Silybin attenuates LPS-induced lung injury in mice by inhibiting NF-κB signaling and NLRP3 activation

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Abstract. Silybin is one of the main flavonoids produced by milk thistle, which has been used in the treatment of liver diseases. In this study, we examined the protective effects and possible mechanisms of action of silybin in lipopolysaccharide (LPS)-induced lung injury and inflammation. Pre-treatment of mice with silybin significantly inhibited LPS-induced airway inflammatory cell recruitment, including macrophages, T cells and neutrophils. The production of cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in bronchoalveolar fluid and serum was also decreased following treatment with silybin. Elevated cytokine mRNA levels induced by LPS in lung tissue were all suppressed by silybin and lung histological alterations were also improved. In addition, experiments using cells indicated that silybin significantly decreased the mRNA levels and secretion of IL-1β and TNF-α in THP-1 cells. Moreover, the mechanisms responsible for these effects were attributed to the inhibitory effect of silybin on nuclear factor-κB (NF-κB) signaling and NLR family pyrin domain containing 3 (NLRP3) inflammasome activation. The data form our study thus support the utility of silybin as a potential medicine for the treatment of acute lung injury-associated inflammation and pathological changes. Silybin exerts protective effects against lung injury by regulating NF-κB signaling and the NLRP3 inflammasome activation.

Introduction

Inflammatory lung diseases, such as acute lung injury (ALI), acute respiratory distress syndrome and pneumonia are life-threatening diseases which cause high morbidity and mortality worldwide (1,2). ALI is most often seen as part of a systemic inflammatory process, particularly systemic sepsis, where the lung manifestations parallel those of other tissues, such as the widespread destruction of the capillary endothelium, extravasations of protein-rich fluid and interstitial edema. In addition, when the alveolar basement membrane is damaged and fluid seeps into the airspaces, it stiffens the lungs and causes ventilation-perfusion mismatch (3). A major cause for the development of ALI is sepsis, of which Gram-negative bacteria are a prominent cause (4). In mice, the inhalation of endotoxins, such as lipopolysaccharide (LPS) can be used to mimic human Gram-negative bacteria-induced ALI, leading to neutrophil recruitment, pulmonary edema and finally in the impairment of gas exchange; this model has been used extensively for testing new anti-ALI drugs. Despite extensive investigations revealing the pathogenetic factors of ALI (5,6), current treatments do not significantly reduce lung injury and mortality (5). Therefore, new and effective treatment strategies are required for patients with ALI.

The excessive production of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β, is a key event in the development of LPS-induced ALI (7). Hence, these pro-inflammatory mediators and the upstream nuclear factor-κB (NF-κB) signaling pathway may play critical roles in the pathogenesis of ALI (8). The injury and inflammation of the lung tissues in ALI can be attenuated by inhibiting the activation of NF-κB (9-11). NF-κB has been considered as a promising pharmacological target in inflammatory diseases, including sepsis, ALI and asthma (12).

Apart from NF-κB, the production of IL-1β is also controlled by NLR family pyrin domain containing 3 (NLRP3). NLRP3, which binds the adaptor apoptosis-associated speck-like protein containing a CARD domain (ASC) to induce procaspase-1 recruitment, autoactivation and pro-IL-1β processing, responds to highly diverse stimuli, including ATP, bacterial toxins, microcrystalline substances, lipid particles, bacteria and viruses (13). It has also been identified as an important target for sepsis, asthma or chronic obstructive pulmonary disease (COPD) (14-16).

Silybin is the major flavonolignan from the extracts of milk thistle seed, Silybum marianum. The whole extract, known as silymarin, as well as silybin, has been found to protect the liver from both acute and chronic toxicity and injury (17-20). Previous studies have demonstrated that silybin inhibits various inflammatory responses by suppressing the NF-κB pathway. For example,
silybin has been shown to effectively suppress tumorigenesis by attenuating oxidative stress and deregulating the activation of inflammatory mediators (21). Silybin also inhibits the production of pro-inflammatory cytokines through the inhibition of the NF-κB signaling pathway in HMC-1 human mast cells (22). In addition, silybin has been shown to attenuate asthma by decreasing antigen-specific IgE production through the modulation of the Th1/Th2 balance in ovalbumin (OVA)-sensitized mice (23). However, the effects of silybin in LPS-induced ALI remain unclear. Thus, in this study, we aimed to assess the possible protective effects of silybin against LPS-induced lung injury and to elucidate the possible underlying mechanisms. The results from our in vivo and in vitro experiments suggest that silybin attenuates LPS-induced ALI through the inhibition of NF-κB signaling and NLPR3 inflammasome activation.

Materials and methods

Animals. Female C57/BL6 mice, 6-8 weeks of age, were purchased from Shanghai Laboratory Animal Centre at the Chinese Academy of Sciences, Shanghai, China. The mice were maintained in a temperature-controlled room (22±2°C) with a 12-h light/dark cycle and a relative humidity of 40-60%. The mice were given free access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tianjin Key Laboratory of Cerebral Vascular and Neurodegenerative Diseases (no. SKL20160021) and the animal protocol was designed to minimize the pain and discomfort of the animals.

Reagents and antibodies. Silybin and LPS (Escherichia coli: Serotype O55:B5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α and IL-1β were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Anti-actin (4970), anti-phosphorylated (p)-p65 (3033) and anti-p65 (8242) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-NLRP3 (ab4270) and anti-caspase-1 (ab179515) antibodies were purchased from Abcam (Burlingame, CA, USA). Anti-ASC (sc-514444) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-mouse CD3-FITC (11-0032), CD11b-PE (12-0112) and Gr1-APC (53-5931) antibodies were obtained from eBioscience (San Diego, CA, USA). Alexa Fluor 488 anti-mouse IgG (A-11001) was from Invitrogen, Thermo Fisher Scientific (Waltham, MA, USA). The DAB staining kit was from KeyGen (Nanjing, China). All other chemicals were obtained from Sigma-Aldrich.

Induction of ALI by LPS. The mice were randomly divided into 4 groups (n=8/group): the control group receiving saline, the model group receiving LPS only, and the 2 experimental groups receiving silybin followed by LPS. The animals inhaled 0.9% NaCl, or 1,000 µg/ml LPS for 1 h at 3 h intervals over a period of 8 h. The mice received silybin (50, 100 mg/kg), dissolved in the vehicle (0.5% carboxy methyl cellulose) via intragastric (i.g.) administration once per day for 3 consecutive days prior to LPS sensitization. We selected the dose of silybin according to the findings of previous studies in which the dose of silybin for OVA-sensitized mice ranged from 25-200 mg/kg (19,23,24). The mice were sacrificed at 6 h post-LPS administration under anaesthesia by an intraperitoneal injection of 30 mg/kg pentobarbital to collect bronchoalveolar lavage fluid (BALF), blood plasma and tissue samples. Serum was isolated from whole blood to assess cytokine levels. After the trachea was cannulated and the chest cavity was opened via a midline incision, the lung was lavaged 3 times with 1 ml of ice-cold sterile saline. The total BALF cell number was counted and the BALF composition was evaluated by FACS analysis as follows: BALF cells were resuspended with 100 µl PBS containing 1 µl CD3-FITC, 1 µl CD11b-PE and 1 µl Gr1-APC antibodies for 30 min on ice. The cells were then washed twice and subjected to FACS analysis. The remaining BALF was centrifuged at 1,000 x g for 5 min at 4°C, and the cell-free supernatant was stored at -80°C for ELISA.

Histological analysis. Lungs from 4 animals in each experimental group were fixed by 10% buffered formalin, embedded in paraffin, and then sectioned to reveal the maximum longitudinal view of the main intrapulmonary bronchus of the left lung lobe. Histopathological analysis was carried out using hematoxylin and eosin (H&E)-stained lung sections.

Cell culture. RAW264.7 mouse macrophages and THP-1 human monocyes were purchased from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences and cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (both from Gibco, Paisley, UK), 100 U/ml penicillin G, and 100 µg/ml streptomycin at 37°C with 5% CO₂. The RAW264.7 cells were treated with silybin in the absence or presence of 500 µg/ml LPS for 24 h and RNA and cell culture supernatant was collected for use in reverse transcription-quantitative PCR (RT-qPCR) and ELISA for the analysis of cytokine levels. For the determination of the phosphorylation levels of NF-κB and its nuclear translocation by western blot analysis and immunofluorescence staining, the RAW264.7 cells were treated with silybin (50 and 100 µM) for 3 h and stimulated with 500 µg/ml LPS for 30 min. For the analysis of NLRP3 inflammasome activation, the THP-1 cells were first induced to differentiate by 500 nM PMA for 3 h and then stimulated with 100 ng/ml LPS for 3 h, followed by treatment with various concentrations of silybin for 1 h, and subsequent incubation with 5 mM ATP for a further 1 h. The cells and cell culture supernatant were then collected for use in western blot analysis, immunoprecipitation and FACS analysis.

RT-qPCR. Total RNA from the lung tissue of each animal and the cells using TRIZol reagent (Takara, Dalian, China). RNA samples were reverse transcribed into cDNA and analyzed by qPCR, and the relative expression of specific genes was determined using the real-time PCR master mix (Roche, Bromma, Sweden) on an ABI 7500 Fast Real-Time PCR system. The program for amplification was 1 cycle of 95°C for 2 min followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec and 72°C for 30 sec. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control to normalize for differences in the amount of total RNA present in the samples. The primer sequences used in this study were as follows: IL-1β, 5′-CTTCAGGCAGGCAGTATCACTC-3′, 5′-CAGTGAGGAGGAGTATCACA-3′; TNF-α, 5′-GAGGGCTGCAGGTCACTGCTG-3′, 5′-GAATGCAGGCTTGGGTCGTC-3′; IL-6, 5′-ATGAGGACAGGGTGAAGGTC-3′, 5′-TGGTGAGCGTTTATTGGGAC-3′; and IL-10, 5′-GGTGGAGCAGACACCTGCT-3′, 5′-ACCACCTGTTGCCAAGATG-3′. All qPCR reactions were performed in a 25 µl reaction volume using the SYBR Green master mix (Thermo Fisher Scientific, Sweden).
Western blot analysis. Freshly isolated lung tissues were homogenized in the presence of protease inhibitors and protein content of the supernatant was determined by BCA protein assay kit (Pierce, Rockford, IL, USA). The protein sample (20 µg) from the lung homogenates was loaded per lane on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Electrophoresis was then performed. The proteins were then transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature. The blocked membranes were then incubated with the indicated primary antibodies overnight at 4°C, followed by incubation with HRP-coupled secondary antibody (7074, Cell Signaling Technology). The binding of all the antibodies was detected using an ECL detection system. For the extraction of nucleoprotein, the cells were collected and lysed in lysis buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 2% NP-40 and 1 mM PMSF) for 30 min. The homogenate was centrifuged for 10 min at 3,000 rpm at 4°C. The supernatant was collected as the nuclear fraction. The pellet was washed twice before being resuspended in 50 µl lysis buffer containing Triton X-100 and 50 or 100 mg/kg silybin (Fig. 1A). Next, the infiltration of inflammatory cells in BALF was examined. As shown in Fig. 1B, the number of total cells, macrophages (CD11b⁺) and neutrophils (Gr1⁻) in BALF was significantly increased following LPS stimulation, while treatment with silybin dose-dependently reduced the infiltration of inflammatory cells.

Immunohistochemical analysis. Immunohistochemical analysis was performed on paraffin-embedded colonic tissue sections (5-µm-thick). Briefly, the sections were deparaffinized, rehydrated and washed in 1% phosphate-buffered saline (PBS)-Tween-20, and they were then treated with 2% hydrogen peroxide, blocked with 3% goat serum and incubated overnight at 4°C with monoclonal rabbit anti-p-NF-κB antibody (1:200). The slides were then processed using the DAB staining kit from KeyGen according to the manufacturer's instructions. Images were obtained using an Olympus IX51 light microscope (Olympus, Tokyo, Japan). The settings for image acquisition were identical for the control and experimental tissues.

Immunofluorescence staining. The cells grown on cover glasses were fixed with 4% paraformaldehyde (20 min, room temperature), stained with the anti-NF-κB antibody (1:100), and detected with secondary antibody (Alexa Fluor 488 anti-mouse IgG; Invitrogen). The coverslips were counterstained with DAPI and imaged using a confocal laser scanning microscope (Olympus).

Co-immunoprecipitation assay. For co-immunoprecipitation, the cells were lysed in lysis buffer containing Triton X-100 and cell lysates were immunoprecipitated with antibody to ASC or control IgG with protein A/G-Sepharose (sc-2003; Santa Cruz Biotechnology, Inc.). The beads were washed, separated by SDS-PAGE and analyzed by western blot analysis with antibodies to caspase-1 and NLRP3. Protein bands were visualized using the western blotting detection system (Chemidoc™ MP Imaging System 17001402; Bio-Rad, Hercules, CA, USA).

Measurement of intracellular ROS levels. LPS-primed THP-1 cells were treated with various concentrations of silybin for 1 h, and then incubated with 5 mM ATP for 1 h. The cells were then harvested and incubated with 2',7'-dichlorofluorescein diacetate (DCFH-DA, Life Technologies, Carlsbad, CA, USA) at 37°C for 20 min and washed twice with cold PBS. DCF fluorescence level was detected by FACS.

Statistical analysis. The data are presented as the means ± SD. The significance of differences between experimental groups was assessed using the two-tailed Student's t-test. The value of statistical significance was set at p<0.05.

Results

Silybin attenuates disease progression and pathological changes in mice with LPS-induced lung injury. In the present study, we used a mouse model of LPS-induced ALI to evaluate the therapeutic effects of silybin. Mice were challenged with LPS and lung tissue samples were collected, and the sections stained with H&E as shown in Fig. 1A. The lung tissues from the LPS group exhibited significant pathological alterations, including notable inflammatory cell infiltration, interstitial and intra-alveolar edema, and patchy hemorrhage, inter-alveolar septal thickening, hyaline membrane formation and some collapsed alveoli. By contrast, a marked attenuation of these responses was observed in the lungs of mice treated with either 50 or 100 mg/kg silybin (Fig. 1A). Next, the infiltration of inflammatory cells in BALF was examined. As shown in Fig. 1B, the number of total cells, macrophages (CD11b⁺), T cells (CD3⁺) and neutrophils (Gr1⁺) in BALF was significantly increased following LPS stimulation, while treatment with silybin dose-dependently reduced the infiltration of inflammatory cells.

Silybin suppresses inflammatory cytokine levels in mice with LPS-induced lung injury. Next, the anti-inflammatory properties of silybin were evaluated by determining the mRNA and protein levels of inflammatory cytokines, including TNF-α and IL-1β. LPS stimulation significantly increased the levels of TNF-α and IL-1β in BALF, as well as the serum levels, whereas treatment with silybin significantly decreased these cytokine levels (Fig. 2A and B). In addition, LPS stimulation significantly elevated the mRNA levels of TNF-α and IL-1β in BALF, as well as the serum levels, whereas treatment with silybin significantly decreased these cytokine levels (Fig. 2A and B). In addition, LPS stimulation significantly elevated the mRNA levels of TNF-α and IL-1β in BALF, as well as the serum levels, whereas treatment with silybin significantly decreased these cytokine levels (Fig. 2C).
Silybin inhibits the secretion of inflammatory cytokines in vitro. To further investigate the protective effects of silybin against LPS-induced lung injury and the underlying mechanisms, we used RAW264.7 cells. LPS stimulation significantly elevated the mRNA and protein levels of TNF-α and IL-1β in the RAW264.7 cells (Fig. 4). Co-incubation with silybin and LPS dose-dependently decreased the mRNA expression levels of TNF-α and IL-1β (Fig. 4A), as well as the protein levels in the cell culture medium (Fig. 4B).

Silybin inhibits NF-κB activation in vitro. As observed in our mouse model, treatment with silybin inhibited the phosphorylation of NF-κB. Thus, we wished to determine whether it can also inhibit NF-κB signaling in vitro. Upon exposure to LPS, the levels of phosphorylated NF-κB (p65) were significantly increased. However, pre-treatment with silybin for 3 h dose-dependently suppressed the phosphorylation of NF-κB (Fig. 5A). Moreover, silybin decreased the nuclear translocation of NF-κB (p65) (Fig. 5B). The results of western blot
analysis of the cytoplasmic and nuclear content revealed that the level of NF-κB (p65) in the nucleus was reduced by silybin treatment (Fig. 5B). Using immunofluorescence staining, strong NF-κB (p65) staining in the nucleus was observed following stimulation with LPS, whereas in the silybin-treated cells, NF-κB (p65) was mainly located in the cytosol (Fig. 5C). These results suggest that the anti-inflammatory effects of silybin may result from the inhibition of NF-κB.

Silybin inhibits the activation of the NLRP3 inflammasome in THP-1 cells by reducing the production of intracellular ROS.

It is worth noting that there is evidence to indicate that IL-1β is processed as an inactive cytoplasmic precursor (pro-IL-1β and pro-IL-18) that requires cleavage by caspase-1 to produce the mature active forms (25,26). As caspase-1 needs to be activated by the NLRP3 inflammasome, we then evaluated the effects of silybin on caspase-1 activation in THP-1 cells. Treatment with silybin inhibited caspase-1 activation in a concentration-dependent manner (Fig. 6A). Furthermore, immunoprecipitation analysis revealed that the process of NLRP3 inflammasome formation was also interrupted by silybin (Fig. 6B). It has been reported that in the presence of signal I (NF-κB signaling), the
Figure 5. Silybin inhibits the phosphorylation of nuclear factor-κB (NF-κB) and its nuclear translocation. (A) RAW264.7 cells were treated with silybin for 3 h and stimulated with 500 µg/ml lipopolysaccharide (LPS) for 30 min. The levels of phosphorylated (p-)NF-κB (p65) were determined by western blot analysis. (B) Cytoplasmic and nuclear proteins were extracted and assayed by western blot analysis. The expression levels of actin and histone 3 were used as loading controls. (C) RAW264.7 cells were treated with silybin for 3 h and stimulated with LPS for 30 min. The localization of NF-κB (p65) was analyzed by immunofluorescence staining.

Figure 6. Silybin inhibits NLRP3 inflammasome activation in THP-1 cells. Lipopolysaccharide (LPS)-primed THP-1 cells were treated with various concentrations of silybin for 1 h, and then incubated with 5 mM ATP for 1 h. (A) Protein levels of pro-caspase-1, cleaved caspase-1, ASC and NLRP3 were determined by western blot analysis. (B) NLRP3 inflammasome complex assembly was analyzed by immunoprecipitation. (C) Measurement of intracellular ROS production by DCF fluorescence.
NLRP3 inflammasome is activated by ROS signaling which leads to the production of caspase-1 (27). Treatment with LPS and ATP led to ROS generation, whereas treatment with silybin significantly scavenged ROS (Fig. 6C). These results indicate that silybin may reduce ROS generation and interrupt the activation of the inflammasome in macrophages, thereby inhibiting the cleavage of caspase-1 and the production of cytokines.

Discussion

Despite the development of innovative therapy and intensive care, ALI induced by bacteria or microbe infection remains an unresolved issue. Silybin, the major active molecule of silymarin, is a very potent antioxidant compound capable of scavenging free radicals and ROS, and has been shown to exert neuroprotective (28-30), hepatoprotective (31,32) and anti-carcinogenic effects (33-35). It is now being used as a traditional medicine for the treatment of various liver disorders in China. In the current study, our data revealed that silybin inhibited the LPS-induced production of TNF-α and IL-1β, and the activation of NF-kB and the NLRP3 inflammasome, thus protecting mice against LPS-induced ALI.

ALI is a disorder of acute inflammation that causes the disruption of the lung endothelial and epithelial barriers. Following infection or trauma, the increased permeability of the alveolar-capillary barrier, and the excessive production of inflammatory mediators from inflammatory cells, particularly cytokines, chemokines and adhesion molecules occurs as a direct response and/or as a marker of ongoing cellular injury. These symptoms can be mimicked in experimental animals by LPS inhalation (36,37).

The treatment of ALI is based in both ventilatory and non-ventilatory strategies. To date, the most significant advances in the supportive care of patients with lung injury have been associated with improved ventilator management (38). The results of this study demonstrated that silybin induced a significant decrease in thickened intra-alveolar septa, and a decrease in the infiltration of prominent inflammatory cells (including alveolar macrophages, neutrophils and T cells) (Fig. 1). Overall, these results suggest that silybin has potential clinical applications for use in the treatment of ALI.

It is well known that NF-κB is important in terms of directing the transcription of many inflammatory genes following exposure to LPS, playing a crucial role in a number of inflammatory disease processes (12,39,40). In unstimulated cells, NF-κB is sequestered into the cytoplasm by IκBα. Upon stimulation, such as with LPS or Toll like receptor (TLR), signal transduction events rapidly lead to the degradation of IκBα, resulting in the nuclear translocation of NF-κB (41). NF-κB binds to specific DNA to initiate the transcription of inflammatory genes. Our results revealed that silybin suppressed the nuclear translocation of NF-κB in vivo and in vitro, which then inhibited the expression of pro-inflammatory cytokines, such as TNF-α and IL-1β. In this study, silybin was concluded to successfully attenuate acute inflammation by inhibiting the release of cytokines (such as TNF-α and IL-1β) in BALF and serum (Fig. 2). Additionally, the in vitro experimental results revealed that silybin inhibited the transcription and protein levels of TNF-α and IL-1β.

The production and secretion of pro-inflammatory cytokines is governed not only by TLR-NF-κB signaling, but also by the protein family containing a nucleotide-binding domain and a leucine-rich repeat motif (NLR) (42). The NLRP3 inflammasome is a cytosolic multiprotein complex and is increasingly being recognized due to its clinical importance in autoimmune, infectious and metabolic diseases (43,44). Previous studies have suggested that ROS derived from damaged mitochondria are the major factor that activate the NLRP3 inflammasome (45). A critical role for the NLRP3 inflammasome in the development of allergic airway inflammation in mice has also been described. NLRP3−/− ASC−/− and caspase-1−/− mice have been found to have significantly attenuated airway inflammation and cytokine release in airway inflammation (14). Our in vitro experiments suggested that silybin inhibited NLRP3/ASC/caspase-1 complex formation, thus suppressing the activation of caspase-1. Treatment with silybin also effectively prevented the LPS- and ATP-induced ROS generation. We hypothesized that the beneficial effects of silybin against LPS-induced lung inflammation may be attributed to its inhibition of the inflammasome activation.

Taken together, our data suggest that the anti-inflammatory effects of silybin attenuate LPS-induced cytokine production via the negative regulation NF-κB and NLRP3 inflammasome activation in mice with ALI.

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Zhang et al.: Silybin attenuates LPS-induced lung injury in mice

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