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Guggulsterone Suppresses the Activation of NF-κB and Expression of COX-2 Induced by Toll-like Receptor 2, 3, and 4 Agonists

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Abstract Toll-like receptors (TLRs) induce innate immune responses recognizing conserved microbial structural molecules. All TLR signaling pathways culminate in the activation of nuclear factor-κB (NF-κB). The activation of NF-κB leads to the induction of inflammatory gene products such as cyclooxygenase-2 (COX-2). Guggul has been used for centuries to treat a variety of diseases. Guggulstreone, one of the active ingredients in guggul, has been used to treat many chronic diseases. However, the mechanism as to how guggulsterone mediate the health effects is largely unknown. Here, we report biochemical evidence that guggulsterone inhibits the NF-κB activation and COX-2 expression induced by TLR2, TLR3, and TLR4 agonists. Guggulsterone also inhibits the NF-κB activation induced by downstream signaling components of TLRs, myeloid differential factor 88 (MyD88), IκB kinase β (IKKβ), and p65. These results imply that guggulsterone can modulate the immune responses regulated by TLR signaling pathways.

Key words: guggulsterone, toll-like receptor, lipopolysaccharide, cyclooxygenase-2, nuclear factor-κB

Introduction

Toll-like receptors (TLRs) play an important role in induction of innate immune responses that are essential for host defense against invading microbial pathogens (1-4). Currently, at least 13 TLRs in mammalian cells are identified with different types of agonists. The best characterized TLR4 recognizes lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria (5). TLR2 dimerized with TLR1 or TLR6 recognizes triacyl or diacyl lipopeptides of Gram-positive bacteria (6-8). TLR4 and TLR2/6 dimer recognizes zymosan for anti-fungal responses (9). TLR5 recognizes bacterial flagellin (10). TLR11 recognizes yet unknown components of uropathogenic bacteria (11) and a profilin-like molecule of the protozoan parasite *Toxoplasma gondii* (12). The antiviral TLR3 recognizes double-stranded RNA (dsRNA) (13). TLR7 and TLR8 recognize single-stranded viral RNA (ssRNA) (14) and TLR9 recognizes viral and bacterial unmethylated cytosine phosphate guanine DNA (CpG DNA) motifs (15,16).

The binding of agonists to TLRs activates intracellular signaling cascades that involves the recruitment of myeloid differential factor 88 (MyD88) and interleukin-1 (IL-1) receptor associated kinase (IRAK) leading to the activation of nuclear factor-κB (NF-κB) and the expression of inflammatory gene products, including cyclooxygenase-2 (COX-2), cytokines, and chemokines (1). MyD88 is the immediate downstream adaptor molecule recruited by activated TLRs through their TIR domain. The interaction of MyD88 with TIR domain of TLR recruits IRAK-4 and induces IRAK-4-induced phosphorylation and degradation of IRAK-1. Phosphorylated IRAK-1 associates with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) leading to the activation of IκB kinase (IKK) complex resulting in the activation of NF-κB transcription factor. The activation of this transcription factor then up-regulates the expression of numerous pro-inflammatory gene products including cytokines and COX-2 (17).

Many pharmaceutical drugs in use for cancer treatment are derived from plant species. The phytochemicals have been shown to have anti-inflammatory properties both *in vivo* and *in vitro*.

Oleogum resin (known as guggul) from the guggul tree, *Commiphora mukul*, found in India, Bangladesh, and Pakistan has been used in Ayurveda, the ancient Indian medical system, for centuries to treat a variety of diseases, including cardiovascular disease, bone fractures, lipid disorders, arthritis, obesity, and inflammation (18,19). The anti-inflammatory activity of guggul was first demonstrated in 1960 (20), and subsequently in 1977 by Sharma (21). In 1986, guggul was approved as a hypolipidemic drug with proven efficacy and safety in India (22). In the middle 1990s, guggul was introduced into the Western medical literature as a remedy for treating or preventing hypercholesterolemia and related cardiovascular diseases (23). An understanding of the molecular mechanisms responsible for the diverse pharmacological effects of guggul is just now emerging. The bioactive constituent of guggul has known to be guggulsterone [4,17(20)-pregnadiene-3,16-dione] (Fig. 1). Several studies found that guggulsterone is a potent antagonist at the mineralocorticoid receptor (MR), glucocorticoid receptor (GR), and androgen receptor (AR), and an agonist at pregnane receptor (PXR), progesterone receptor (PR), and estrogen receptor α (ERα) (24-27). In addition, guggulsterone has been found to suppress NF-κB activation induced by inflammatory agents and carcinogens (28-30). A growing evidence in the association of NF-κB activation and

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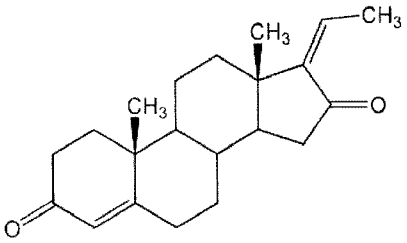

Fig. 1. The chemical structure of guggulsterone.

inflammation suggests that the modulation of NF-κB signaling pathway could be the main target for the treatment of inflammation (31,32). Therefore, the aim of this study was to determine whether guggulsterone can suppress NF-κB activation induced by several TLR agonists and block NF-κB regulated gene expression including COX-2 that mediates inflammation and carcinogenesis.

Materials and Methods

Reagents Guggulsterone was purchased from Sigma-Aldrich (St. Louis, MO, USA) and diluted in dimethyl sulfoxide (DMSO). Macrophage-activating lipopeptide 2-kDa (MALP-2) was purchased from Alexis Biochemical (San Diego, CA, USA). Polyriboinosinic polyribocytidylic acid (Poly[I:C]) was purchased from Amersham Biosciences (Piscataway, NJ, USA). Purified LPS was obtained from List Biological Lab., Inc. (Campbell, CA, USA). All other reagents were purchased from Sigma-Aldrich unless otherwise described.

Cell culture RAW264.7 cells (a murine monocytic cell line, ATCC TIB-71) and 293T cells (human embryonic kidney) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10%(v/v) fatal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37°C in a 5% CO_2/air environment.

Transfection and reporter gene luciferase assay These were performed as described in our previous studies (33,34). Cells were co-transfected with a luciferase plasmid and HSP70-β-galactosidase plasmid as an internal control using SuperFect transfection reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Various expression plasmids or equal amounts of empty vector for signaling components were co-transfected. Luciferase enzyme activities were determined using the Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activity was normalized by β-galactosidase activity.

Immunoblotting These were performed the same as previously described (35,36). Equal amounts of cell extracts were subjected to 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and electrotransferred to polyvinylidene difluoride membrane for COX-2 and β-actin immunoblot analysis. The membrane was blocked to prevent nonspecific binding of antibodies in phosphate buffered saline (PBS) containing 0.1% Tween 20 and 3% nonfat dry milk. Immunoblotting was performed with the indicated antibodies and secondary antibodies conjugated to horseradish peroxidase (Amersham Biosciences, Arlington Heights, IL, USA). The reactive bands were visualized with ECL Western blot detection reagents (Amersham Biosciences).

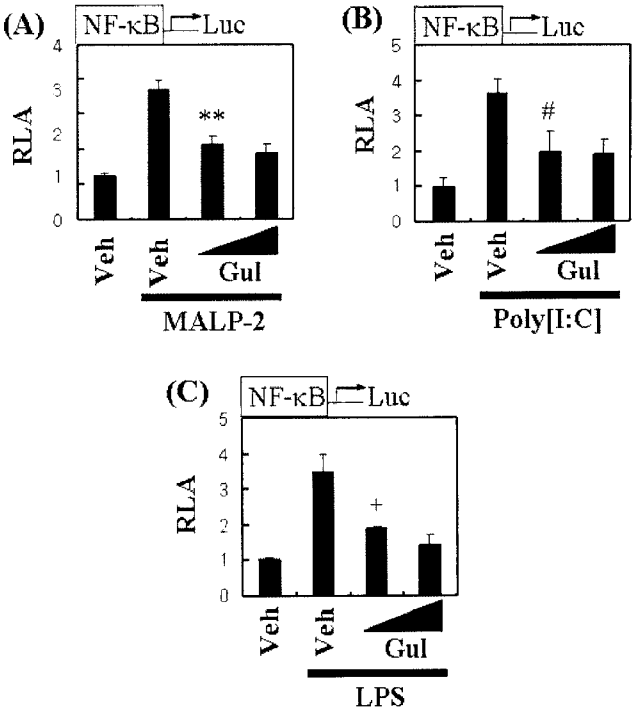

Fig. 2. Guggulsterone suppressed the NF-κB activation induced by MALP-2, poly[I:C], or LPS. RAW264.7 cells were transfected with NF-κB luciferase reporter plasmid and pre-treated with guggulsterone (10, 20 μM) for 1 hr and then treated with MALP-2 (5 ng/mL) (A), poly[I:C] (10 μg/mL) (B), or LPS (5 ng/mL) (C) for an additional 8 hr. Cell lysates were prepared and luciferase enzyme activities were measured using the Luciferase Assay System. Relative luciferase activity (RLA) was normalized with β-galactosidase activity. Values are mean±SEM (n=3). **, Significantly different from MALP-2 alone, $p<0.01$. #, Significantly different from poly[I:C] alone, $p<0.05$. +, Significantly different from LPS alone, $p<0.05$. Veh, vehicle; Gul, guggulsterone.

Statistical analysis Data were obtained from triplicate experiments. Values were expressed as mean±standard error mean (SEM).

Results and Discussion

NF-κB is the common downstream signaling component for all TLRs TLR signaling pathways can trigger the activation of NF-κB through the MyD88-dependent and -independent (TRIF-dependent) pathways. MyD88 is the immediate adaptor molecule which is common to all mammalian TLRs except for TLR3. TLR3 only use the TRIF-dependent pathways to activate NF-κB. NF-κB is the common downstream signaling component for all TLRs, because both MyD88-dependent and -independent signaling pathways can lead to NF-κB activation. Therefore, to identify whether guggulsterone modulates TLR-mediated signaling pathways, several TLR agonists-induced NF-κB activation was used as readout for the activation of TLRs.

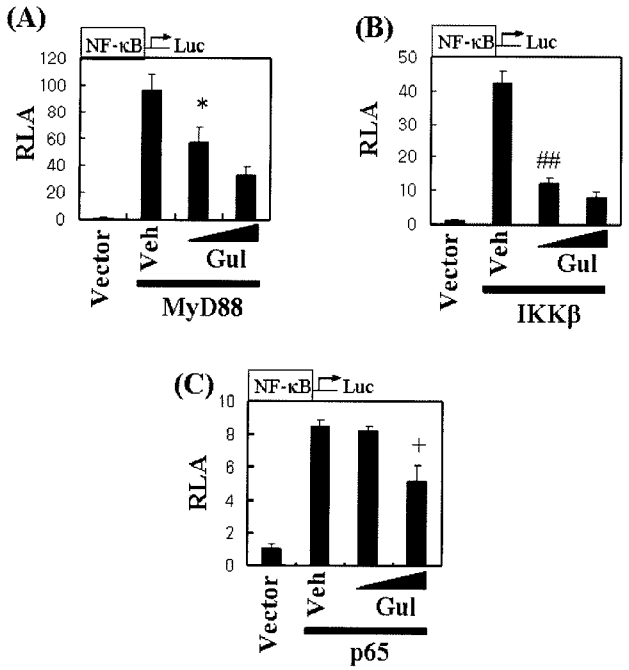

Fig. 3. Guggulsterone inhibits the activation of NF-κB induced by the overexpression of MyD88 (A), IKKβ (B), or p65 (C). 293T cells were co-transfected with NF-κB-luciferase reporter plasmid and expression plasmids. pcDNA was used as vector control for plasmids. After 24 hr, cells were treated with guggulsterone (10, 20 μM) for 8 hr. Relative luciferase activity (RLA) was determined by normalization with β-galactosidase activity. Values are mean±SEM (n=3). *, Significantly different from MyD88 plus vehicle ($p<0.05$). ##, Significantly different from IKKβ plus vehicle ($p<0.01$). +, Significantly different from p65 plus vehicle ($p<0.05$). Veh, vehicle; Gul, guggulsterone.

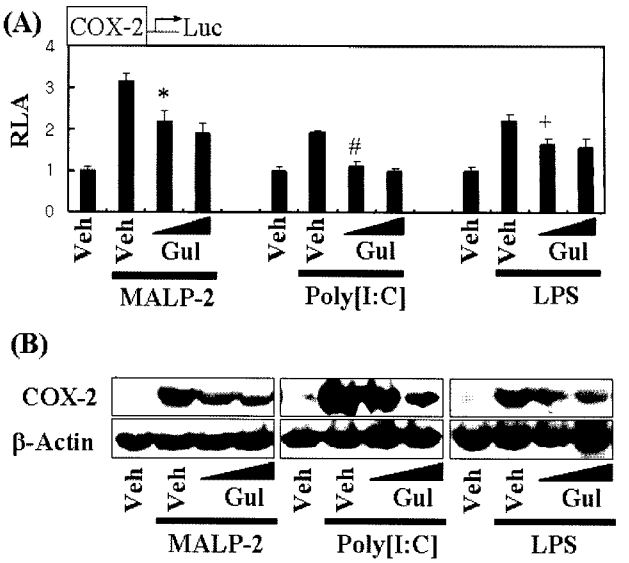

Fig. 4. Guggulsterone suppressed the COX-2 expression induced by MALP-2, poly[I:C], or LPS. (A) RAW264.7 cells were transfected with COX-2 luciferase reporter plasmid and pretreated with guggulsterone (10, 20 μM) for 1 hr and then treated with MALP-2 (5 ng/mL), poly[I:C] (10 μg/mL), or LPS (5 ng/mL) for an additional 8 hr. Cell lysates were prepared and luciferase enzyme activities were measured using the Luciferase Assay System. Relative luciferase activity (RLA) was normalized with β-galactosidase activity. Values are mean±SEM (n=3). *, Significantly different from MALP-2 alone ($p<0.05$). #, Significantly different from poly[I:C] alone ($p<0.05$). +, Significantly different from LPS alone ($p<0.05$). (B) RAW264.7 cells were pretreated with guggulsterone (10, 20 μM) for 1 hr and then further stimulated with MALP-2 (5 ng/mL), poly[I:C] (10 μg/mL), or LPS (5 ng/mL) for 8 hr. Cell lysates were analyzed for COX-2 and β-actin protein by immunoblots. Veh, vehicle; Gul, guggulsterone.

Guggulsterone suppressed MALP-2, Poly[I:C], and LPS induced the activation of NF-κB and the expression of COX-2 Guggulsterone suppressed MALP-2 (TLR2 agonist), Poly[I:C] (TLR3 agonist), and LPS (TLR4 agonist) induced the activation of NF-κB in a dose-dependent manner as determined by the luciferase reporter gene assay (Fig. 2A-2C). The activation of TLRs induced by agonists leads to the recruitment of MyD88. MyD88 recruits IRAK-4 that phosphorylates IRAK-1 leading to its degradation. IRAK-1 associates with TRAF6 leading to the activation of IKK complex resulting in the activation of NF-κB transcription factor. It was further investigated the differential regulation of MyD88-dependent signaling pathways by guggulsterone. Guggulsterone suppressed the agonist-independent activation of NF-κB induced by MyD88, IKKβ, or p65 in 293T cells (Fig. 3A-3C). Next, it was determined whether guggulsterone inhibits COX-2 expression induced by MALP-2, Poly[I:C], or LPS. COX-2 is one of the target genes regulated through the activation of NF-κB in macrophages. Guggulsterone inhibited MALP-2-, Poly[I:C]-, or LPS-induced COX-2 expression in RAW264.7 cells as determined by the COX-2 luciferase reporter assay (Fig. 4A) and COX-2 immunoblotting (Fig. 4B). These results suggested that guggulsterone inhibited several TLR agonists-induced NF-κB activation resulting in the inhibition of the target gene expression, such as COX-2.

COX-2 is the molecular target to alleviate the symptoms of inflammation COX is an enzyme that is responsible for formation of prostanoids (including prostaglandins, prostacyclin, and thromboxane) from arachidonic acid by perooxidation. COX is the molecular target for relief from the symptoms of inflammation and pain (37). COX enzyme consists of at least 2 distinct isoforms, COX-1 and COX-2 (38). COX-1 and COX-2 have similar molecular weight (approximately 70 and 72 kDa, respectively), and 65% amino acid sequence homology. However, the most significant difference between 2 isoenzymes is the substitution of isoleucine at position 523 in COX-1 with valine in COX-2. COX-1 is constitutively expressed in nearly all cells and tissues at relatively stable levels, but COX-2 is almost undetectable in most tissues. COX-1 is believed to have some housekeeping functions, such as production of PG precursors for thromboxane in platelets, and regulates important physiological processes such as gastrointestinal cytoprotection and electrolyte homeostasis in kidneys. In contrast to COX-1, the expression of COX-2 is greatly increased in most normal mammalian tissues in response to physical, chemical, and biological stimuli, such as ultra violet (UV), H_2O_2, and LPS (39). Many research suggested that the toxic effects of chemicals are due to inhibition of COX-1, while the therapeutic effects of those are due to inhibition of COX-2. Therefore, these results

have led to pharmaceutical companies to develop drugs to inhibit COX-2.

So far, only one study has attempted to determine the relative activity of inhibition by guggulsterone on NF-κB activation and COX-2 expression induced by TNF (28). Therefore, this study for the first time demonstrated that guggulsterone inhibited TLR2, TLR3, and TLR4 agonists-induced NF-κB activation and targeted gene expression, COX-2. These results suggest that guggulsterone can modulate TLR-mediated inflammatory responses and the risk of chronic diseases.

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