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Original Article

Salvianolic acid B attenuates lung ischemia-reperfusion injury in rat possibly by inhibiting the P2X7/NLRP3 signaling pathway

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Abstract: Lung ischemia-reperfusion injury (LIRI) is a morbid complication that frequently occurs following lung transplantation, pulmonary artery resection, pulmonary embolism thrombolysis, cardiac surgery, and cardiopulmonary resuscitation. LIRI has a high incidence and risk of death; however, current therapies have limited efficacy for treating LIRI. Salvianolic acid B (Sal B) is a natural active product extracted from *Salvia miltiorrhiza* Bge*,* and protects cardiac myocytes as well as neurons from ischemia-reperfusion injury. However, protective effects of Sal B against LIRI have not yet been reported. In this work, we demonstrated that Sal B attenuated lung injury induced by LIRI in rat in a dose-dependent manner as well as reduced the lung W/D ratio and MPO expression in lung tissues. In addition, Sal B inhibited expression of P2X7, NLRP3, ASC and caspase-1 in lung tissue and decreased the concentration of IL-1β and IL-18 in serum. We propose that Sal B improves LIRI in rats by inhibiting the P2X7/NLRP3 signaling pathway, suggesting that Sal B is an emerging and efficient natural product candidate for the treatment of LIRI.

Keywords: Salvianolic acid B, lung ischemia-reperfusion injury, P2X7, NLRP3 inflammasome

Introduction

Lung tissue is extremely susceptible to injury. Lung ischemia-reperfusion injury (LIRI) is a leading cause of death after pulmonary artery resection, pulmonary embolism thrombolysis, cardiopulmonary bypass cardiac surgery, and cardiopulmonary resuscitation, especially following lung transplantation [1]. In the case of lung transplantation, the incidence of LIRI is as high as 25%, and the occurrence of acute and chronic cellular rejection or obliterative bronchiolitis (OB) is associated with the extent of LIRI [2]. LIRI clinically manifests through pulmonary interstitial edema, pulmonary hypertension and gas exchange disorders, and severe cases manifest with development of acute lung injury (ALI) and/or acute respiratory distress syndrome (ARDS) [3]. ALI occurs in the initial stage of ischemia, but is exacerbated during reperfusion, which can induce a systemic inflammatory response and multiple organ dysfunctions [4]. LIRI has a complex pathogenesis, involving either intracellular injury processes or injurious inflammatory responses. Studies have shown that inhibition of inflammatory responses can significantly reduce LIRI, however, the underlying mechanism of the inflammatory response induced by LIRI is not yet fully understood. Thus, understanding the underlying mechanism of LIRI is necessary for the development of novel drugs to manage this disease.

Recent studies have described that a new type of innate immunity involving the purinergic receptor P2X, ligand-gated ion channel, 7 (P2X7) and NOD-like receptor protein 3 (NLRP3) inflammasome plays an important role in renal, brain, liver and myocardial ischemia-reperfusion injury [5-7]. P2X7 is one of the most important ATP receptor subtypes currently known, and is mainly expressed in immune cells and epithelial cells, where it acts as an immune regulatory receptor that is considered to be a new therapeutic target of ischemia-reperfusion injury [8]. Cumulative evidence has shown that the P2X7 signaling pathway couples with pathways that generate interleukin (IL)-1β, IL-18 and other inflammatory factors, and is closely related to inflammation-related diseases [9]. Inhibition of

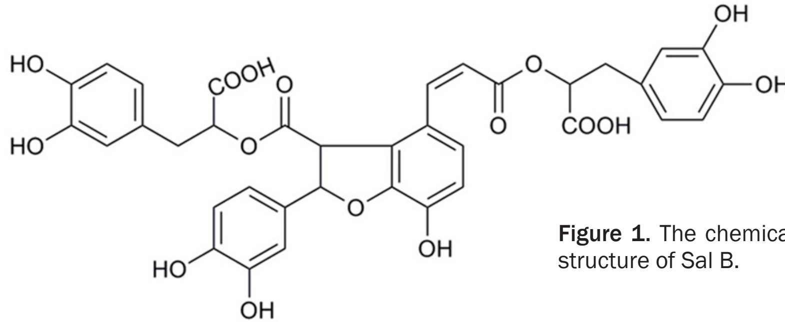

Figure 1. The chemical structure of Sal B.

P2X7 has been reported to regulate the inflammatory response, and to improve heart and cerebral ischemia/reperfusion injury in experimental models [5, 6]. NLRP3 is not only an important pattern recognition receptor in cytoplasm, but also the core component of an inflammasome. NLRP3 recognizes pathogen-associated molecular patterns (PAMPs) and endogenous risk-related molecular patterns (DAMPs), such as ATP and reactive oxygen species (ROS) [10]. After activation, NLRP3 recruits the downstream adapter protein, ASC (apoptosis-related spots-like protein containing CARD), and the effector protein caspase-1 to assemble into the NLRP3 inflammasome complex, which promotes the cleavage of caspase1 precursors into active caspase1. Activated-caspase1 contributes to the cleavage and maturation of IL-18 and IL-1β precursors, as well as release of mature IL-18 and IL-1β. Mature IL-1β binds to IL-1β receptors in peripheral tissues, regulating the modification and activation of proinflammatory cytokines, such as IL-8, tumor necrosis factor (TNF) and IL-17, and triggering inflammatory cascades [11]. Previous studies have demonstrated that blocking the P2X7/NLRP3 pathway can relieve lipopolysaccharide-induced acute lung injury in mice [9]. However, the presence of P2X7 and the NLRP3 inflammasome in LIRI remains still unknown.

A large number of active natural products, such as astragaloside from *Leguminosae [12]*, resveratrol from red grapes [13], and chrysophanol from *Rheumpalmatum* L. [14], confer resistance to ischemia-perfusion injury. Salmonic acid B (Sal B, **Figure 1**), is a natural phenolic acid active product extracted from the Chinese herb *Salvia miltiorrhiza* Bge. The anti-oxidation, anti-inflammatory and anti-tumor activities of Sal B have been confirmed by pharmacological studies over the past decades [15-17]. Sal B ameliorates heart and cerebral ischemia-reperfusion injury, as reported in previous studies. However, there are no reports about the therapeutic effects of Sal B for LIRI. In this study, we explore the protective effect and mechanism of Sal B on LIRI via investigating the dynamic expression of P2X7 and NALP3 inflammasome in the lung tissue of LIRI rat model. The results suggest that Sal B is an emerging and efficient natural product candidate for the treatment of LIRI.

Materials and methods

Reagents

Sal B (purity > 99%) was purchased from the China Materials Research Center (Beijing, China). MPO assay kit was obtained from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The tissue protein extraction kit and BCA protein assay kit were provided by Beyotime Biotechnology Research Institute (Shanghai, China). Rabbit anti-NLRP3 and P2X7 antibodies, and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Proteintech Group, Inc. (Chicago, USA). Rabbit anti-ASC and caspase-1 antibodies were purchased from Abcam (Cambridge, UK). IL-1β and IL-18 enzyme-linked immunosorbent assay kits were obtained from Langdun Biotech (Shanghai, China). RNAiso Plus reagent, PrimeScript®RT reagent and SYBR® PremixEx Taq™II were purchased from Takara Biotechnology Co., Ltd. (Dalian, China).

Animals

Healthy adult male Sprague-Dawley (SD) rats (SPF grade) weighing 200 ± 20 g were purchased from the Experimental Animal Center of North China University of Science and Technology. Rats were fed for one week with a 12-hour light-dark cycle (temperature 22 ± 3°C, humidity 50%), and free access to standard laboratory feed and water. The animal experiments were approved by the ethical committee for Laboratory Animal Care and Use of North China University of Science and Technology. All the experimental processes are in accordance with the National Institutes of Health Criteria.

Table 1. Primer sequences used for the real-time PCR assay

Gene	GenBank accession	Primers (5'-3')
IL-1β	NM_031512.2	Forward: CCCTGAACTCAACTGTGAAATAGCA
		Reverse: CCCAAGTCAAGGGCTTGGAA
IL-18	NM_ 019165.1	Forward: CGGAGCATAAATGACCAAGTTCTC
		Reverse: TGGGATTCGTTGGCTGTTC
GAPDH	NM_017008.3	Forward: GGCACAGTCAAGGCTGAGAATG
		Reverse: ATGGTGGTGAAGACGCCAGTA

Forty SD rats were randomly divided into four groups (n=10 for each group): Group I, sham operation group (SO); Group II, LIRI group (LIRI), Groups III-IV, Sal B pre-treated groups, in which the rats were administered Sal B at the doses of 60 and 30 mg/kg/d, respectively. Both the SO and LIRI groups were administered normal saline at equivalent volumes. After 7 days of continuous administration, the LIRI model was carried out as previously reported. Briefly, rats were fasted for 12 hours, and then anesthetized with 3% sodium pentobarbital (60 mg/kg body weight) intraperitoneal injection. For mechanical ventilation, the trachea of the rat was slit and intubated. Parameters were set with a respiratory rate of 60-70 times/min, a suction ratio of 1:1.5 and an inhalation oxygen concentration of 1.0. Rats were injected with heparin sodium (250 U/kg) through the right jugular vein, after which the left hilum was exposed by an anterior lateral incision located at the 5th intercostal space of the left thoracic, and then was closed by vascular clip for 30 min. After blocking blood flow, lung tissues begin to shrink and change into a dark purple, the rapid expansion of the lungs and color recovery into light red were observed along with the vascular clip removal, suggesting that the model was established successfully. Samples were obtained after 120 min of reperfusion. The SO group underwent exposure of the left hilar, but without clipping treatment after thoracotomy. Blood samples were obtained by abdominal aorta for biochemical analyses. A portion of the left lungs were fixed in 10% buffered formalin for histopathological examination. The others were immediately frozen and maintained at -80°C for western blotting analyses and quantitative real-time PCR assay.

Histopathological examination

Formalin-fixed lung tissue samples obtained from each group were sliced, dewaxed and stained with hematoxylin/eosin (H&E). Images were collected using a light microscope (Leica DM4000B, Germany) with 200× magnification.

Lung W/D weight ratio

The same portions of fresh left lung tissues were obtained, and the surface moisture was dried by a filter paper. Lung tissue wet weight was measure with an electronic saidscale, and tissue was then dehydrated in a roaster at 80°C for 6 h and dry weight was measured. The ratio of the two weights served as the W/D weight ratio.

MPO activity determination

Lung tissue weighing 50 mg was taken to prepare tissue homogenate, and centrifuged for 4 min at 20000 rpm and 4°C. The supernatant was collected into 96 well plates, (7 μl per well), MPO activity was detected according to the MPO assay kit manufacturer's instructions. Absorbance changes were detected at 470 nm within 1 min, and the MPO activity was calculated as the change of absorbance value units per gram of lung tissue over 1 min, expressed as U/g.

Western blotting assay

The expression levels of P2X7, NLRP3, ASC and caspase-1 in lung tissue were observed by western blotting assay. Total protein was extracted from the lung tissues using a protein extraction kit, and the protein concentration was determined using a BCA protein assay kit. Samples were separated by SDS-PAGE (10-15%) and transferred onto a PVDF membrane (Millipore, USA). Membranes were blocked in 5% skim milk for 30 min and incubated overnight at 4°C with anti-P2X7, NLRP3, ASC and caspase-1 antibodies. The membranes were then washed and incubated with secondary antibody at room temperature. After being washed, proteins were exposed using an enhanced chemiluminescence (ECL) method. Protein bands were imaged using a ChemiDoc XRS bioimaging system (Bio-Rad, USA) and normalized with GAPDH as an internal control.

Quantitative real-time PCR assay

According to the manufacturer's protocol, RNA samples of rats were extracted using the RNAiso Plus reagent. Then, reverse transcripttion polymerase chain reaction (RT-PCR) was per-

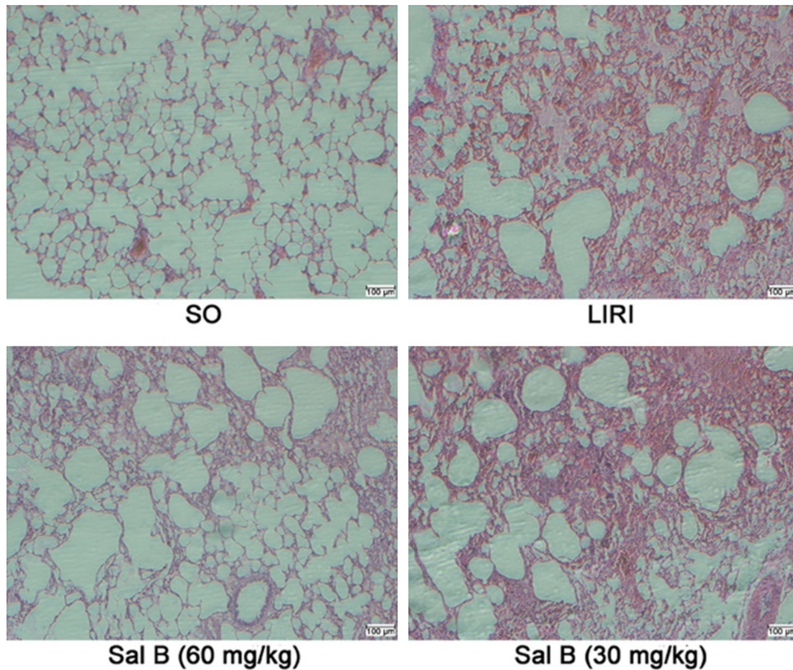

Figure 2. Sal B alleviated lung injury in LIRI rats. Representative images of H&E staining of lung tissues in the SO group, LIRI group, high-dose Sal B-pretreated (60 mg/kg) group, and low-dose Sal B-pretreated (30 mg/kg) group. Images are presented at a 200× magnification.

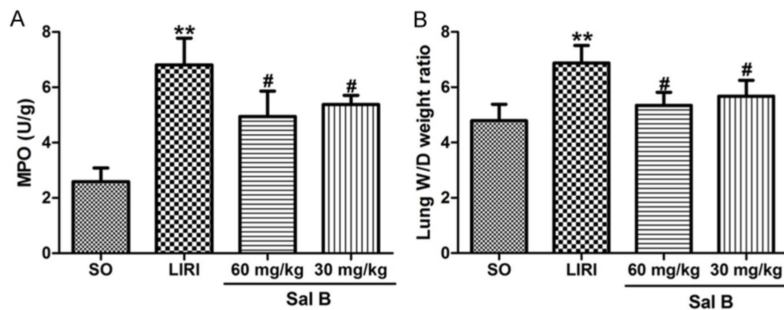

Figure 3. Sal B inhibited MPO activity (A) and decreased the lung W/D weight ratio (B) in LIRI rats. The data are presented as the mean ± S.D., $^{**}p < 0.01$ vs. SO; $^{\#}p < 0.05$ vs. LIRI.

formed with the PrimeScript® RT reagent Kit and TC-512 PCR system (TECHNE, UK) after determining RNA purity. Eventually, mRNA levels were quantified through real-time PCR with SYBR® PremixEx Taq™II (Tli RNaseH Plus) and ABI 7500 Real Time PCR System (Applied Biosystems, USA). The sequences of the primers are listed in **Table 1**. GAPDH gene was selected as the house-keeping gene, and a no-template control was analyzed in parallel for each gene.

Plasma IL-1β and IL-18 assays

Plasma IL-1β and IL-18 levels were detected by enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. The absorbance in each group was measured at 450 nm by Thermo Scientific Multiskan FC (Massachusetts, USA). The concentration of plasma IL-1β and IL-18 were calculated with reference to a standard curve.

Statistical analysis

Statistical analysis was executed with SPSS 18.0 statistical software. All data are expressed as the mean ± standard deviation (S.D.) from three or more independent experiments, and the differences between multiple groups were compared with one-way ANOVA/Kruskal-Wallis tests followed by post-hoc Tukey's/Dunn's (according to homogeneity of variances). Post hoc tests were performed when ANOVAs indicated that a significant difference existed between groups. $P < 0.05$ or < 0.01 indicates that the difference was statistically significant.

Results

Sal B improved pathological damage of the lung tissue in LIRI rats

Typical HE staining results of lung tissue are shown in **Figure 2**. The structure of lung tissues in the SO group is complete, and no obvious pathological changes were observed. Obvious pulmonary interstitial edema, serious capillary congestion and alveolar wall fracture and structure disorder were observed in the LIRI group, compared with the SO group. Moreover, epithelial cell degeneration and necrosis, and a large number of inflammatory cells infiltrating into alveolar, pulmonary interstitial and capillary regions also occurred in the LIRI group. After pretreatment with Sal B (60 and 30 mg/kg/d), the pathological changes of the lung tissue in LIRI rats were significantly improved, and the protective effe-

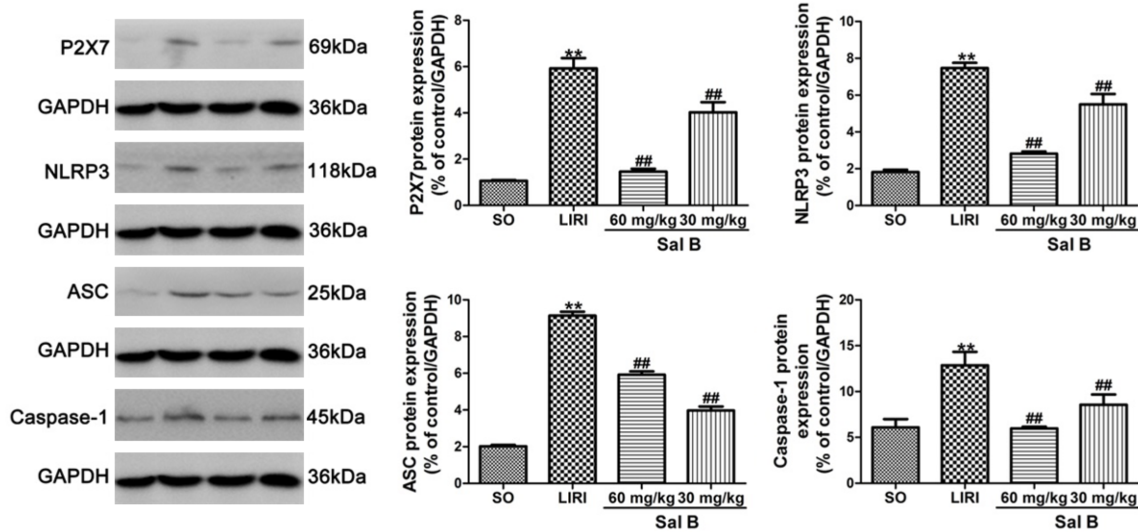

Figure 4. Sal B down-regulated expression of P2X7, NLRP3, ASC and caspase-1 in lung tissue of rats with LIRI (n=3). The data are presented as the mean ± S.D., $^{**}p < 0.01$ vs. SO; $^{\#\#}p < 0.01$ vs. LIRI.

cts of high-dose Sal B (60 mg/kg/d) pretreatment were the most significant.

Sal B inhibited MPO activity in the lung tissue of LIRI rats

Pulmonary MPO activity can indirectly reflect the degree of neutrophil infiltration in the lung tissue, that is, the severity of lung injury and inflammation. Compared with the SO group, MPO activity in the lung tissue in the LIRI group was significantly increased (P < 0.01), and the MPO activity in rats pretreated with Sal B was decreased in a dose-dependent manner (**Figure 3A**), indicating that Sal B can reduce the amount of neutrophils infiltrating into the lung tissue, thereby reducing the degree of lung injury.

Sal B down-regulated lung W/D weight ratio in LIRI rats

As shown in **Figure 3B**, the lung W/D weight ratio (6.87 ± 0.52) was significantly increased in the LIRI group compared with the SO group (4.79 ± 0.48, P < 0.05), whereas Sal B pretreatment groups (60 and 30 mg/kg/d) could decrease this ratio in a dose dependent manner, and the differences were statistically significant (p < 0.05) compared with the LIRI group. The lung W/D weight ratios in high- and low-dose Sal B pretreatment groups were 5.35 ± 0.47 and 5.68 ± 0.58, respectively.

Sal B decreased the expression of proteins related to the P2X7/NLRP3 pathway

As shown in **Figure 4**, the protein expression levels of P2X7, NLRP3, ASC and caspase-1 in lung tissue in the LIRI group were significantly higher than that of the SO group (P < 0.01), and Sal B pretreatment significantly down-regulated the expression of the above proteins, especially in the high-dose Sal B pretreatment group. In addition, as shown in **Figure 5**, the mRNA expression levels of IL-1β and IL-18 in the LIRI group were markedly increased compared with the SO group (P < 0.01), however, Sal B also could notably decrease the IL-1β and IL-18 mRNA. Therefore, the results showed the inhibitory activity of Sal B towards the P2X7/NLRP3 signaling pathway.

Sal B lowered plasma IL-1β and IL-18 levels in LIRI rats

In the current study, enzyme-linked immunosorbent assay kits were used to detect the levels of IL-1β and IL-18 in the plasma of rat models. The results from our study showed that the plasma levels of IL-1β and IL-18 in the LIRI group were significantly higher than those in the SO group (p < 0.05). The changes in the plasma IL-1β and IL-18 levels could be obviously reduced by Sal B with different doses (60 and 30 mg/kg), but were still higher than normal levels (**Figure 6**), indicating that Sal B can sup-

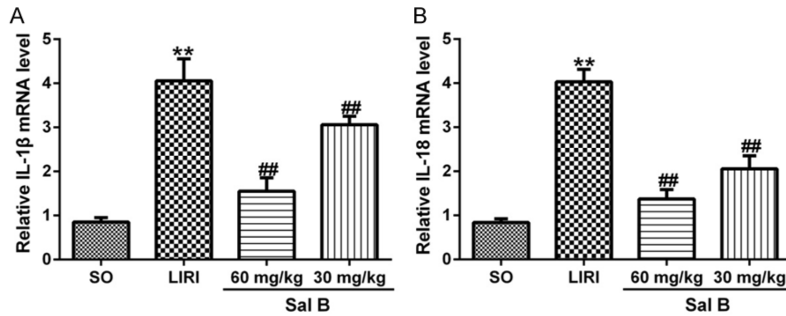

Figure 5. Sal B reduced the relative mRNA levels of IL-1β (A) and IL-18 (B). The data are presented as the mean ± S. D., $^{**}p < 0.01$ vs. SO; $^{\#\#}p < 0.01$ vs. LIRI.

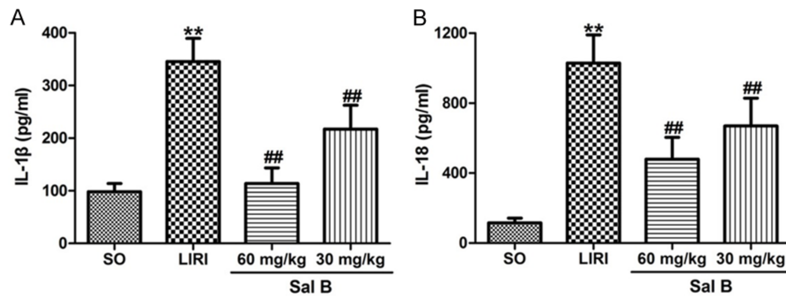

Figure 6. Sal B reduced the plasma concentrations of IL-1β (A) and IL-18 (B). The data are presented as the mean ± S. D., $^{**}p < 0.01$ vs. SO; $^{\#\#}p < 0.01$ vs. LIRI.

press inflammatory cytokines produced by a series of mechanisms in LIRI rats.

Discussion

Like the two sides of a coin, LIRI not only maintains lung function but also triggers a complex chain reaction [1]. Considerable advancements in lung transplantation during recent years have led to use of this last-resort treatment option for more patients with various end-stage lung diseases. However, despite improvements in techniques, transplanted lungs remain extremely susceptible to ischemia-reperfusion injury. Sal B is the most critical and abundant bioactive component of the traditional Chinese herb Salvia miltiorrhiza. In this research, a rat model of LIRI was used to demonstrate the effect of Sal B on LIRI. The results showed that Sal B could effectively improve the pathological injury of the lung tissue in LIRI rats in a dose-dependent manner, and reduce the lung W/D weight ratio, suggesting that Sal B has a protective effect in a rat model of LIRI.

Inflammation in response to infection is critical for pathogen eradication and tissue repair. Recent studies have demonstrated that neutrophils play an important role in the pathophysiology of lung reperfusion injury, and MPO level was determined to be an indicator of neutrophil sequestration in the lung tissue after LIRI. In this work, we found that Sal B could inhibit MPO activity in the lung tissue of LIRI rats, thereby reducing the number of neutrophils infiltrating into the lung tissue.

The innate immune system-induced post-ischemic inflammatory response is thought to play a crucial role in the progress of LIRI [18]. Previous studies have suggested that the immune system can identify pathogens through pattern recognition receptors and consecutively initiate inflammatory responses [19]. Recent studies point to a non-pattern recognition receptor, the purine receptor, the activation of which can trigger inflammation signal transduction. Activated P2X7 stimulates multiple signal pathways, including ROS, MAPK pathway, and the production, activation and metastasis of transcription factors, to result in the release of a large number of inflammatory mediators [6]. Studies have shown that P2X7 also participates in the activation of the NLRP3 inflammasome [20]. NLRP3 is a member of the NOD-like receptor family, and is a cytoplasmic recognition receptor, which can be involved in regulation of the maturation and secretion of inflammatory factors such as IL-1β and IL-18, resulting in an inflammatory response cascade [21]. As a core protein, NLRP3 can recognize intrinsic hazards and external microbial stress signals, and plays an important role in innate immune responses. Activated NLRP3 undergoes self-oligomerization, and recruits the downstream effector protein ASC and caspase-1 to form the NLRP3 inflammasome, which cleaves the inactive precursor of caspase-1 into active caspase-1, further promoting IL-18 and IL-1β maturation and release [21]. Mature IL-18 and IL-1β are important inflammatory factors involved in a variety of inflammatory response pro-

cesses [22, 23]. To investigate the molecular mechanism by which Sal B attenuates LIRI *in vivo*, we detected the protein expression of P2X7, NLRP3, ASC and caspase-1 in rat lung tissue. The results showed that these four proteins were significantly enhanced in the LIRI group compared with the SO group. The expression of P2X7, NLRP3, ASC and caspase-1 decreased after pretreatment with different doses of Sal B (60 and 30 mg/kg) in a dose-dependent manner. Therefore, we believe that Sal B may play a protective role against LIRI by inhibiting the P2X7/NLRP3 pathway.

IL-1β and IL-18 are two of the most frequently studied preinflammatory factors in models of acute inflammatory injury regulated by the NLRP3 inflammasome. IL-1β has been studied in isolation and in combination in multiple models of ischemia-reperfusion injury in a variety of vascular beds. Functional roles for IL-1β have been identified in liver, heart, brain, kidney, hind limb, and gut ischemia and reperfusion. It is also closely related to the severity of LIRI, and investigations of LIRI have demonstrated that protection is afforded by IL-1 receptor blockade [24]. IL-18 is a novel proinflammatory factor that has a structure and function similar to that of IL-1β. It can induce the synthesis and secretion of IL-1, TNF-α, and other chemokines [25]. In this study, we analyzed the plasma concentrations and relative mRNA levels of IL-1β and IL-18 after pretreatment with Sal B in LIRI rats. The concentrations and relative mRNA levels of IL-1β and IL-18 in the LIRI group were significantly higher than those of the SO group, and pretreatment with Sal B reduced the levels of IL-1β and IL-18 in a dose-dependent manner. The results confirmed that Sal B down-regulated IL-1β and IL-18 protein and mRNA expression via inhibition of the NLRP3 inflammasome activation and function.

This study shows that the immunoregulatory effect of Sal B on LIRI rats may be to reduce the expression of IL-1β and IL-18 by inhibiting P2X7 and the NLRP3 inflammasome, thereby delaying the progression of this disease and improving the systemic inflammatory status. Therefore, Sal B is expected to be an emerging and efficient natural product candidate for the control of LIRI. However, due to the extremely complex mechanisms of LIRI, it is necessary to further elucidate the protective mechanism of Sal B for clinical applications to lung tissue damage induced by ischemia-reperfusion.

Disclosure of conflict of interest

None.

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