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Ginkgo biloba extract suppresses endotoxin-mediated monocyte activation by inhibiting nitric oxide- and tristetraprolin-mediated toll-like receptor 4 expression

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Abstract

Monocytes expressing toll-like receptor 4 (TLR4) play a major role in regulating the innate immune response and are involved in systemic inflammation. Previous studies have shown that *Ginkgo biloba* extract (GBE) may act as a therapeutic agent for some cardiovascular and neurological disorders. The objective of this study was to determine whether GBE could modulate immunity in human cells. The monocytic cell line THP-1 was used. Enzyme-linked immunosorbent assay results showed that lipopolysaccharide (LPS) induces the expression of monocyte chemotactic protein-1 (MIP-1), tumor necrosis factor-α, stromal cell-derived factor-1, and MIP-1α, and this induction may be repressed by GBE treatment due to TLR4 blockade. The Griess reagent assay and western blot analysis showed that GBE-mediated inhibition of TLR4 expression was associated with the activation of mitogen-activated protein kinase and production of nitric oxide (NO). Actinomycin D chase experiments demonstrated that GBE decreased the TLR4 mRNA stability in cells. Confocal microscopy and real-time polymerase chain reaction showed that GBE induced the expression of intracellular tristetraprolin (TTP). Transfection with TTP siRNA reversed the effects of GBE in naïve or TLR4-overexpressing cells. Treatment with SNAP (an NO donor) may increase intracellular TTP expression in cells. Immunoprecipitation analysis showed that GBE mediates TTP activation and increases the interaction of TTP with the 3′ untranslated region (UTR) of TLR4 mRNA by regulating NO production. Our findings indicate that GBE could decrease the sensitivity of monocytes to LPS. Utilizing TTP to control TLR4 expression may be a promising approach for controlling systemic inflammation, and GBE may have potential applications in the clinical treatment of immune diseases.

Keywords: Toll-like receptor 4; *Ginkgo biloba* extract; Tristetraprolin; Nitric oxide

1. Introduction

Toll-like receptors (TLRs) are Type I transmembrane receptors that were identified as a *Drosophila* gene to administer ontogenesis and against microbial attach [1]. To date, more than 10 kinds of TLRs have been identified [2]. The intracellular domain of TLR4 is similar to that of interlukin-1 receptor (IL-1R) and is known as the Toll/IL-1R domain. Endotoxin-induced dimerization of TLR4 is followed by the recruitment of the IL-1R accessory protein, binding of MyD88, activation of interleukin-1 receptor-associated kinase and the phosphorylation of tumor necrosis factor receptor-associated factor 6 [3]. This pathway subsequently triggers the activation of nuclear factor-κB and the phosphorylation of mitogen-activated protein kinases (MAPKs), which regulate the induction of inflammation- and innate immune-related gene expression [4,5]. In addition to responding to endotoxins, TLR4 also recognizes a broad spectrum of microbes [6,7] such as *Saccharomyces cerevisiae*, *Candica albicans* and *Crytococcus neoformans*. TLR4 is also capable of responding to various endogenous ligands, including heat shock protein 60 [8,9], high mobility group box 1 [10], hyaluronate [11], minimally modified low-density lipoprotein [12] and heparin sulfate [13], in endothelial and epithelial cells as well as leukocytes. TLR4 recognizes various potential host-derived components that appear as key mediators of innate immunity [14]. It has been shown that TLR4 antagonists and TLR4 ligand-based therapies can be used to treat diseases and may serve as potential drug candidates.

TLR4 is abundantly expressed in monocytes and macrophages and mediates the expression of tumor necrosis factor-α (TNF-α) and

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manganese superoxide dismutase [15]. The regulation and expression of TLR4 may be involved in altering the actual immune capacity of monocytes and macrophages [16]. Monocytes express TLR4, which plays a key role in maintaining healthy function and differentiation [17], directly or indirectly. Correlation of TLR4 levels and mobility in patients with cardiovascular diseases indicated that monocytic TLR4 levels were elevated in patients with coronary artery disease [18], heart failure after acute myocardial infarction [19], and stable angina [18]. Recent investigations have revealed that in addition to its interactions with endotoxins and heat shock proteins from bacteria, TLR4 also interacts with endogenous factors and cytokines [20]. Therefore, up-regulated TLR4 may increase the cellular sensitivity to antigens and thereby mediate the progression of atherosclerosis.

Ginkgo biloba has been used in traditional Chinese medicine for thousands of years. Recently, *G. biloba* extract (GBE), a defined complex mixture extracted from *G. biloba* leaves and containing 24% Ginkgo flavone glycoside and 6% terpenlactones (ginkgolides and bilobalide), was used as a therapeutic agent for some cardiovascular and neurological disorders [21,22]. Several mechanisms have been proposed for the beneficial effects of GBE, including increased blood flow [23], inhibition of platelet aggregation [24], suppression of nitric oxide (NO) production [25], and potential antioxidant activity [26]. In addition, GBE provides protection in model systems of oxidative stress, including cardiac [23,27] and retinal [28] ischemia-reperfusion injury. In a recent study, we presented considerable evidence for the activation of nicotinamide adenine dinucleotide phosphate oxidase and MAPK signaling pathways as well as the stabilization of TLR4 mRNA in lipopolysaccharide (LPS)-stimulated human aortic smooth muscle cells (HASMCs) and endotoxin-induced peripheral arterial system injury [29,30]. GBE may down-regulate TLR4, thereby attenuating the progression of atherosclerosis or restenosis after angioplasty [31]. However, the effects of GBE on immunity are largely unknown. In this study, we investigated whether GBE regulates the immune capacity and sensitivity of monocytes to endotoxins by regulating the TLR4 levels. The results may provide a basis for future applications of GBE in the clinical treatment of immune diseases.

2. Materials and methods

2.1. Cell culture

THP-1 cells, a human promyelomonocytic cell line, were obtained from the American Type Culture Collection (ATCC, VA, USA) and grown in RPMI 1640 medium with 2 mM L-glutamine, 4.5 g/L glucose, 10 mmol/L HEPES, 1.0 mmol/L sodium pyruvate, 10% fetal bovine serum, and 1% antibiotic-antimycotic mixture. The cell density was maintained between 5×10^4 to 8×10^5 viable cells/ml, and the medium was renewed every 2–3 days. The GBE stock solution was purchased from Dr. Willmar Schwabe (Karlsruhe, Forschungszenstrum, Germany).

2.2. Cell viability assessment by the MTT assay and flow cytometry

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to measure cell viability. Cells were grown in 96-well plates and incubated with various concentrations (12.5–400 μg/ml) of GBE for 2 or 24 h. Subsequently, 0.5 μg/ml of MTT was added to each well, and incubation was continued at 37°C for an additional 4 h. Dimethyl sulfoxide was added to each well, and the absorbance was recorded at 530 nm using a DIAS Microplate Reader (Dynex Technologies, Chantilly, VA, USA).

Using flow cytometry to measure the cytotoxicity of GBE. Prepare cell and resuspend cell at 2×10^6 cells/ml in PBS+0.1% bovine serum albumin (BSA). Add propidium iodide (PI) staining solution to cells, mix gently and incubate 1 min. PI fluorescence was analyzed by flow cytometry.

2.3. Enzyme-linked immunosorbent assay

THP-1 cells were seeded in 24-well plates at a density of 10^6 cells/ml/well, and these were then pretreated with various concentrations of GBE for 2 or 24 h followed by LPS (*E. coli* serotype 0127:B8; Sigma-Aldrich, MA, USA) stimulation (25 ng/ml). After 24 h, the culture medium was collected to quantify the levels of monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor alpha, stromal cell-derived factor-1 (SDF-1), and macrophage inflammatory protein-1 (MIP-1) alpha [MIP-1α ; using the DuoSet enzyme-linked immunosorbent assay (ELISA) development kits (R&D Biosystems, San Jose, CA, USA)].

2.4. Quantitative real-time polymerase chain reaction

Total RNA was isolated using the TRIZOL reagent kit (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from total RNA using Superscript II reverse transcriptase. Quantitative real-time polymerase chain reaction (PCR) was performed using a FastStart DNA Master SYBR Green I kit and LightCycler (Roche, Pleasanton, CA, USA). The FastStart *Taq* DNA polymerase was activated by incubation at 95°C for 2 min. This was followed by 40 cycles at 95°C for 1 s, 60°C for 5 s and 72°C for 7 s. Fluorescence was measured at 86°C after the extension step at 72°C. Calculate the cross point values to detect the presence of the TLR4 mRNA and normalize TLR4 mRNA expression against an internal control, such as glyderaldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The PCR primers used for the amplification of TLR4 and GAPDH were as follows. The TLR4 forward primer was 5′-AAG CCG AA GGT GAT TGT TG-3′, and the reverse primer: was 5′-CTG TCC TCC CAC TCC AGGTA-3′. The GAPDH forward primer was 5′-TGC CC CTC TGC TGA TGC C-3′, and the reverse primer was 5′-CCT CCG ACG CCT GCT TCA CA C-3′. All specific primers were synthesized by Sigma-Aldrich (St. Louis, MO, USA).

2.5. Flow cytometry analysis

To examine membrane TLR4 expression in THP-1 cells, the cells were incubated with a PE-conjugated mouse anti-hTLR4 antibody (Biolegend, San Diego, CA, USA) or with mouse IgM/IgG2a isotype controls (DakoCytomation, Hamburg, Germany). After washing with the staining buffer [phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and 0.1% sodium azide], TLR4 expression was analyzed by flow cytometry. Sorting 10^4 cells and analyzing the cell surface fluorescent intensity. The mean fluorescent intensity in experimental groups were compared with that in untreated group and the data were represented as percentage of control.

2.6. Western blot analysis

Western blot analysis was used to determine the changes in the cytosolic activation of p38 MAPK, extracellular signal-regulated kinase 1 and 2 (ERK1/2), and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) in THP-1 cells stimulated with GBE. Total cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gels were transferred onto polyvinyl difluoride (PVDF) membranes. The PVDF membranes were first probed with the rabbit anti-p38 antibody, rabbit anti-phospho-p38 antibody, rabbit anti-SAPK/JNK antibody, rabbit anti-phospho-SAPK/JNK antibody, rabbit anti-p44/p42 MAPK antibody or mouse anti-phospho-p44/p42 MAPK antibody (all anti-MAPK antibodies were purchased from Cell Signaling Technology, Beverly, MA, USA) at a dilution of 1:1000. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. The proteins were visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Piscataway, NJ, USA).

2.7. Measurement of NO production

After incubation of THP-1 cells with GBE for 4–18 h, the nitrite levels of the conditioned media were measured using the Griess reagent [1% sulfanilamide and 0.1% *N*-(1-naphthyl) ethylenediamine in 2% phosphoric acid], as described previously [32].

2.8. Actinomycin D chase experiments

To determine the steady-state dynamic balance between the rate of transcription and the message stability of TLR4 mRNA, an actinomycin D chase experiment [30] was conducted. Actinomycin D (20 μg/ml) was added to the cells for 1 h following treatment under various experimental conditions. Total RNA was extracted at 0, 30, 60, 120 and 240 min after the addition of actinomycin D, and quantitative real-time PCR was then performed. The mRNA decay curves were constructed, and the half-life (t1/2) was calculated from the curves.

2.9. TLR4 plasmid transfection

The full-length wild-type TLR4 plasmid was purchased from Origene (Cambridge Bioscience, Cambridge, UK). Exponentially growing THP-1 cells (3×10^6 cells) were transiently transfected with the plasmids using the Lipofectamine transfection reagent, according to the manufacturer's instructions (Sigma, MO, USA). The cells were seeded in six-well plates immediately after transfection for further experiments at 48 h after transfection.

2.10. Knockdown of gene expression by RNA interference

Knockdown of tristetraprolin (TTP) gene expression was performed by siRNA transfection. Cells (3×10^6) were suspended in 2.5 ml of serum-free medium, and 25 nM of TTP siRNA duplexes (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were transfected, as described in the instruction manual. Silencer Validated siRNA