



Cordycepin alleviates lipopolysaccharide-induced acute lung injury via Nrf2/HO-1 pathway

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ABSTRACT

Aims: The present study is to investigate the protective effect of cordycepin on inflammatory reactions in rats with acute lung injury (ALI) induced by lipopolysaccharide (LPS), as well as the underlying mechanism.

Methods: Wistar rat model of ALI was induced by intravenous injection of LPS (30 mg/kg body weight). One hour later, intravenous injection of cordycepin (1, 10 or 30 mg/kg body weight) was administered. The wet-to-dry weight ratio of lung tissues and myeloperoxidase activity in the lung tissues were measured. The contents of nitrite and nitrate were measured by reduction method, while chemiluminescence was used to determine the content of superoxide. Quantitative real-time polymerase chain reaction and Western blotting were used to determine the expression of mRNA and protein, respectively. Colorimetry was performed to determine the enzymatic activity of heme oxygenase-1 (HO-1). Nuclear translocation of Nrf2 was identified by Western blotting. The plasma contents of cytokines were measured by enzyme-linked immunosorbent assay.

Results: Cordycepin enhanced the expression and enzymatic activity of HO-1 in ALI rats, and activated Nrf2 by inducing the translocation of Nrf2 from cytoplasm to nucleus. In addition, cordycepin regulated the secretion of TNF- α , IL-6 and IL-10 via HO-1, and suppressed inflammation in lung tissues of ALI rats by inducing the expression of HO-1. HO-1 played important roles in the down-regulation of superoxide levels in lung tissues by cordycepin, and HO-1 expression induced by cordycepin affected nitrite and nitrate concentrations in plasma and iNOS protein expression in lung tissues. Cordycepin showed protective effect on injuries in lung tissues.

Conclusion: The present study demonstrates that cordycepin alleviates inflammation induced by LPS via the activation of Nrf2 and up-regulation of HO-1 expression.

1. Introduction

Acute lung injury (ALI) is a common critical complication in patients with sepsis or infection [1]. Pathologically, ALI is mainly characterized by injury in alveolar capillary system and increase in pulmonary vascular permeability, leading to overactivation of macrophages and neutrophils, excessive release of inflammation-associated proteases and reactive oxygen species (ROS), and pulmonary hemorrhage [1]. Afterwards, microvascular and tissue damages, edema and fibrin deposition occur, finally leading to injury in respiratory function [1]. A variety of cytokines and inflammatory mediators are involved in the occurrence and development of ALI, including tumor necrosis factor (TNF)- α , interleukin (IL)-1, IL-6, high-mobility group box-1 (HMGB1) protein, inducible nitric oxide synthase (iNOS), and nitric oxide (NO) [2]. It is reported that iNOS/NO play important roles in the pathological process of ALI [3]. Persistent and uncontrolled inflammatory responses of the lungs are believed to play an important

role in the pathogenesis of ALI. Therefore, inhibiting inflammatory responses mediated by innate immune system may be a potential mechanism for slowing down ALI progression. Lipopolysaccharide (LPS) is a main component of the outer membrane of Gram-negative bacteria, and is considered as a key molecule that triggers innate immune response and acute inflammation. LPS induces excessive expression of pro-inflammatory cytokines, chemokines, ROS and NO, and finally causes ALI. By now, there has been no ideal animal model of ALI. However, symptoms of ALI induced by LPS in rats are very similar to those observed in human. An ideal model of ALI can be constructed by injecting LPS into the trachea, without leading to systemic inflammatory response and multiple organ failure.

Heme oxygenase-1 (HO-1) is a rate-limiting enzyme that catalyzes the decomposition of heme into Fe²⁺, biliverdin and CO. It is reported that HO-1 and its metabolites have a certain inhibitory effect on inflammatory responses mediated by neutrophils, macrophages and lymphocytes [4]. Proinflammatory cytokines, HMGB1 and oxidative

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stress can up-regulate the expression of HO-1. HO-1 deficient mice tend to be associated with higher levels of oxidative stress and higher mortality. By contrast, HO-1 expression induced by low dose of CO significantly reduces lung injury and lethal endotoxin shock of rats induced by LPS [5], suggesting that HO-1 has negative regulation on inflammatory reaction and oxidative stress of ALI.

Although the treatment of ALI has been greatly improved, its mortality rate is still as high as 30%. By now, there has been no effective cure for ALI. Cordycepin is a kind of purine alkaloid extracted from *Cordyceps sinensis*, a precious traditional Chinese herbal medicine that has regulatory effect on the immune system [6]. It is reported that cordycepin has inhibitory effects on synthesis of DNA or RNA, post-transcriptional modification of hnRNA and mRNA, adenosine cyclase activation and specific protein synthesis at molecular level; at cellular level, cordycepin promotes cell differentiation, enhances anti-tumor activity, and increases chemotaxis of macrophages [7]. In addition, cordycepin promotes the release of IL-10 from human peripheral blood mononuclear cells, and inhibits the production of IL-2 [8]. Mature dendritic cells (DC) induce the proliferation of regulatory T cells (Tregs) under the treatment by cordycepin [9]. In addition, cordycepin affects toll-like receptor 4 (TLR4)/MAPKs pathway in macrophages and the release of a variety of proinflammatory cytokines and inflammatory mediators [10,11], suggesting that cordycepin is a kind of effective immunomodulator. However, it is still unclear whether cordycepin has protective effects against ALI. In the present study, we investigate the effect of cordycepin on inflammatory reactions in ALI animal model induced by LPS, as well as the underlying mechanism.

2. Materials and methods

2.1. Animals

Specific pathogen-free male Wistar rats (8–10 weeks) were obtained from Laboratory Animal Center of Shaoyang University and raised under $23 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ humidity with normal feedstuffs. After being anesthetized by intraperitoneal injection of 1.5 g/kg urethane, in vitro ALI model of rats was established according to a previously published literature [12]. The rats were divided into 7 groups of 10 rats. For control group, the rats were infused with 9 ml saline via jugular vein for 4 h; at 1 h after the infusion, the rats were infused with 2 ml Ringer's solution through femoral vein. For LPS group, the rats were infused with 9 ml saline containing LPS (30 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) via jugular vein; at 1 h after the infusion, the rats were infused with 2 ml Ringer's solution through femoral vein. For three cordycepin groups, the rats were first infused with 9 ml saline containing LPS (30 mg/kg body weight) via jugular vein; at 1 h after the infusion, the rats were infused with Ringer's solution containing cordycepin (1, 10 and 30 mg/kg body weight, respectively; Sigma-Aldrich, St. Louis, MO, USA) [13]. For SnPP group, the rats received intraperitoneal injection of SnPP (30 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) at 6 h before LPS infusion, while other conditions were the same with cordycepin group (30 mg/kg body weight). For CoPP group, the rats received intraperitoneal injection of CoPP (10 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) at 6 h before LPS infusion, while other conditions were the same with cordycepin group (30 mg/kg body weight). All animal experiments were conducted according to the ethical guidelines of Shaoyang University.

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

Lung tissues (100 mg) of rats were ground into powder in liquid nitrogen and mixed with 1 ml Trizol (Thermo Fisher Scientific, Waltham, MA, USA) for lysis. Then, total RNA was extracted using phenol chloroform method. The purity of RNA was determined by A260/A280 using ultraviolet spectrophotometry (Nanodrop ND2000, Thermo Scientific, Waltham, MA, USA). Then, cDNA was obtained by

reverse transcription using reverse transcription system (Thermo Fisher Scientific, Waltham, MA, USA) from 2 μg RNA and stored at -20°C . MiniOpticon Real-Time PCR System (Bio-Rad, Hercules, CA, USA) was used to detect mRNA expression of HO-1, using β -actin as internal reference. The reaction system (20 μl) was composed of 10 μl SYBR EX Taq-Mix, 0.5 μl forward primer (HO-1, 5'-CGTGCAGAGAATTCTGAG TTC-3'; β -actin, 5'-CCTGTATGCCTCTGGTCGTA-3'), 0.5 μl reverse primer (HO-1, 5'-AGACGCTTTACGTAGTGCTG-3'; β -actin, 5'-CCATCT CTTGCTCGAAGTCT-3'), 1 μl cDNA and 8 μl ddH₂O. PCR condition was: initial denaturation at 95°C for 10 min; 35 cycles of denaturation at 95°C for 10 s, annealing at 61°C for 20 s and elongation at 72°C for 10 s. The $2^{-\Delta\Delta\text{Ct}}$ method was used to calculate the relative expression of iNOS or HO-1 mRNA against β -actin. Each sample was tested in triplicate.

2.3. Western blotting

Lung tissues were mixed with cocktails containing protease inhibitor (Roche, Basel, Switzerland) and homogenized. Then, the samples were thoroughly lysed with Radio-Immunoprecipitation Assay (RIPA) lysis buffer (25 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP-40, and 1% sodium dodecyl sulfate) containing protease inhibitor and phosphatase inhibitor. Protein concentration was determined using Bradford method at 595 nm. Protein samples (20 μg) were then mixed with sodium dodecyl sulfate loading buffer before denaturation in boiling water bath for 5 min. Afterwards, the samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (100 V, 2 h) and blocked with 5% skimmed milk at room temperature for 2 h. Then, the membranes were incubated with rabbit anti-rat HO-1 (1:1000), Nrf2 (1:500), TATA-binding protein (1:500) and β -actin (1:2000) polyclonal primary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C overnight. After extensive washing with phosphate-buffered saline with Tween 20 for 3 times of 15 min, the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10,000; Abcam, Cambridge, UK) for 1 h at room temperature before washing with phosphate-buffered saline with Tween 20 for 3 times of 15 min. Then, the membrane was developed with enhanced chemiluminescence detection kit (Sigma-Aldrich, St. Louis, MO, USA) for imaging. Image lab v3.0 software (Bio-Rad, Hercules, CA, USA) was used to acquire and analyze imaging signals. The relative contents of proteins were calculated against β -actin.

2.4. Determination of HO-1 enzymatic activity

Lung tissues were homogenized and supernatants (400 μl) were mixed with 0.8 mmol/L NADPH, 2 mmol/L glucose-6-phosphate, 0.2 U glucose-6-phosphate 1-dehydrogenase, 2 mg rat liver cytosol, 100 mmol/L phosphate-buffered saline and 10 $\mu\text{mol/L}$ chlorhematin. The mixture was incubated at 37°C in dark for 1 h, and the generated amount of bilirubin was used to reflect HO-1 enzymatic activity (nmol/mg protein \times h). In the end, 1 ml chloroform was added to terminate reactions. The absorbance of each sample was measured at 463 nm on a reader (iMark; Bio-Rad, Hercules, CA, USA), using 530 nm as reference. The absorbance of examination group was expressed relative to that of control group.

Enzyme-linked immunosorbent assay (ELISA).

Serum concentrations of TNF- α , IL-6 and IL-10 were measured using ELISA kits (Neobioscience, Shenzhen, China). In microtiter plates, 100 μl samples or standards were added, followed by incubation at 37°C for 2 h. After washing the plates for 5 times, substrates A (50 μl) and B (50 μl) were added into each well, followed by incubation at 37°C for 15 min. After incubation at 37°C for 15 min, stop solution (50 μl) was added into each well, and absorbance of each well was measured at 450 nm within 15 min. The concentrations of TNF- α , IL-6

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