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Guggulsterone suppresses the activation of transcription factor IRF3 induced by TLR3 or TLR4 agonists

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ABSTRACT

Toll-like receptors (TLRs) are vital in the induction of innate immune responses. The microbial components trigger the activation of the myeloid differential factor 88 (MyD88)- and toll-interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF)-dependent downstream TLR signaling pathways. Guggulsterone, which has been used for centuries to treat many chronic diseases, inhibits the MyD88-dependent pathway by inhibiting the activity of inhibitor-κB kinase. However, it is not known whether guggulsterone inhibits the TRIF-dependent pathway. Presently, we sought to identify the molecular targets of guggulsterone in this pathway. Guggulsterone inhibited nuclear factor-κB and IRF3 activation induced by lipopolysaccharide or poly[I:C] and activation of IRF3 induced by the overexpression of TRIF, TBK1 or constitutively active IRF3. Guggulsterone also suppressed the lipopolysaccharide-induced phosphorylation of IRF3. These results suggest that guggulsterone can modulate both MyD88- and TRIF-dependent signaling pathways of TLRs leading to decreased inflammatory gene expression.

1. Introduction

Many pharmaceutical drugs in use for cancer treatment are derived from plant species. Such phytochemicals possess anti-inflammatory properties both in vivo and in vitro [1]. One anti-inflammatory phytochemical that has aroused considerable interest is the isomer E- and Z-guggulsterone [4,17(20)-pregnadiene-3,16-dione], a plant sterol derived from oleogum resin (known as guggul) from the guggul tree, *Commiphora mukul* [2]. The resin has been used in Ayurvedic, the traditional Indian medicinal system, for thousands of years to treat obesity, arthritis, cardiovascular disease, lipid disorders and inflammation [2,3]. The anti-inflammatory and anti-arthritic activities of guggul were first demonstrated in 1960 [4]. The activity of guggul in experimental arthritis induced by mycobacterial adjuvant was demonstrated in 1977 [5]. Guggul also exhibited anti-inflammatory activities in a carrageenan induced rat paw edema model [6]. In addition, guggulsterone suppresses nuclear factor-κB (NF-κB) activation induced by inflammatory agents [7–9]. An understanding of the molecular mechanisms responsible for the diverse pharmacological effects of guggul is just now emerging.

Toll-like receptors (TLRs) recognize diverse microbial products that are collectively known as pathogen associated molecular patterns. TLRs play an important role in the induction of innate immune responses that are essential for host defense against invading microbial pathogens [10–12]. Currently, at least 13 TLRs in mammalian cells are identified with different types of agonists.

Broadly, TLR signaling involves two downstream pathways: the myeloid differential factor 88 (MyD88)-dependent pathway and the toll-interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF)-dependent pathway [13]. MyD88 is the immediate adaptor molecule common to all mammalian TLRs except TLR3 [11]. MyD88 recruits interleukin-1 receptor-associated kinase-4 (IRAK-4) and induces phosphorylation of IRAK-4. The phosphorylated IRAK-4 induces phosphorylation of IRAK-1 that in turn mediates the recruitment of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) leading to the activation of the canonical I-κB kinase (IKK) complex followed by the activation of the NF-κB transcription factor. Activation of NF-κB induces cytokine gene expression leading to pro-inflammatory responses [14].

Recognition of viral double-stranded RNA (dsRNA) by TLR3 and bacterial lipopolysaccharide (LPS) by TLR4 leads to interferon-regulatory factor 3 (IRF3) activation that is mediated via TRIF-dependent signaling pathways [15,16]. The activation of TLR3 and TLR4 recruits TRIF that in turn activates the downstream kinases TANK-binding kinase1 (TBK1) and inhibitor-κB kinase-ε (IKK-ε). Activation of these kinases preludes the activation of IRF3 [17]. The activation of the TRIF pathway also induces delayed activation of NF-κB mediated through RIP1. The representative target genes regulated through the TRIF-dependent signaling pathway of TLRs include interferon-β (IFN-β) and IFN-inducible genes such as inducible nitric oxide synthase and IP-10 [18,19].

The activation of TLRs by agonists can induce inflammatory responses that are key etiological conditions for the development of

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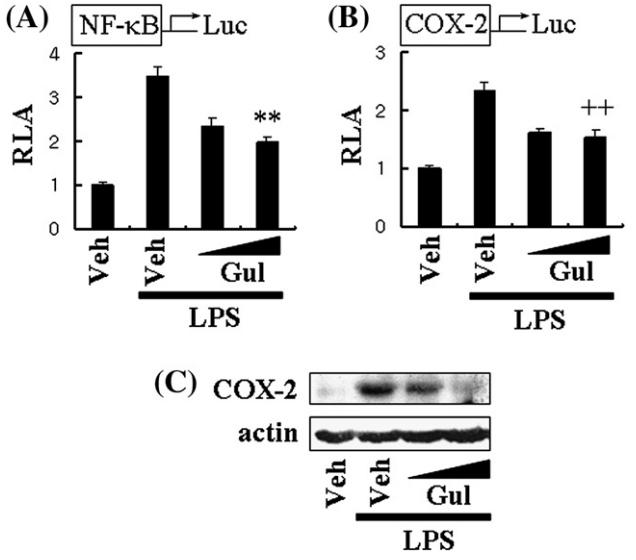

Fig. 1. Guggulsterone suppresses NF-κB activation and COX-2 expression induced by LPS. A, B) RAW264.7 cells were transfected with NF-κB (A) or COX-2 (B) luciferase reporter plasmid and pre-treated with guggulsterone (10, 20 μM) for 1 h and then treated with LPS (5 ng/ml) for an additional 8 h. Cell lysates 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), $p<0.01$. ++, Significantly different from LPS alone (B), $p<0.01$. C) RAW264.7 cells were pretreated with guggulsterone (10, 20 μM) for 1 h and then further stimulated with LPS (5 ng/ml) for 8 h. Cell lysates were analyzed for COX-2 and actin proteins by immunoblotting. Veh, vehicle; Gul, guggulsterone.

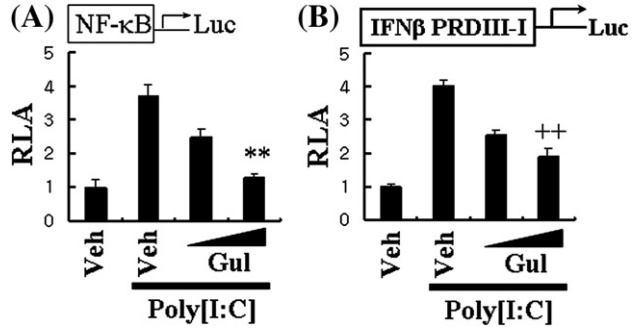

Fig. 3. Guggulsterone inhibits the activation of NF-κB and IRF3 induced by poly[I:C]. A, B) RAW 264.7 cells were transfected with NF-κB (A) or IRF3 binding site (IFNβ PRDIII-I) (B) luciferase reporter plasmid. Cells were treated with guggulsterone (10, 20 μM) for 1 h and further stimulated with poly[I:C] (5 μg/ml) for 8 h. Relative luciferase activity (RLA) was determined as described in the legend of Fig. 1. **, Significantly different from poly[I:C] alone alone (A), $p<0.01$. ++, Significantly different from poly[I:C] alone (B), $p<0.01$.

many chronic inflammatory diseases. Guggulsterone inhibits the MyD88-dependent pathway of TLRs by inhibiting the activity of IKKβ, which is the key kinase in the canonical pathway for NF-κB activation [7]. However, it is not known whether guggulsterone inhibits the TRIF-dependent pathway of TLRs. Presently, we aimed to identify the molecular target of guggulsterone in the TRIF-dependent signaling pathway.

2. Materials and methods

2.1. Reagents

(Z)-Guggulsterone was purchased from Sigma-Aldrich (St. Louis, MO). Purified LPS was obtained from List Biological Lab. Inc. Prior to use, guggulsterone was dissolved in dimethyl sulfoxide and LPS was dissolved in endotoxin-free water. Poly[I:C] was purchased from Amersham Biosciences (Piscataway, NJ). All other reagents were purchased from Sigma-Aldrich unless otherwise described.

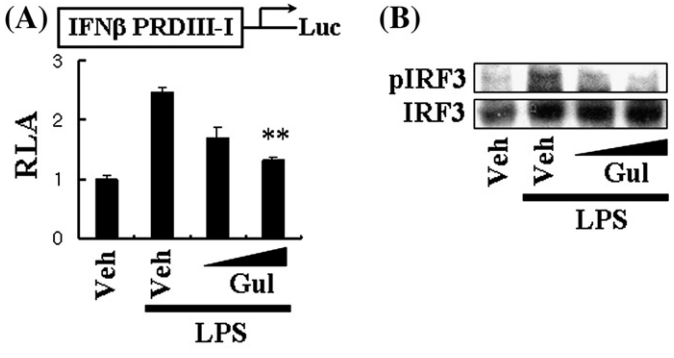

Fig. 2. Guggulsterone suppresses the activation of IRF3 induced by LPS in macrophages. A) RAW 264.7 cells were transfected with IRF3 binding site (IFNβ PRDIII-I)-luciferase reporter plasmid. Cells were treated with guggulsterone (10, 20 μM) for 1 h and further stimulated with LPS (5 ng/ml) for 8 h. Relative luciferase activity (RLA) was determined as described in the legend of Fig. 1. Values are mean±SEM (n=3). **, Significantly different from LPS alone, $p<0.01$. B) RAW 264.7 cells treated with guggulsterone (10, 20 μM) for 1 h and further stimulated with LPS (5 ng/ml) for 1.5 h. Cell lysates were analyzed for phospho-IRF3 (S396) and IRF3 proteins by immunoblotting.

2.2. Cell culture

RAW 264.7 cells (murine monocytic cell line ATCC TIB-71) 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), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Cells were maintained at 37 °C in a 5% CO_2/air environment.

2.3. Plasmids

A NF-κB (2×)-luciferase reporter construct was provided by Frank Mercurio (Signal Pharmaceuticals, San Diego, CA). An IFNβ PRDIII-I-luciferase reporter plasmid and a wild-type TBK1 expression plasmid were kind gifts from Kate Fitzgerald (University of Massachusetts Medical School). Heat shock protein 70 (HSP70)-β-galactosidase reporter plasmid was from Robert Modlin (University of California, Los Angeles, CA). All DNA constructs were prepared in large scale using the EndoFree™ Plasmid Maxi kit (Qiagen, Valencia, CA) for transfection.

2.4. Transfection and luciferase assays

These were performed as described previously [20,21]. Briefly, RAW 264.7 or 293T cells were co-transfected with a 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 corresponding empty vector plasmids for signaling components were co-transfected. The total amount of transfected plasmids was equalized by supplementing with the corresponding empty vector to eliminate transfection-associated experimental error. Luciferase and β-galactosidase enzyme activities were determined using luciferase assay and β-galactosidase enzyme system (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized to β-galactosidase activity.

2.5. Immunoblotting

Immunoblots were performed as previously described [21,22]. Equal amounts of extracts were resolved using sodium dodecyl sulfate-