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Sinomenine protects against *E.coli*-induced acute lung injury in mice through Nrf2-NF-κB pathway

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ABSTRACT

Acute lung injury (ALI) is a common disease characterized by pulmonary inflammation and oxidative stress. Sinomenine (SIN) is an alkaloid originally extracted from the Chinese medicinal plant *Sinomenium acutum*. It has been shown to have anti-inflammatory and anti-oxidative effect. However, it's unclear whether SIN can alleviate ALI. In this study, we assessed the effect of SIN on *Escherichia coli (E.coli)*-induced ALI mouse model. Mice were conditioned with SIN or placebo 1 h before intratracheally instilled with *E.coli*. Lung water content, malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, Myeloperoxidase (MPO) levels and inflammatory cytokines production were measured. Immunohistochemistry and western blot were performed to measure target protein expression. *E.coli* induced histological changes indicating tissues damage and increased W/D ratio, MPO activity, MDA content, and inflammatory cytokines production in the Lung. Whereas in mice pretreated with SIN, these changes were absent. *E.coli*-induced NF-κB activation was also inhibited by SIN. In addition, SIN increased the expression of HO-1, NQO1 and Nrf2 in lung tissues. Our results suggest that SIN attenuates ALI through the inhibition of inflammation and oxidative stress.

1. Introduction

Bacterial pneumonia is the third most common cause of death in the world [1]. It can induce acute lung injury (ALI) [2], which leads to acute respiratory distress syndrome (ARDS), one of the worldwide public health problems [3]. In the United States, more than 100,000 people suffer from ALI each year [4]. ALI is characterized by endothelial and epithelial disruption, which lead to inflammatory responses in the lung, including neutrophilic inflammation, perivascular and interstitial edema, impairment of gas exchange and surfactant dysfunction [5,6] and the persistence of inflammation plays an essential role in the progression of ALI.

The inflammation response against bacterial pathogens in the lung generally consists of resident alveolar macrophages, recruited neutrophils and existed endogenous antimicrobial factors in the respiratory secretions [7]. *Escherichia coli* (*E.coli*), used as a surrogate for infectious exposure, induces experimental ALI when it is introduced into the airway [8].

Treatment of ALI is based on either ventilatory or non-ventilatory strategy. To date, neither strategy is efficient [9]. Therefore, it is important to develop an effective new drug for the treatment of ALI. Sinomenine (SIN, 7, 8-didehydro-4-hydroxy3, 7-dimethoxy-17-methyl-α, 13, 14 -morphinan-6-one) is an active alkaloid originally extracted from the Chinese medicinal plant *Sinomenium acutum* [10]. It possessed immune-regulatory and anti-inflammatory properties [11], and has been used to treat mesangial proliferative nephritis and rheumatoid arthritis (RA) [12,13]. Studies in vitro also suggested that SIN might be effective in the management of various autoimmune and inflammation diseases [14]. Kondo et al. found that SIN inhibited endotoxin-induced TNF production and/or reactive oxygen species generation [15]. In addition, SIN can inhibit IκB phosphorylation and NF-κB translocation, leading to reduced expression of downstream inflammatory genes [16,17].

Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and the capacity of cellular antioxidant [18]. NF-E2-related factor 2 (Nrf2, also defined as HEBP1), a redox-sensitive protein, is required to reduce oxidative stress [19]. Under the basal conditions, Nrf2 is degraded by Nrf2 repressor Kelch-like ECH-associated protein 1 (Keap1)-dependent pathway [20]. Once activated, such as by tissue damage-induced ROS, Nrf2 is dissociated with Keap1, and trans-locates into the nucleus, inducing the expression of its downstream target genes, including hemeoxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), superoxide dismutase (SOD) and glutathione peroxidase(GSH) [12].

A previous study suggested that SIN could protect against kidney

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injury and improved Unilateral ureteral obstruction (UUO)-associated inflammation and pathogenic protein expression via Nrf2-Keap1 pathway [21]. In addition, Jun Li et al found that SIN played a protective role in ALI because of its anti-inflammatory effect [13]. These reports led us to hypothesize that SIN could protect against E.coli-induced ALI via Nrf2-Keap1 pathway. In this study, we explored the mechanism by which SIN prevented *E.coli* induced acute lung injury in mice. Our result indicated that SIN reduced the lung injury in a Nrf2-dependent manner. Therefore, our study has revealed a novel mechanism of SIN's anti-inflammation and anti-oxidant functions.

2. Materials and methods

2.1. Materials

Sinomenine (HPLC ≥ 98%) was purchased from Shanghai Roche Pharmaceutical Co., Ltd. (Shanghai, China). The indicated primary antibodies and β-actin were obtained from Hangzhou HuaAn Biotechnology Co., Ltd. (Hangzhou, China). MDA, MPO and SOD kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Animals

Female ICR mice (20–25 g) were purchased from Medical Experimental Animal Center of Yangzhou University (Yangzhou, China). The mice were kept at constant temperature (21 ± 2 ℃) in a light/dark cycle and received food and water ad libitum. Thirty mice were divided into three groups: control group, *E.coli* group, and *E.coli* + SIN group (100 mg/kg).

2.3. Induction of acute lung injury and drug administration

The *E.coli* (ATCC25922) were grown in Luria-Broth medium overnight at 37 ℃ before being used for the experiment. The mice were anaesthetized with an intraperitoneal injection of pentobarbital. *E.coli* were re-suspended in PBS buffer, then the mice were intratracheally instilled with *E.coli* (CFU 5×10^8/40 μL). The control group who received equal volumes of PBS. SIN (100 mg/kg) was administered 1 h before the *E.coli* challenge. The experimental design used in this study was based on previous study [22]. Study in vivo suggested that SIN-HCL (100 mg/kg) protected against CLP-induced sepsis [23]. Jun Li et al. also found SIN (120 mg/kg) attenuated LPS-induced acute lung injury [13], from the above we chose 100 mg/kg of SIN in our study. The BALF, lung tissues and serum were collected for subsequent experiments at day 1, day 3 and day 7 after being exposed to *E.coli*.

2.4. Assay of lung water content

Mice were sacrificed 1 day, 3 days and 7 days after *E.coli* challenge. The lung tissues were immediately weighed for wet weight, then placed in an oven at 60℃ for 48 h to obtain the dry weight. The ratio of wet weight to dry weight (W/D) was calculated.

2.5. Histopathological evaluation

The lung tissues from each group were collected and fixed in 4% paraformaldehyde fixing solution, embedded in paraffin, cut into 5 μm sections and stained with hematoxylin/eosin (H&E). Finally, the pathological changes of lung tissues were examined with a microscope (Olympus).

2.6. Myeloperoxidase (MPO) assay

1 day, 3 days and 7 days after *E.coli* treatment, serum was collected, MPO activity in mice serum was measured by test kits purchased from Nanjing Jiancheng Bioengineering Institute (China, Nanjing) according to the manufacturer's instructions, and the results were measured in absorbance at 460 nm with a 96-well plate.

2.7. Bronchoalveolar lavage fluid (BALF) collection and analysis

The BALF was collected by flushing the right lung (0.3 mL × 3 times). The cells obtained after centrifugation of the BALF were re-suspended in 100 mL of saline and were stained for 8 min with Wright-Giemsa staining. Cells were counted to obtain the total cells per slide ratio at 40× magnification.

The concentration of BALF protein was measured by using bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Biotechnology, China).

2.8. Measurement of malondialdehyde (MDA) and (SOD)

Levels of malondialdehyde (MDA) and superoxide dismutase (SOD) in serum were determined by commercially available kits (Nanjing Jiangcheng Bioengineering Institute, Nanjing, China) in accordance with the instructions.

2.9. RNA isolation and qRT-PCR analysis

Total RNA was extracted from lung tissues by using TRIzol reagents (Invitrogen, USA) according to the producer's instructions. The sample RNA was reversely-transcribed to cDNA with HiScript Reverse Transcriptase Kit (CWBIO, China). The total cDNA was used as starting material for qRT- PCR with Super Multiplex PCR Mix (CWBIO, China). The specific primers in our study for gene amplification were shown in Table 1. The expression of β-actin was used as an internal standard.

2.10. Western blot

The total protein of lung tissues was extracted according to the standard protocol (Beyotime Biotechnology, China). The protein concentrations were assessed using a BCA Protein Assay Kit (Beyotime Biotechnology, China). Then protein (40 μg) was separated by 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Then the membranes were blocked in 5% skim milk in Tris-buffered saline Tween-20 (TBST) for 2 h at room temperature and were incubated specific antibodies at 4 ℃ overnight. The membranes were washed with TBST for three times and were incubated with secondary antibodies conjugated with horseradish peroxidase (HRP)- for 2 h at room temperature. Protein expression was detected using the ECL Western blotting detection kit (Beyotime Biotechnology, China).

2.11. Statistical analysis

All values were expressed as the mean ± standard error of mean (SEM). The results were analyzed by one-way ANOVA with variance test, using GraphPad Prism 6 (GraphPad Instat Software, USA).

Table 1
Primer sequences of qRT-PCR test.

Name	Forward Primer (5'-3')	Reverse primer (5'-3')
IL-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
IL-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
TNF-α	CATCTTCTCAAAATTCGAGTGAC	TGGGAGTAGACAAGGTACAACCC
HO-1	GATAGAGCGCAACAAGCAGAA	CAGTGAGGCCCATACCAGAAG
NQO1	AGGATGGGAGGTACTCGAATC	TGCTAGAGATGACTCGGAAGG
Keap1	CAGCTACACACTAGAGGATCACA	GTGGATGCCTTCGATGGACA
Nrf2	CTTTAGTCAGCGACAGAAGGAC	AGGCATCTTGTTTGGGAATGTG
β-actin	GTGGGCCGCTCTAGGCACCA	CGGTTGGCCTTAGGGTTCAGG