII-I and IP-10-luciferase plasmids were a kind gift from Katherine A. Fitzgerald (University of Massachusetts Medical School, USA) and Daniel Hwang (University of California, USA), respectively. 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; Park et al., 2009a). RAW264.7 cells were transfected with a luciferase plasmid by using SuperFect transfection reagent (Qiagen, USA). Heat shock protein (HSP)70-β-galactosidase plasmid was co-transfected as an internal control. 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 to determine relative luciferase activity. Data were obtained from triplicate experiments. Values are expressed as mean ± standard error of the mean (SEM).

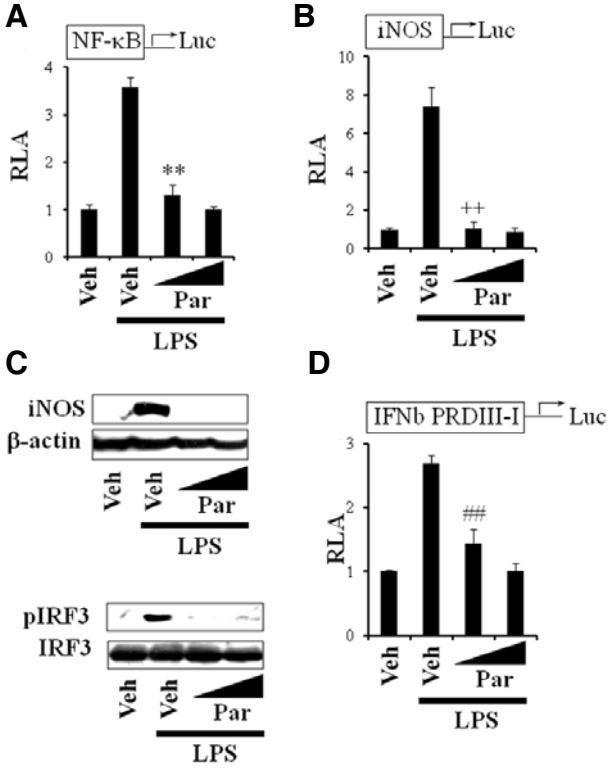

Fig. 2. Parthenolide inhibits LPS-induced NF-κB and IRF3 activation and iNOS expression. (A, B) RAW264.7 cells were transfected with NF-κB (A) or iNOS (B) luciferase reporter plasmid and pre-treated with parthenolide (10, 20 μM) for 1 h and then treated with LPS (10 ng/ml) for an additional 8 h. Cell 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), $p < 0.01$. ++, Significantly different from LPS alone (B), $p < 0.01$. (C) RAW264.7 cells were pretreated with parthenolide (10, 20 μM) for 1 h and then further stimulated with LPS (10 ng/ml) for 8 h. Cell lysates were analyzed for iNOS and β-actin proteins by Western blotting. (D) RAW264.7 cells were transfected with IRF3 binding site (IFNβ PRDIII-I) luciferase reporter plasmid and pre-treated with parthenolide (10, 20 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), $p < 0.01$. (E) RAW264.7 cells were pretreated with parthenolide (10, 20 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. Veh, vehicle; Par, parthenolide.

Western blotting

The procedure was performed the same as previously described (Park et al., 2009b; Youn et al., 2010). Equal amounts of extracts were resolved on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and separated proteins

were electro transferred to a polyvinylidene difluoride membrane. The membrane was 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).

Enzyme-linked immunosorbent assay (ELISA)

For the measurement of IP-10 concentration, cells were pretreated with parthenolide (10, 20 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 Systems, USA).

RESULTS AND DISCUSSION

Parthenolide suppresses LPS-induced NF-κB and IRF3 activation

LPS (a TLR4 agonist) triggers the activation of NF-κB mediated through both MyD88- and TRIF-dependent pathways. Therefore, the activation of NF-κB and expression of its target gene such as iNOS was used as the readout for TLR4 activation induced by LPS. Parthenolide inhibits the activation of NF-κB by targeting IKKβ (Saadane et al., 2007). Parthenolide inhibited LPS-induced NF-κB activation in RAW264.7 cells as determined by a luciferase reporter gene assay (Fig. 2A). Parthenolide also inhibited LPS-induced iNOS expression as determined by a luciferase reporter gene assay and immunoblotting (Figs. 2B and 2C).

The next experiment determined whether parthenolide could inhibit TRIF-dependent signaling pathway of TLR4. Since this pathway induces the activation of IRF3 transcription factor (Fitzgerald et al., 2003), IRF3 activation was used as the readout for the TRIF-dependent pathway. Parthenolide 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. 2D). Parthenolide also inhibited the phosphorylation of IRF3 as determined by Western blotting (Fig. 2E). These results suggest that parthenolide inhibits TRIF-dependent signaling pathway derived from TLR4 activation.

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

The activation of TLR4 by LPS can trigger NF-κB and IRF3 activation mediated through both the MyD88- and TRIF-dependent pathways. However, the activation of TLR3 by poly[I:C] can trigger NF-κB and IRF3 activation only through the TRIF-dependent pathway. Therefore, induction of NF-κB and IRF3 activation by poly[I:C] can be used as the readout for the TRIF-dependent pathway. Parthenolide inhibited poly[I:C]-induced NF-κB and IRF3 activation as determined by a luciferase reporter gene assay (Figs. 3A and 3B). Parthenolide also inhibited poly[I:C]-induced iNOS expression as determined by a luciferase reporter gene assay and immunoblotting (Figs. 3C and 3D).

Parthenolide suppresses LPS or poly[I:C]-induced IP-10 expression

To further investigate if parthenolide modulates TRIF-dependent pathway, the expression of genes associated with the TRIF-dependent pathways such as IP-10 was measured by a luciferase reporter gene assay and ELISA. Parthenolide inhibited LPS or poly[I:C]-induced IP-10 expression (Figs. 4A-4D).

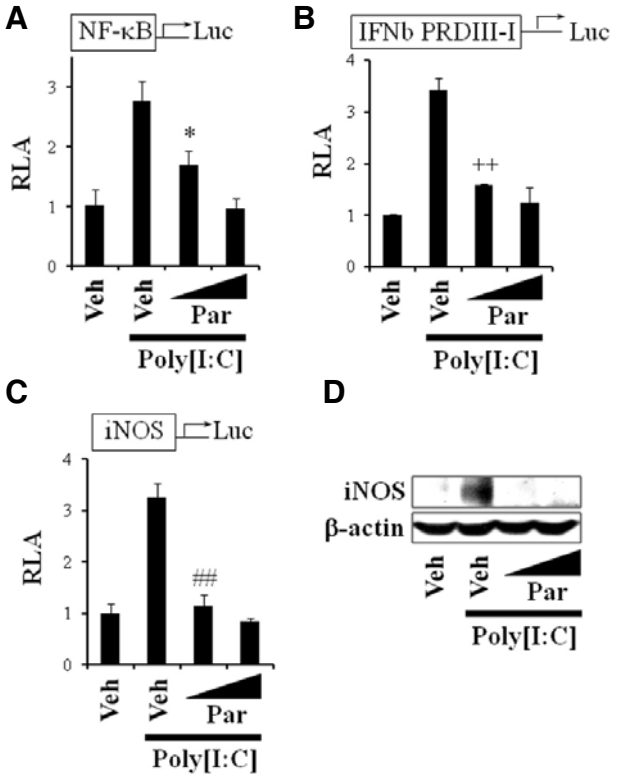

Fig. 3. Parthenolide inhibits poly[I:C]-induced NF-κB and IRF3 activation and iNOS expression. (A-C) RAW264.7 cells were transfected with NF-κB (A), IRF3 binding site (IFNβ PRDIII-I) (B), or iNOS (C) luciferase reporter plasmid and pre-treated with parthenolide (10, 20 μM) for 1 h and then treated with 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), $p < 0.01$. ##, Significantly different from poly[I:C] alone (C), $p < 0.01$. (D) RAW264.7 cells were pretreated with parthenolide (10, 20 μM) for 1 h and then further stimulated with poly[I:C] (10 μg/ml) for 8 h. Cell lysates were analyzed for iNOS and β-actin proteins by Western blotting. Veh, vehicle; Par, parthenolide.

The results indicated that parthenolide suppressed the activation of transcription factor and the expression of target genes by TLR3 or TLR4 agonist.

Broadly, TLR signaling involves two main downstream adaptor pathways (Takeda and Akira, 2005). In one pathway, the main adaptor protein is MyD88, which binds to the cytosolic domain of TLR1, 2, 4, 5, 6, 7, 8, 9, and 10 to induce NF-κB activation. In the other pathway, the main adaptor protein is TRIF, which binds to the cytosolic domain of TLR3 and 4 to induce mainly IRF3 activation, but which can also induce NF-κB activation. TLR4 uses both the MyD88- and TRIF-dependent pathways. The activation of TLR3 or TLR4 by viral double-stranded RNA or LPS, respectively, leads to IRF3 activation mediated through the TRIF-dependent signaling pathway, which is responsible for the expression of IFNβ and IFN-inducible genes (Lin et al., 1998; Navarro and David, 1999). It is well known that the production of IFNβ is the first line of host defense against viral infection.

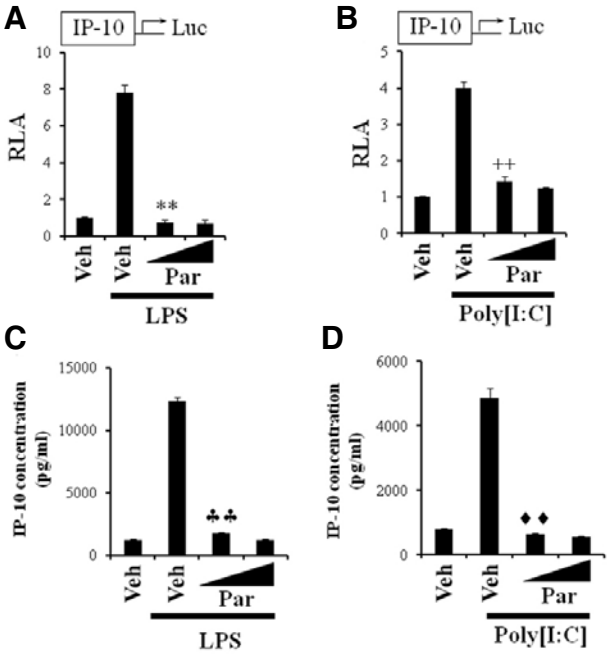

Fig. 4. Parthenolide inhibits LPS or poly[I:C]-induced IP-10 expression. (A, B) RAW264.7 cells were transfected with IP-10-luciferase reporter plasmid and pre-treated with parthenolide (10, 20 μM) for 1 h and then treated with LPS (10 ng/ml) (A) or poly[I:C] (10 μg/ml) (B) for an additional 8 h. Cell 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 parthenolide (10, 20 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 = 3). ♣♣, Significantly different from LPS alone (A), $p < 0.01$. ♦♦, Significantly different from poly[I:C] alone (B), $p < 0.01$. Veh, vehicle; Par, parthenolide.

Accumulating evidence now suggests that the TRIF-dependent signaling pathway of TLRs is important in inflammatory responses and development of certain chronic diseases. The TRIF-dependent pathway is responsible for more than 70% of LPS-inducible genes. Therefore, the modulation of the TRIF-dependent pathway of TLRs might be a useful anti-inflammatory strategy.

TRIF induces the activation of IRF3 mediated through the downstream kinases TBK1 and IKKε (Fitzgerald et al., 2003). Activated IRF3 becomes readily phosphorylated resulting in IRF3 dimerization. The IRF3 complex then translocates into the nucleus and binds to its target DNA sequences IFN-stimulated responses elements (ISRE) found in the promoter regions of genes such as those encoding IFNβ, IP-10, and RANTES (Lin et al., 1999; Schafer et al., 1998). Viral and bacterial infection induces both the NF-κB and IRF3 activation, but target genes regulated by those are distinct. Indeed, NF-κB activation induces proinflammatory cytokines, but IRF3 activation induces type I IFN genes. These IRF3-regulated genes play an important role for anti-viral and anti-bacterial activities.

It is already known that parthenolide inhibits NF-κB activation by inhibiting MyD88-dependent signaling pathway of TLRs (Saadane et al., 2007). Presently, we showed that parthenolide suppressed the target gene expression, IP-10, induced by TLR3 or TLR4 agonists and the transcriptional activation of IRF3 by inhibiting TRIF-dependent signaling pathway. Therefore, the modulation of the TRIF-dependent pathway of TLRs provides new insight to understand the mode of action of parthenolide for its anti-viral, anti-bacterial, and anti-inflammatory activities.

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