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Madecassoside suppresses LPS-induced TNF-α production in cardiomyocytes through inhibition of ERK, p38, and NF-κB activity

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ABSTRACT

Madecassoside (MA) is a major triterpenoid component of *Centella asiatica* that has a wide range of biological activities, including wound-healing and antioxidative activities. In the present study, we evaluated the therapeutic effect of MA on rat cardiac dysfunction during sepsis induced by lipopolysaccharide (LPS), as well as the possible mechanism. Pretreatment of the neonatal rat cardiomyocytes with MA inhibited LPS-induced TNF-α production in a concentration-dependent manner. In addition, pretreatment of the rats with MA (20 mg/kg, *i.g.*) significantly inhibited the elevation of plasma TNF-α, delayed the fall of mean arterial blood pressure, and attenuated the tachycardia induced by LPS. We further observed that MA prevented the LPS-induced nuclear factor-kappa B (NF-κB) translocation from the cytoplasm into the nucleus, and inhibited the LPS-induced phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38. These results suggest that MA inhibits LPS-stimulated TNF-α production through the blocking of ERK1/2, p38 and NF-κB pathways in cardiomyocytes. MA may have cardioprotective effects in LPS-mediated sepsis.

1. Introduction

Stimulation of local inflammatory mediator production plays an important role in the progressive pathological development of ischemic myocardial injury and heart failure. It is well known that the release of LPS from Gram-negative bacteria into the bloodstream causes dramatic pathophysiological reactions such as tachycardia, decreased blood pressure, and multiorgan failure. Recent studies have suggested that exposure of mammalian cells to LPS can lead to release of pro-inflammatory cytokines and in turn activate a second level of inflammatory cascades including cytokines, lipid mediators, and adhesion molecules [1]. Among them, tumor necrosis factor α (TNF-α) is a major pro-inflammatory cytokine that has been implicated in the pathogenesis of cardiovascular diseases, including acute myocardial infarction, chronic heart failure, atherosclerosis, viral myocarditis, cardiac allograft rejection, and sepsis associated cardiac dysfunction [2]. The production of LPS-induced myocardial TNF-α is nearly evenly distributed between cardiomyocytes and resident cardiac macrophage cell types [3,4]. Therefore, targeted therapies to reduce cardiac TNF-α production may improve cardiac performance in failing hearts [2].

Madecassoside (MA), a triterpenoid, is a major component of *Centella asiatica* which is a traditional herb used in Asiatic countries. MA has been used as a wound-healing agent and for the prevention of cicatrisation, because it has been shown to effectively promote fibroblast proliferation and collagen synthesis [5,6]. Recently, some studies demonstrated that MA could protect rat hearts and isolated cardiomyocytes against reperfusion injury both *in vitro* and *in vivo* [7]. In addition, the anti-inflammatory effects of MA were assessed in an LPS-induced macrophage model and collagen-induced arthritis [8]. It was demonstrated that treatment with MA inhibited the production of nitric oxide (NO), prostaglandin E_2 (PGE_2), TNF-α, interleukin-1β (IL-1β), and IL-6 [9,10]. However, it is unknown if MA possesses an anti-inflammatory property on LPS-induced cardiomyocytes and the beneficial effects of MA on myocardial dysfunction have not been evaluated.

In the present study, we examined the effect of MA on TNF-α production in LPS-induced cardiomyocytes and explained the possible mechanisms. Our findings suggest that MA has anti-inflammatory and cardioprotective effects and exerts beneficial effects on endotoxemia.

2. Materials and methods

2.1. Reagents

MA ($C_{48}H_{78}O_{20}$, CAS#: 34540-22-2, HPLC >98%, structure shown in Fig. 1) was purchased from Chengdu Mansite Pharmaceutical Co.

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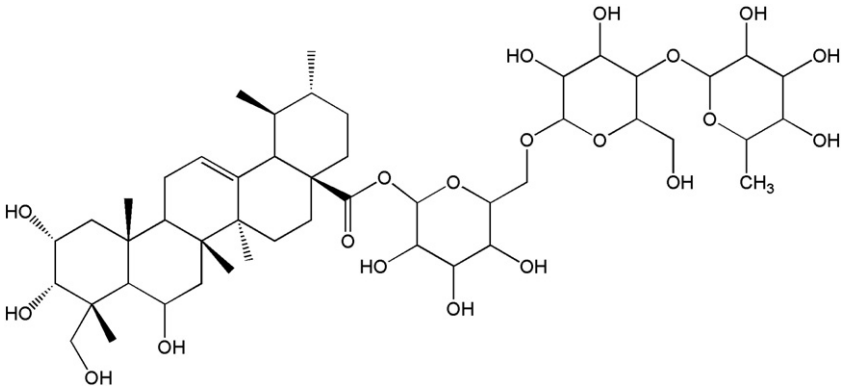

Fig. 1. Structure of madecassoside.

Ltd. (Chengdu, China). LPS (*Escherichia coli* serotype 026:B6), 5-bromo-2′-deoxyuridine (BrdU), and β-actin antibody were purchased from Sigma Co. (St Louis, MO, USA). Rat TNF-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Superscript III reverse transcriptase was purchased from Invitrogen (Gaithersburg, MD, USA). dNTP mixture, Tag DNA polymerase and RNAase inhibitor were obtained from Takara Bio Inc. (Otsu, Shiga, Japan). The rabbit polyclonal anti-ERK and anti-p38, mouse monoclonal anti-phospho-ERK and anti-phospho-p38 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit polyclonal antibodies against phospho-IκB-α and IκB-α were purchased from Cell Signaling Technology (Beverly, MA, USA). Supersignal ECL kit was purchased from Pierce Chemical Inc. (Rockford, IL, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and Trizol reagent were obtained from GIBCO-BRL (Carlsbad, California, USA).

2.2. Cell culture and stimulation with LPS

Neonatal rat cardiomyocytes were isolated from the ventricles of one to three-day-old Sprague–Dawley rats, essentially according to the procedure described previously [11]. Cells, enriched for cardiomyocytes were plated on 6- or 12-well plates and maintained in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. BrdU (0.1 mM) was added throughout the culture period. The medium was changed every 2 days. After 4 days, the medium was replaced with serum-free medium for 24 h, and then cells were incubated with various concentrations of MA and stimulated with LPS (100 ng/ml) for the indicated time. MA dissolved in dimethyl sulfoxide (DMSO) was diluted in the medium immediately before use (the final DMSO concentration in all assays did not exceed 0.05%).

2.3. RT-PCR

After treated with varying concentrations of MA (0–300 μmol/l) for 2 h and then stimulated with 100 ng/ml of LPS for 12 h [12], cells (1×10^6 cells/well) were collected immediately and total RNA was extracted using Trizol reagent. One microgram of total RNA was reverse-transcribed into cDNA with 0.2 U/ml Superscript III reverse transcriptase according to the manufacturer's protocol. The following oligonucleotide primers were used: TNF-α, sense 5′-TACTGAACTTCGGGGTGATTGGTCC-3′, antisense 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′ [13], producing a 295 bp fragment; β-actin, sense 5′-GCTCGTCGTCGACAACGGCTC-3′, antisense 5′-CAAACATGATCTGGGTCATCTTCTC-3′, producing a 353 bp fragment. PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination.

2.4. TNF-α assay

The cardiomyocytes (2×10^5 cells/well) were plated onto 24-well plates and treated with MA and LPS for 0–4 h. The supernatants were collected, immediately frozen in liquid nitrogen, and stored at −70 °C until analysis. The concentrations of TNF-α were measured by ELISA kits according to the manufacturer's instructions.

Blood samples (0.5 ml) were collected at different times after the injection of LPS for measurement of the TNF-α level in plasma by ELISA.

2.5. Animals and drug administration

Male Sprague–Dawley rats (weight 200 to 250 g) were purchased from the Center of Experimental Animals of the Fourth Military Medical University and were housed for six days in standard cages. To determine the effect of different doses of MA in plasma TNF-α production, animals were randomly divided into the following six groups ($n=6$): vehicle group (0.5% carboxymethylcellulose (CMC)-saline), model group (LPS group) and MA groups (5, 10, 20 or 40 mg/kg MA + LPS). MA was dissolved in 0.5% CMC-saline. The animals in test groups were administered MA at different doses by intragastric (*i.g.*) once daily from days 1 to 5. For the groups of vehicle and model, rats were given an equal volume of vehicle at the same time. At 2 h after the last application of MA, the model and MA animals received intravenous injection of 5 mg/kg body weight LPS. 2 h later, 0.5 ml of blood was collected from each rat and was centrifuged (6000 *g* for 5 min). The plasma samples were stored at −70 °C for TNF-α analysis.

To determine the effect of MA on the mean arterial blood pressure (MAP) and the heart rate (HR), animals were randomly divided into three groups ($n=6$): vehicle group (0.5% CMC-saline), model group (5 mg/kg LPS) and MA groups (20 mg/kg MA + LPS). All the groups were treated (*i.g.*) with vehicle or MA once daily for 5 days. After the last application of MA, the rats were anesthetized by intraperitoneal injections of sodium pentobarbital (7.5 mg/kg). The trachea was cannulated to facilitate respiration. The environmental temperature was maintained at about 25 °C. The right femoral artery was cannulated and connected to PowerLab/4SP recording system (AD Instruments Co., Australia) by a pressure transducer for the measurement of MAP and HR. The left femoral vein was cannulated for the administration of LPS. After the completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 30 min. After recording the baseline hemodynamic parameters (rats were given MA 2 h later), animals received saline or LPS (5 mg/kg) in normal saline to induce endotoxemia. The MAP and HR were reassessed at 0.5, 1, 2 and 4 h after saline or LPS injection. Prior to and at 1, 2 and 4 h after saline or LPS administration, 0.5 ml of blood was collected. Any blood withdrawn was immediately replaced by the injection of an equal amount of saline. After the blood samples were