Gambogic Acid Disrupts Toll-like Receptor4 Activation by Blocking Lipopolysaccharides Binding to Myeloid Differentiation Factor 2

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Our body’s immune system has defense mechanisms against pathogens such as viruses and bacteria. Immune responses are primarily initiated by the activation of toll-like receptors (TLRs). In particular, TLR4 is well-characterized and is known to be activated by gram-negative bacteria and tissue damage signals. TLR4 requires myeloid differentiation factor 2 (MD2) as a co-receptor to recognize its ligand, lipopolysaccharides (LPS), which is an extracellular membrane component of gram-negative bacteria. Gambogic acid is a xanthonoid isolated from brownish or orange resin extracted from *Garcinia hanburyi*. Its primary effect is tumor suppression. Since inflammatory responses are related to the development of cancer, we hypothesized that gambogic acid may regulate TLR4 activation. Our results demonstrated that gambogic acid decreased the expression of pro-inflammatory cytokines (TNF-α, IL-6, IL-12, and IL-1β) in both mRNA and protein levels in bone marrow-derived primary macrophages after stimulation with LPS. Gambogic acid did not inhibit the activation of Interferon regulatory factor 3 (IRF3) induced by TBK1 overexpression in a luciferase reporter gene assay using IFN-β-PRD III-I-luc. An in vitro kinase assay using recombinant TBK1 revealed that gambogic acid did not directly inhibit TBK1 kinase activity, and instead suppressed the binding of LPS to MD2, as determined by an in vitro binding assay and confocal microscopy analysis. Together, our results demonstrate that gambogic acid disrupts LPS interaction with the TLR4/MD2 complex, the novel mechanism by which it suppresses TLR4 activation.

Key words: Phytochemical, Toll-like receptor, LPS, MD2, Bacterial infection, Immune-suppression

INTRODUCTION

Our immune system contains many host-defense mechanisms against pathogens such as viruses and bacteria. Toll-like receptors (TLRs) especially are critical to the induction of innate and adaptive immune responses during pathogenic invasion. Each TLR recognizes pathogen-associated molecular patterns in a ligand-specific way. Their activation leads to the activation of two major signaling pathways dependent on the myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter inducing interferon-beta (TRIF). TLR interaction with MyD88 and TRIF initiates the NF-kB and IRF3 signaling cascades, leading to the production of pro-inflammatory cytokines and type I interferon (IFN), which exert immune and antiviral responses (1). Whether these adaptors are required differs between TLR isotypes. The signaling pathways of TLR4 are well-characterized as having both MyD88- and TRIF-dependent pathways. TLR4 requires myeloid differentiation factor 2 (MD2), a co-receptor, in order to be fully expressed on the cell surface as a complex and capable of interacting with its ligands. Lipopolysaccharides (LPS), an extracellular membrane component of gram-negative bacteria, is a representative ligand for TLR4. It is the main surface antigen among all the various molecules on the bacterial outer membrane, and plays the critical role of activating host immunity against the gram-negative bacteria (2). Recognition occurs when LPS lipid
Fig. 1. Chemical structure of gambogic acid.

chains with hydrophobic residues interact with the internal surface of the MD2 pocket (3). Therefore, the modulation of LPS interaction with MD2 can be considered an efficient target in regulating TLR4 activation.

Gambogic acid (Fig. 1) is a xanthonoid that is isolated from the brownish or orange resin extracted Garcinia hanburyi. In vitro and animal studies indicated that it has suppressive effects on tumor growth, in addition to anti-inflammatory activities against endotoxic shock (4). However, the details of how gambogic acid regulates TLR4 remain unclear. The results from our study show that gambogic acid reduces the expression of various inflammatory cytokines in macrophages stimulated with a TLR4 ligand, LPS. We demonstrated that gambogic acid prevented the engagement of LPS to the TLR4/MD2 complex using an in vitro binding assay and cellular imaging analysis. Our results suggest that gambogic acid treatment would make the host more susceptible to bacterial infection due to the down-regulation of TLR4 activation.

MATERIALS AND METHODS

Animals and cell culture. Animal care and study protocols were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Catholic University of Korea (permission # 2012-5-001). Mice were purchased from Orient Bio (Seoul, Korea) and acclimated under specific pathogen-free conditions in the animal facility for at least a week before the experiments. They were housed in a room controlled for temperature (23 ± 3°C) and relative humidity (40–60%). Bone marrow cells were isolated from C57BL/6 mice and differentiated into macrophages as described previously (5). Bone marrow cells were cultured for 6 days in Dulbecco Modified Eagle Medium (DMEM) (Hyclone, Logan, UT, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (Hyclone), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM Hepes buffer, and 20% L929 cell-conditioned medium. Adherent cells were used as macrophages. HEK293T cells were cultured in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37°C in a 5% CO2/air environment.

Reagents and plasmids. Gambogic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide. Cells treated with dimethyl sulfoxide were used as a vehicle-treated control. Purified LPS from Escherichia coli was obtained from List Biological Laboratory Inc. (Campbell, CA, USA) and dissolved in endotoxin-free water. Biotin-labelled LPS was purchased from Invivogen (San Diego, CA, USA).

The expression plasmid of Tbk1 and the IFNβ PRDIII-I-luciferase plasmid were kind gifts from Katherine Fitzgerald (University of Massachusetts Medical School, Worcester, MA, USA). The NF-κB(2x)-luciferase reporter construct was provided by Frank Mercurio (Signal Pharmaceuticals, San Diego, CA, USA). The expression plasmid of Tlr4 was obtained from A. Hajjar (University of Washington, Seattle, WA, USA). A constitutively active form of Myd88[Myd88(AToll)] was provided by Jurg Tschopp (University of Lausanne, Switzerland). All DNA constructs for transfection were prepared using an EndoFree Plasmid Mid Kit (Qiagen, Valencia, CA, USA).

Transfection and luciferase assay. HEK293T cells were co-transfected with various plasmids together with a β-galactosidase plasmid using Superfect Transfection Reagent according to the manufacturer’s instructions (Qiagen, Valencia, CA, USA). Luciferase and β-galactosidase enzyme activities were determined using the Luciferase Assay System and β-galactosidase Enzyme System (Promega, Fitchburg, WI, USA). Luciferase activity was normalized by β-galactosidase activity to determine relative luciferase activity.

Cytokine measurement using enzyme-linked immunosorbent assay. Levels of tumor necrosis factor (TNF-α), interleukin (IL)-1β, IL-6, and IL-12 in culture media were determined using enzyme-linked immunosorbent assay (ELISA) kits for each cytokine (TNF-α and IL-1β, R&D Systems, Minneapolis, MN, USA; IL-6 and IL-12, BD biosciences, Franklin Lakes, NJ, USA). The concentration ranges for the standard curves of TNF-α are 10.9 to 1,000 pg/mL and the minimum detectable dose ranged from 0.36 to 7.21 pg/mL; the concentration ranges for the standard curves of IL-1β are 12.5 to 1,000 pg/mL and the minimum detectable dose ranged from 0.46 to 4.8 pg/mL; the concentration ranges for the standard curves of IL-6 are 15.6 to 1,000 pg/mL and the minimum detectable dose ranged from 0.36 to 7.21 pg/mL; the concentration ranges for the standard curves of IL-12 are 62.5 to 4,000 pg/mL.

Reverse transcription and quantitative PCR analysis. Reverse transcription and PCR were performed as described previously (6). Total RNAs were isolated with Welprep™ reagent (Jeil Biotechservices Inc., Daegu, Korea). RNAs were reverse-transcribed with ImProm-II™ Reverse Transcriptase (Promega). For real-time quantitative PCR analy-
sis, SYBR Premix Ex Taq™ (TaKaRa, Shiga, Japan) was used. Primers were as follows: inf-α, 5'-AAATTCGAGTGAACGCCCTGTAG-3' and 5'-CCCTTGAAGAGAAGGGGTGGAGTAG-3'; il-6, 5'-TTCCCTCTGCAAGGGGGGAG-3'; tnf-α, 5'-GGGTGTCGTTAGTCCAGATGTTG-3'; il-1β, 5'-CCTGTGGCCTTGGGGCCTAA-3' and 5'-GAGGTGCTGATGTACCAGTTGG-3'; actin, 5'-TCATGAAGTGTGACGTTGACATCCGT-3' and 5'-TTGCCTGTCGATGGAAGGGGCGGA-3'. The specificity of the amplified PCR products was assessed using a melting curve analysis.

In vitro assay for LPS binding to MD2. The assay was performed as previously described (5). A 96-well microplate was coated overnight with polyclonal anti-human MD2 antibody at 4°C (Abnova, Taipei, Taiwan) in 50 mM Na₂CO₃ in buffer (pH 9.6). The plate was washed with phosphate buffered saline (PBS) and blocked with 2% bovine serum albumin in PBS at room temperature for 1 hr. Recombinant human MD2 (0.1 μM per well; R&D systems) in 10 mM Tris-HCl buffer (pH 7.5) with gambogic acid was incubated at 37°C for 1 hr, and then this mixture was added to a pre-coated plate and incubated at room temperature for 2 hr. After washing with PBS, the biotin-LPS was incubated at room temperature for 30 min, and then streptavidin conjugated to horseradish peroxidase was added at room temperature for 1.5 hr. Horseradish peroxidase activity was determined using the EzWay™ TMBsubstrate kit (Koma Biotech, Seoul, Korea). Optical density of each well was measured at 450 nm.

In vitro TBK1 kinase assay. This assay was performed according to methods described in a previously published paper (7) using the TBK1 kinase enzyme system (Promega) and ADP-Glo™ kinase assay (Promega). Gambogic acid and recombinant active TBK1 protein (20 ng; SignalChem, Richmond, BC, Canada) were pre-incubated in buffer A with DTT (0.1 M) at 30°C for 30 min. Then the ATP assay solution with MBP protein (1 mg/mL) was added to the tube at 30°C for 15 min. ADP-Glo™ Reagent was further added, and the solution was incubated at room temperature for 40 min. Kinase detection substrate was added to the plate and incubated at room temperature for 30 min. Luminosensor intensity was determined with a luminometer (Centrino XS LB 960, Berthold Technologies, Bad Wildbad, Germany).

Immunostaining and confocal imaging. These procedures were performed as previously described (8). BMDMs were grown on glass cover slips (18-mm diameter; Marienfeld laboratory Glassware, Lauda-Königshofen, Germany). After pre-treatment with gambogic acid for 1 hr, the cells were incubated with Alexa Fluor 594® conjugated with LPS ( Molecular Probes Inc., Eugene, OR, USA). Cells were fixed with 4% paraformaldehyde and washed three times with PBS. Then, cells were blocked with 1% bovine serum albumin for 1 hr and further incubated with anti-MD2 antibody in blocking buffer overnight at 4°C. After washing three times with PBS, the cells were incubated with FITC-conjugated anti-rabbit IgG secondary antibody (Sigma-Aldrich) and mounted with anti-fade solution (Molecular Probes Inc.) in blocking buffer at room temperature. The samples were examined with an LSM710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with 40X objectives. Images were obtained with ZEN2011 software (Carl Zeiss).

Statistical analysis. Data are expressed as means ± SEM (n = 3). Comparisons of data between groups were performed by one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test. Values of p < 0.05 were considered significant.

RESULTS
Gambogic acid suppresses the expression of cytokines at both mRNA and protein levels in LPS-stimulated bone marrow-derived primary macrophages. We investigated the effects of gambogic acid on TLR4 Fig. 2. Gambogic acid suppresses the production of pro-inflammatory cytokines induced by LPS, a TLR4 ligand, in bone marrow-derived macrophages. Bone marrow-derived macrophages were pre-treated with gambogic acid (1 and 2 μM) for 1 hr and then stimulated with lipopolysaccharide (LPS) (10 ng/ mL) for an additional 16 hr. Culture media were collected and analyzed for tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and IL-12 levels by enzyme-linked immunosorbent assay (ELISA). Values are means ± SEM (n = 3). *Significantly different from LPS alone, p < 0.05.
Gambogic acid decreases ligand-independent activation of NF-κB induced by TLR4-signaling components.

It is well reported that the expression of pro-inflammatory mediators is associated with NF-κB activation. We investigated whether the inhibitory effect of gambogic acid on TLR4 activation was related to blockade of NF-κB activation. NF-κB activation was induced by an overexpression of components in the TLR4 signaling cascade in HEK293T cells, and activation of NF-κB was determined by a luciferase reporter gene assay. Gambogic acid inhibited NF-κB activation induced by the overexpression of TLR4 (Fig. 4A). Similarly, NF-κB activation induced by the overexpression of the constitutively active form of MyD88, an adaptor protein of TLR4, was suppressed by gambogic acid (Fig. 4B). Gambogic acid also suppressed NF-κB activation induced by the overexpression of IKKβ (Fig. 4C). These suggest that gambogic acid blocks NF-κB activation in TLR4 signaling pathway downstream of IKKβ, which is consistent with other previous reports on the inhibition of NF-κB activation by gambogic acid.

Gambogic acid does not inhibit TBK1 kinase activity.

Tank-binding kinase 1 (TBK1), another recognition factor for viral infection, is an important kinase that conveys TLR4 activation signals to activate IRF3 (9). Our previous studies presented TBK1 as the target of certain anti-inflammatory phytochemicals in the TLR4-signaling pathways (7,10,11). Therefore, our current study investigated whether gambogic acid suppressed TBK1 kinase activity. IRF3 activation induced by the overexpression of TBK1 was not suppressed by gambogic acid as determined by an IFNβ PRDIII-I-luciferase reporter assay (Fig. 5A). In vitro kinase assay using a recombinant TBK1 protein also demonstrated that gambogic acid did not suppress the kinase activity of TBK1 (Fig. 5B). These results indicate that TBK1 is not an anti-inflammatory target of gambogic acid in modulating TLR4 signaling pathways.

Gambogic acid disrupts LPS binding to MD2.

The interaction in the ligand and receptor complex was pre-
Suppression of TLR4 by Gambogic Acid

We investigated whether gambogic acid inhibited the interaction between LPS and the TLR4/MD2 complex by using an *in vitro* binding assay to examine the level of LPS bound to recombinant MD2. Gambogic acid reduced the association of biotinylated LPS with recombinant MD2 (Fig. 6A), and consistently blocked the co-localization of LPS with MD2 in BMDMs as determined by confocal microscopy analysis (Fig. 6B).

Collectively, the results demonstrate that gambogic acid interferes with LPS interaction with MD2, leading to the suppression of TLR4 activation.

Fig. 5. TBK1 kinase activity was not inhibited by gambogic acid. (A) 293T cells were transfected with the expression plasmid for Tank-binding kinase 1 (TBK1) and a luciferase reporter plasmid of IFN-β PRD III-luc as indicated. Cells were treated with gambogic acid (1 and 2 μM) for 8 hr. Relative luciferase activity was determined. (B) *In vitro* kinase assay for TBK1 was performed using recombinant active TBK1 (rTBK1) in the absence or presence of gambogic acid (1 and 2 μM) or luteolin (20 μM). Values are means ± SEM (n = 3). Significantly different from rTBK1 alone, p < 0.05.

Fig. 6. Gambogic acid interrupts LPS binding to the TLR4/MD2 complex. (A) *In vitro* binding assay for lipopolysaccharides (LPS) binding to myeloid differentiation factor 2 (MD2) using recombinant MD2 (rMD2) and biotinylated LPS were performed. (B) Bone marrow-derived macrophages were pre-treated with gambogic acid (1 and 2 μM) for 1 hr and treated with Alexa Fluor 594 conjugated with LPS (1.5 μg/group) for 30 min. Cells were stained with anti-MD2 antibody together with FITC-conjugated anti-rabbit IgG secondary antibody. DIC, differential interference contrast.

Fig. 7. Diagram of the inhibition of TLR4 activation by gambogic acid. Gambogic acid disrupts the interaction of lipopolysaccharides (LPS, ligand) with toll-like receptor 4/myeloid differentiation factor 2 complex (TLR4/MD2 complex, receptor).
decrease of cytokine expression in macrophages and the modulation of immune responses against bacterial infection (Fig. 7).

**DISCUSSION**

It has been shown that sulforaphane (1-isothiocyanato-4-(methylasulfinyl)-butane), abundant in cruciferous vegetables such as broccoli and cauliflower, interferes with the binding of LPS to MD2 (8). In addition, caffeic acid phenethyl ester found in natural materials such as propolis, is able to interrupt LPS interaction with the TLR4/MD2 complex by forming a covalent adduct with Cys133 in the MD2 hydrophobic pocket (5). These findings suggest that LPS binding with MD2 is an efficient therapeutic target in the regulation of TLR4 activation. In this study, we identified MD2 as a new anti-inflammatory target of gambogic acid, and demonstrated that gambogic acid interferes with LPS binding to the MD2 complex, leading to down-regulation of TLR4 activation. This was demonstrated by an in vitro binding assay using biotinylated LPS and recombinant MD2 as well as co-localization of LPS and MD2 through confocal microscopic analysis. In contrast, kinase activity of TBK1 as determined by an in vitro binding assay using recombinant TBK1 was not reduced by gambogic acid. Our study suggests that gambogic acid could be used as a valuable agent in preventing inflammatory diseases associated with TLR4 activation by LPS.

Down-regulation of TLR4 activation by gambogic acid may culminate in decreased host immunity, resulting in greater susceptibility to gram-negative bacterial infection. Therefore, the immune status of individuals should be considered when gambogic acid is given, especially in cancer patients who, under anti-cancer therapeutics, show reduced immune system activity. These patients would be highly vulnerable to infection by multidrug-resistant gram-negative bacteria and are likely to receive inadequate treatments. A higher mortality rate was observed in gram-negative bacteria and are likely to receive inadequate treatments. A higher mortality rate was observed in gram-negative bacteria infections in patients with febrile neutropenia from cancer chemotherapy (12-14). These suggest a need to pay particular attention to maintaining an immune balance when developing anti-cancer and anti-inflammatory drugs, to prevent an enhanced susceptibility to bacterial infection.

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