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Sophoraflavanone G from *Sophora alopecuroides* inhibits lipopolysaccharide-induced inflammation in RAW264.7 cells by targeting PI3K/Akt, JAK/STAT and Nrf2/HO-1 pathways

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ABSTRACT

Sophoraflavanone G (SG), a prenylated flavonoid from *Sophora alopecuroides*, has been reported to have many pharmacological activities including anti-inflammation. However, the molecular mechanisms of its anti-inflammatory activity remain largely unclear. In this study we investigated the effects and the underlying molecular mechanisms of SG on lipopolysaccharide (LPS)-induced inflammation in RAW264.7 cells. Pretreatment with SG inhibited LPS-induced production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) through reducing the expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). SG also decreased the expressions of pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β), both in the protein and gene levels. Further experiments demonstrated that SG downregulated the LPS-induced upregulation of phosphorylated phosphoinositide-3-kinase and Akt (PI3K/Akt). SG also attenuated the expression of phosphorylated Janus kinase signal transducer and activator of transcription (JAK/STAT). In addition, SG upregulated heme oxygenase-1 (HO-1) expression via nuclear translocation of nuclear factor E2-related factor 2 (Nrf2). Taken together, SG may act as a natural agent to treat some inflammatory diseases by targeting PI3K/Akt, JAK/STAT and Nrf2/HO-1 pathways.

1. Introduction

Increasing studies show that inflammation accounts for a wide range of progressive diseases, including cancer, neurological disease, metabolic disorder and cardiovascular disease [1–4]. Inflammation is a complex process regulated by an array of inflammatory mediators and cytokines. As the main pro-inflammatory cells, macrophages are responsible for invading pathogens by releasing large amounts of pro-inflammatory mediators including NO, PGE_2 and various pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 [5]. LPS, an outer membrane component of Gram-negative bacteria, is a potent activator of macrophages [6]. Many researchers use LPS-activated macrophages for inflammation experiments [5,6].

Flavonoids affect the inflammatory process and possess anti-inflammatory as well as immunomodulatory activities in vitro and in vivo [7]. Among the flavonoid derivatives, prenylated flavonoids are the minor, yet unique classes of flavonoids, which are distributed mainly among several plant families such as Leguminosae and Moraceae [8]. Previous investigations concerning some prenylated flavonoids have demonstrated their anti-inflammatory potential [8,9].

Sophoraflavanone G (SG) (Fig. 1A), a prenylated flavonoid, was separated from *Sophora alopecuroides* [10]. *S. alopecuroides*, a traditional Chinese herbal, has been widely used to treat various kinds of inflammation for many years [11,12]. However, the vast majority of previous studies focused mainly on the phytochemical constituents of alkaloids [11–16]. To this day, it has been reported that SG can inhibit 5-LOX, 12-LOX and COX-1 activity [17], reduce pro-inflammatory mediators and cytokines production in vitro [18,19], and shows in vivo anti-inflammatory activity against mouse croton oil-induced ear edema and rat carrageenan paw edema [20]. Although these studies have addressed the anti-inflammatory activities of SG, the detailed molecular mechanisms of SG remain largely unclear. So this study aimed to further explore the mechanism in LPS-induced RAW264.7 cells. We here

Abbreviations: SG, Sophoraflavanone G; LPS, lipopolysaccharide; NO, nitric oxide; PGE_2, prostaglandin E_2; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1β; PI3K, phosphoinositide-3-kinase; JAK, Janus kinase; STAT, signal transducer and activator of transcription; PIAS, protein inhibitor of activated Stat; HO-1, heme oxygenase-1; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor E2-related factor 2; NF-κB, nuclear factor-κB.

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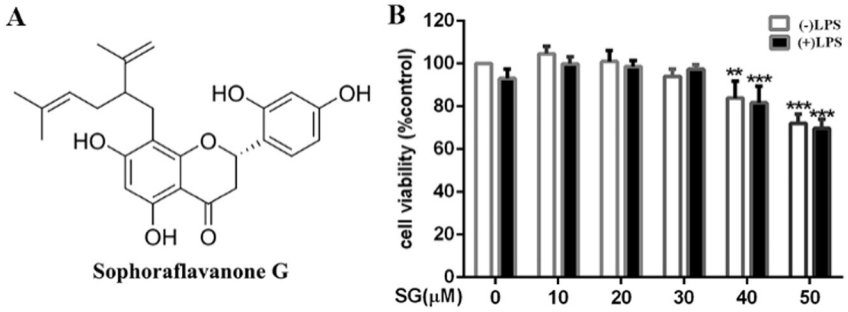

Fig. 1. A) Chemical structure of Sophoraflavanone G (SG). B) Cytotoxicity of SG in RAW264.7 cells. Cells were treated with SG at 10–50 μM for 24 h in the presence or absence of LPS, and then cell viability was examined by the MTT assay. Data are expressed as the means ± S.D. of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ in comparison with control group.

unraveled that SG suppressed the inflammatory response by targeting PI3K/Akt, JAK/STAT and Nrf2/HO-1 pathways.

2. Materials and methods

2.1. Materials

SG (>95%) was separated from *S. alopecuroides* in our laboratory [10]. It was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50 mM and further diluted in cell culture media so that the final DMSO concentration was below 0.1% v/v. LPS (*Escherichia coli* serotype 055: B5) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were bought from Gibco BRL (Gaithersburg, MD, USA). All antibodies, except for HO-1 and Keap1 (Bioworld Technology Co. Ltd., Nanjing, China) were obtained from Cell Signaling Technology (Danvers, MA). Primers for Real-time PCR were synthesized by Generay Biotech Co., Ltd. (Shanghai, China). EASYspin Plus Cell/Tissue RNA Isolation Kit was bought from Aidlab Biotechnologies Co. Ltd. (Beijing, China). ReverTra Ace qPCR RT Kit and SYBR Green Real-Time PCR Master Mix were from TOYOBO Ltd. (Osaka, Japan). All other chemicals were obtained from Sigma-Aldrich unless otherwise stated.

2.2. Cell culture

RAW264.7, a mouse macrophage cell line, was purchased from the Cell Bank of the Shanghai Institute of Cell Biology and Biochemistry, Chinese Academy of Sciences (Shanghai, China) and cultured in high glucose DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a 37 °C humidified incubator containing 5% CO_2.

2.3. Cell viability assay

MTT assay was used to evaluate the effect of SG on cell viability. In Brief, RAW264.7 cells were seeded in 96-well plates (Corning Inc., Corning, NY, USA) at a density of 10^4 cells/well. After overnight growth, cells were treated with various concentrations of SG (10–50 μM) for 1 h, followed in the presence or absence of LPS (100 ng/mL) for the next 24 h. 20 μL of MTT solution (5 mg/mL) was added and the cells were further cultured for 4 h. After that, the supernatant was carefully removed and then the resulting formazan crystals were dissolved in 150 μL DMSO with horizontal shaking. The absorbance at 570 nm (ref 630 nm) was measured with a microplate reader (Molecular Devices, California, US).

2.4. Measurement of NO, PGE_2, TNF-α, IL-6 and IL-1β release

RAW264.7 cells were seeded into 24-well plates at a density of 10^6 cells/mL and cultured overnight. After pretreatment with SG of various concentrations for 1 h, the cells were stimulated with LPS (100 ng/mL) for indicated time periods. The concentration of NO in the conditioned culture medium was examined with the Nitric Oxide Assay Kit (Beyotime institute of Biotechnology. Jiangsu, China) and the release of PGE_2, TNF-α, IL-6 and IL-1β in the cell supernatants were assayed using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The concentrations were calculated from the standard curves.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

RAW264.7 cells (2×10^6 cells in a 6-well plate) were treated with SG for 1 h and then were stimulated with LPS for 6 h. Total RNA was extracted with an EASYspin Plus Cell/Tissue RNA Isolation Kit and 1 μg total RNA was reverse-transcribed using a ReverTra Ace qPCR RT Kit according to the manufacturer's instructions. Aliquots of diluted cDNA (1:5) were amplified with SYBR Green Real-Time PCR Master Mix in a final volume of 20 μL. QRT-PCR was carried out using the LightCycler 480 (Roche Molecular Biochemicals, Mannheim, Germany). PCR cycles consisted of initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 15 s. The Δ cycle threshold method was used for the calculation of relative differences in mRNA abundance with the LightCycler 480. Data were normalized to the expression of GAPDH. The results were expressed as fold-changes. The normalized value of the target mRNA of the LPS control group is arbitrarily presented as 1. The sequences of primers used were listed in Table 1.

2.6. Protein samples preparation

RAW264.7 Cells (1×10^6 cells/mL) were treated or left untreated with SG (10, 20 and 30 μM) for 1 h and stimulated with LPS for indicated time. Then cells were rinsed, scraped off and collected in ice-PBS. After centrifugation the PBS was pipetted completely and Cell lysis buffer for Western and IP (Beyotime institute of Biotechnology. Jiangsu, China) containing a cocktail of protease and phosphatase inhibitors

Table 1
Primers used for the qRT-PCR study.

Gene	Sequence (5′ to 3′)
iNOS	F: GAATCTTGGAGCGAGTTGTGGA
	R: GTGAGGGCTTGGCTGAGTGAG
COX-2	F: CTGGTGCCTGGTCTGATGATGT
	R: AGTCTGCTGGTTTGGAATAGTTGCT
TNF-α	F: CTTGTTGCCTCCTCTTTTGCTTA
	R: CTTTATTTCTCTCAATGACCCGTAG
IL-6	F: AAGGAGTGGCTAAGGACCAAGAC
	R: AGTGAGGAATGTCCACAAACTGATA
IL-1β	F: TGTGTTTTCCTCCTTGCCTCTGAT
	R: TGCTGCCTAATGTCCCCTTGAAT
GAPDH	F: CTTTGGCATTGTGGAAGGGCTC
	R: GCAGGGATGATGTTCTGGGCAG