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An anti-sepsis monomer, 2′,5,6′,7-tetrahydroxyflavanonol (THF), identified from *Scutellaria baicalensis* Georgi neutralizes lipopolysaccharide *in vitro* and *in vivo*

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ABSTRACT

Lipopolysaccharide (LPS) is a known trigger in the pathogenesis of sepsis, lipid A being the toxic component. One of several adjuvant therapeutic approaches for severe sepsis is currently focusing on the neutralization of LPS. In order to obtain the components from traditional Chinese herbs that can neutralize the endotoxin, aqueous extractions of twelve herbs were tested using affinity biosensor technology. From twelve herbs, *Scutellaria baicalensis* Georgi (Huang Qin) found to possess high lipid A-binding abilities, and was selected in subsequent experiments. After subjected to macroporous adsorptive resins and HPLC, we obtained 2′,5,6′,7-tetrahydroxyflavanonol (THF) from *S. baicalensis* Georgi under the direction of neutralization of LPS and reducing proinflammatory cytokines. *In vitro*, THF directly bound to LPS and neutralized its activity. THF not only down-regulated TNF-α mRNA expression but also decreased TNF-α and IL-6 release from RAW264.7 cells induced by LPS in a dose-dependent manner. THF-mediated inhibition on proinflammatory cytokine release is probably associated with downregulation of LPS-induced TLR4 mRNA augmentation. *In vivo*, THF could significantly protect mice against a lethal challenge with heat-killed *E. coli* 35218 (*E. coli* 35218) in a dose-dependent manner, and decreased the plasma LPS level in endotoxemia mice. These findings provide compelling evidence that THF may be an important potential drug for sepsis treatment. Considering the inhibitory effects of THF on LPS-induced cytokine release are unlikely due to its nonspecific cellular toxicity, THF should be considered as a safe putative candidate for development as a drug for sepsis treatment.

1. Introduction

Sepsis results in the activation of numerous proinflammatory mediators such as TNF-α, IL-6 and IL-12. This condition may result in multiple organ dysfunction syndrome (MODS), septic shock and ultimately death [1,2]. An epidemiological study found that the incidence of sepsis was approximately three cases per 1000 people, and the morbidity is increasing in recent years. The overall mortality rate is about 30%, rising to 40% in the elderly and more than 50% in patients with septic shock [3].

Lipopolysaccharide (LPS/endotoxin), the major constituent of the outer membrane of gram-negative bacteria, is a common trigger of sepsis. The overproduction of LPS-induced cytokines such as TNF-α, IL-6 and IL-12, has been considered central to the pathophysiologic derangement associated with sepsis and septic shock. Despite improved care, the hospital mortality rate from severe sepsis and septic shock has not improved significantly over recent decades [4]. There are currently few effective adjuvant therapies in clinical use except activated protein C, which targets the coagulation system [5–7]. Therefore, it is important to investigate new anti-LPS drugs to potentially identify a clinically relevant anti-sepsis drug.

Lipid A, an evolutionarily conserved region of LPS, has been identified as the toxic component of LPS and hence represents an ideal target for anti-sepsis drug development. The mechanism of biological activity of LPS probably involves in specific binding of lipid A moiety to the cationic residues of a receptor molecule. Several agents such as polymyxin B (PMB), bactericidal/permeability increasing protein (BPI) and Limulus anti-LPS factor (LALF) bind to lipid A and antagonize the effects of LPS exhibit extensive physicochemical properties (i.e., hydrophobicity and cationic charge) within their binding domains [8].

Medicinal plants have been used as traditional remedies for hundreds of years in China. Recently, clinical trials have shown a lot of traditional Chinese herbs possess anti-sepsis function [9]. Therefore, it is promising to explore anti-sepsis drug form these herbs. However, these herbs usually have a large number of complex constituents, which extremely difficult to identify the anti-sepsis component, which limit their clinical uses. Therefore, the platform to screen anti-sepsis component from traditional Chinese herbs was established using biosensor affinity technology in 2004, and an anti-sepsis

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monomer, 1, 2, 3, 4, 6-β-D-pentagalloylglucose (PGG), was obtained using silica gel chromatography and HPLC [10].

In present experiments, we describe the screening and isolation of anti-sepsis monomer from traditional Chinese herbs using affinity technology. An anti-sepsis monomer was isolated from *S. baicalensis* Georgi (Huang Qin) and its anti-sepsis activity was investigated *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

LPS from *E. coli* O111:B4, lipid A from Salmonella Re 595 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were purchased from Sigma Chemicals (St. Louis, MO, USA). Macroporous adsorptive resins AB-8 was purchased from Qingdao Marine Chemical Factory (Qingdao, China). Mouse TNF-α and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biosource International (Camarillo, CA, USA). The kinetic turbidimetric *Limulus* amebocyte lysate (LAL) kit was purchased from Zhanjiang A & C Biological Ltd. (Zhanjiang, China). The RNA easy kit (ReverTra Ace$^{-\alpha-}$) and Real-time PCR Master Mix (SYBR Green) were obtained from Toyoboco Ltd. (Osaka, Japan).

2.1.2. Traditional Chinese herbs

Twelve traditional Chinese herbs were purchased from Sichuan Province, and identified in the Chongqing Academy of the Chinese Materia Medica (Chongqing, China).

2.1.3. Animals

One hundred and twenty KM mice (4–6 weeks old), with equal numbers of male and female, were obtained from the Experimental Animal Center of the Third Military Medical University (Chongqing, China). The weight of mice on the day of the experiments was (20.6±1.5) g. They were maintained in specific pathogen free (SPF) condition until used. All the experiments were conducted in accordance with the national guidelines for the care and use of laboratory animals.

2.1.4. Preparation of bacterial strains

Bacterial strains of *E. coli* ATCC 35218 were maintained in our laboratory and used for the mouse sepsis model. Single colonies from viable, growing Luria–Bertani (LB) agar plates were transferred to sterile liquid LB medium (containing 10 g tryptone, 10 g NaCl, and 5 g yeast extract per liter) and cultivated aerobically in 50-ml volumes at 37 °C in a heated, shaking environmental chamber for 12 h. These cultures were then transferred to 500 ml of fresh LB medium for another 12 h. When bacteria were in the log phase of growth, the suspension was centrifuged at 9391 ×*g* for 5 min at 4 °C; the supernatant was discarded, and the bacteria were resuspended and diluted in sterile NS to achieve a concentration of approximately 1×10^{10} colony-formation units (CFU) per milliliter. Finally, the bacterial suspensions were incubated in a water bath at 100 °C for 30 min in order to inactivate the cells [11].

2.1.5. Cell line and culture

Murine macrophage RAW264.7 cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% low endotoxin fetal calf serum (HyClone, Logan, UT), 2 μM glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml in a 37 °C humid atmosphere with 5% CO_2. The cells were diluted with 0.4% trypan blue in phosphate-buffered saline (PBS; 0.1 mM [pH 7.2]), and live cells were counted with a hemacytometer. In each experiment, 10^6 cells/ml were used, except where otherwise indicated.

2.2. Methods

2.2.1. Screening and isolation of lipid A-binding components/monomer from Chinese herbs

2.2.1.1. Screening of lipid A-binding herbs using biosensor technology. Lipid A was immobilized on the surface of a hydrophobic cuvette according to the manufacturer's instructions (Thermo Labsystem, USA), and as described previously [12]. Twelve herbs were each soaked in water for 24 h, after washing thoroughly with distilled water, and then boiled in water at 100 °C for 45 min. After filtration, the material was centrifuged at 4000 ×*g* for 30 min and the supernatants collected, and are termed here "aqueous extract". Five microlitres of aqueous extract (15 g/L) from each herb was added into a cuvette containing 60 μl PBS/AE. After 5 min, the cuvette was washed seven times with 60 μl PBS/AE and alternately washed with 0.1 M HCl, PBS/AE, and 10 mM NaOH, respectively. Data analyses were performed using the FASTplot software package (Thermo Labsystem, USA).

2.2.1.2. Isolation lipid A-binding components from S. baicalensis Georgi using macroporous adsorptive resins technology. An aqueous extract from *S. baicalensis* Georgi was subjected to column chromatography on macroporous adsorptive resins AB-8 (5 cm i.d.×60 cm) eluted with distilled water and gradient ethanol (10%, 20%, 40% and 60%). The elute was collected and concentrated by rotary-evaporation (BUCHI Rotavapor R205, Switzerland), respectively, to yield a total five fraction and named SbG-1~5. Five microlitres of SbG-1~5 (2.0 g/L) was assayed for its binding activity to lipid A in turn as described as above, Thus, the compound (SbG-4) which has the highest binding activity to lipid A was screened among them.

2.2.1.3. Isolation lipid A-binding monomer from S. baicalensis Georgi using HPLC technology. The fraction (SbG-4) screened in experiment 2.2.1.2 was injected onto HPLC system (Agilent, USA) eluted with methanol/0.5% acetic acid (20:80 to 60:40 gradient) to yield a total five HPLC fraction and named 5KL-1–5. Five microlitres of 5KL-1–5 (1.0 g/L) was assayed for its binding activity to lipid A as described as above. The fraction (5KL-1) which has the highest binding activity to lipid A was screened among them and further purified by HPLC. Thus, a HPLC purified product with lipid A-binding activity was obtained. Furthermore, purity factor calculation was performed for its peak purity evaluation as described in Agilent operating manual. After analyzed by mass spectrometry, infrared and NMR (nuclear magnetic resonance) at the National Center of Biomedical Analysis (Beijing, China), a monomer was harvested.

2.2.2. In vitro studies

2.2.2.1. Neutralization of endotoxin. The ability of the monomer to neutralize LPS was assayed using the LAL test, which is an extremely sensitive indicator of the presence of free, non-neutralized LPS [9]. Different concentrations of the monomer (0–4.0 mg/L) were incubated with LPS (0.1 μg/L) at 37 °C for 30 min. Subsequently, 100 μl of this mixture was added to an equal volume of the LAL reagent. The kinetic turbidity was measured using EDS-99 Tube Reader (Zhanjiang A & C Biological Ltd., China).

2.2.2.2. Cytokine release induced by LPS. RAW264.7 (0.2 ml) were incubated in 96-well plate for 4 h, and the supernatants were then discarded and replaced with 0.2 ml serum-free DMEM. The cells were pretreated with the monomer at the indicated dose (0, 10, 20, 40, 80, 160 mg/L) for 30 min, and then stimulated with LPS (100 μg/L) for 4 h and 12 h. The supernatants were collected to assess TNF-α and IL-6 levels using the appropriate ELISA kits, respectively.