Salvianolic acid B inhibits myofibroblast transdifferentiation in experimental pulmonary fibrosis via the up-regulation of Nrf2

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ABSTRACT
Salvianolic acid B (SaB) is one of the most bioactive components extracted from Salvia miltiorrhiza, and its antioxidant capacity corresponds with its protective effects against cell injury from oxidative stress. The aim of the present study was to evaluate the effect of SaB on experimental pulmonary fibrosis and its ability to ameliorate the oxidative/antioxidative imbalance during fibrosis pathogenesis. The anti-fibrotic activity of SaB was first confirmed in Transforming growth factor β1(TGF-β1)-stimulated MRC-5 cells. The protection of SaB against oxidative stress during fibrogenesis in vitro was verified by detecting ROS production, the levels of glutathione (GSH) and malondialdehyde (MDA). The Western blot and PCR results indicated that SaB could up-regulate nuclear factor erythroid-derived 2-like 2 (Nrf2) at both the protein and mRNA levels and induce Nrf2 nuclear translocation in vitro, which may be the mechanism underlying the anti-fibrotic capacity of SaB. Furthermore, the anti-fibrotic and antioxidant capacities of SaB in vivo were confirmed in rats with BLM-induced pulmonary fibrosis. The immunohistochemistry results showed that Nrf2 was absent in fibroblast transdifferentiation in highly contractile, synthetic alpha-smooth muscle actin (α-SMA)-positive myofibroblasts, which are considered the key effector cells in pulmonary fibrosis [2,3]. Transforming growth factor β (TGF-β) has been reported to have profound effects on epithelial cells and fibroblasts by promoting EMT, collagen synthesis and fibroblast proliferation and transformation into myofibroblasts. Therefore, TGF-β-induced epithelial cells or fibroblasts are commonly used as an experimental fibrosis model in vivo. It has also been reported that the pathogenesis of IPF is associated with oxidative stress and antioxidant imbalance in the lung. As it is directly involved in respiration, it is not surprising that the lungs are exposed to higher oxygen tensions than most other organs and is the primary organ that comes into contact with inhaled oxidants. Inflammatory cells are activated to produce many reactive oxygen species (ROS), intensifying the local oxidative stress in the lung and leading to the activation not only of defense mechanisms but also of growth factors, which results in fibrogenesis [1,4]. This oxidant challenge to the lungs is normally neutralized by the antioxidants in the lung tissue. The complex antioxidant network is disturbed in IPF, which means that the antioxidant levels in the lung are inadequate to neutralize the inhaled oxidants or free radicals. The levels of some antioxidants,
such as glutathione (GSH), are decreased, while the levels of some oxidant markers, such as malondialdehyde (MDA), are increased. Therefore, the regulation of oxidative stress may be an effective approach for pulmonary fibrosis therapy.

Recently, much attention has been focused on natural antioxidants for the treatment of cancer, neurological disorders, cardiovascular diseases and inflammation. These compounds may have a therapeutic effect on IPP as well. Salvinanolic acid B (SalB) is one of the most bioactive component extracted from **Salvia miltiorrhiza**, which shows higher free radical scavenging activity than vitamin C, and its antioxidant capacity corresponds with its protective effects against cell injury from oxidative stresses [5]. In our previous study, SalB was screened to have a noticeably preventive effect on bleomycin (BLM)-induced pulmonary fibrosis in Wistar rats by characterizing the biophysical properties of TGF-β1-induced A549 cells [6]. In this study, we focused on the effect of SalB on myofibroblast transdifferentiation in TGF-β1-induced MRC-5 cells, the ability of SalB to protect against oxidative stress, and the possible mechanism of this protection. Wistar rats with BLM-induced pulmonary fibrosis were used to verify the protection in vivo.

2. Materials and methods

2.1. Cell culture and treatment

The MRC-5 human embryonic lung fibroblast line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in MEM (HyClone, Logan, UT) containing 10% fetal bovine serum (HyClone), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO) in an incubator at 37 °C with 5% CO₂. The medium was replaced every 2 days, and the cells were passaged every 4 days.

The cells were rendered quiescent by incubating in serum-free medium for 24 h and then treated with 10 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN), 40 μM SalB with a purity of >98% (Shanghai Winherb Medical Technology Co., Ltd, China), or both TGF-β1 and SalB for another 48 h. MRC-5 cells simulated with PBS were used as a control.

2.2. Immunofluorescence cell staining

Cells were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min and then incubated with 1% BSA (Sigma) in PBS at 37 °C to block nonspecific sites for 1 h. For staining, the cells were incubated with the following rabbit polyclonal primary antibodies overnight at 4 °C: α-SMA (dilution 1:200), vimentin (dilution 1:500) and fibronectin (dilution 1:500) (Proteintech Group, Chicago, IL). The cells were then incubated with a tetramethylrhodamine (TRITC)-labeled goat anti-rabbit IgG antibody (Proteintech Group) and Hoechst 33258 (Sigma) at room temperature for 1 h. Fluorescent images were taken using an Olympus fluorescence microscope.

2.3. Western blot analysis

The MRC-5 cell lysates and lung tissue lysates were denatured and electrophoresed on SDS-PAGE gels. The separated proteins were transferred onto Hybond-P PVDF membranes (Millipore, Bedford, MA). The membranes were incubated with 1% BSA (Sigma) diluted in TBS for 2 h at room temperature and then incubated with primary antibodies against α-SMA (dilution 1:1000), vimentin (dilution 1:1000), fibronectin (dilution 1:1000), nuclear factor erythroid-derived 2-like 2 (Nrf2) (dilution 1:1000, Proteintech Group) and GAPDH (dilution 1:2000, Hangzhou Goodhere Biotechnology Co., Ltd, China) or lamin B1 (dilution 1:1000, Proteintech Group) overnight at 4 °C. The membranes were washed with 0.1% Tween-20 diluted in TBS (0.1% TBST) three times for 10 min. The primary antibodies were conjugated with an HRP-labeled secondary IgG antibody (Santa Cruz Biotechnology (Shanghai) Co., Ltd, China) and visualized using an ECL-Plus detection kit (Beyotime Institute of Biotechnology, China). The results were normalized to GAPDH or lamin B1.

2.4. ROS detection assay

ROS were detected by the cell-permeable fluorescent probe dihydrodihorhodamine-123 (DHR-123) (Sigma). MRC-5 cells were co-incubated with 10 ng/L DHR-123 for 1 h at 37 °C in the dark. Then, the cells were rinsed, and the signal was detected by a fluorescence microscope and flow cytometer.

2.5. GSH and MDA assay

The GSH and MDA contents in MRC-5 cells and the lung tissues of rats were determined using a reduced glutathione assay kit (Nanjing Jiancheng Bioengineering Institute, China) and an MDA assay kit (TBA method) (Nanjing Jiancheng Bioengineering Institute, China). The MRC-5 cell lysates and lung tissue lysates were denatured and treated according to the manufacturer’s instructions.

2.6. Polymerase chain reaction (PCR)

Total RNA was isolated from MRC-5 cells using 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, cDNA was synthesized from the total RNA from each sample using a Superscript pre-amplification system (Invitrogen) according to the manufacturer’s instructions. The PCR amplification of the cDNA products was performed on an AC1244 PCR instrument (Bio-Rad Laboratories, Hercules, CA, USA). The mRNA levels were normalized using GAPDH as an internal control. The PCR amplifications were performed as follows: 94 °C for 5 min (hot start) and 30 cycles of denaturation for Nrf2 or 18 cycles for GAPDH at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 60 s, with a final step at 72 °C for 3 min. The primer sequences used to amplify the human genes are as follows: Nrf2: 5'-TCACCGACGAGGAAGGATGATA-3' and 3'-CCACTGTTTTCTGACTGATGT-5'; GAPDH: 5'-GGACGCGATCCTCCTCAAAAT-3' and 3'-GGCTGGTCATATCTCTCATGGG-5'. The PCR products were electrophoresed on 1% agarose gels and visualized after staining with 0.05 mg/ml ethidium bromide.

2.7. Animals and model establishment

SPF Wistar rats (male, 180–200 g, 6–7 weeks old) were provided by the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). All the rats were fed standard rat chow with food and water ad libitum and kept in a temperature-controlled (20–22 °C) animal room with an alternating cycle of 12 h light and 12 h dark. All experimental procedures were carried out according to the **Guideline for Animal Experimentation of Shenyang Pharmaceutical University**, and the protocol was reviewed and approved by the Animal Ethics Committee of the institution.

The rats were randomly divided into three groups (n = 6) as follows: control, BLM and SalB. Bleomycin sulfate (0.2 ml, 5 mg/kg, diluted in saline solution, Japanese Medicine Co., Ltd, Japan) or an equal volume of saline as a control were slowly instilled intratracheally into the rats. The rats in the SalB group were injected intravenously with SalB at 20 mg/kg/d from the 14th day to the...
28th day after bleomycin instillation. The dose of SalB used in this study was based on our previous study. All the rats were euthanized on the 28th day. The lung tissues were either fixed in 4% polymethylaldehyde for histochemical and immunohistochemical analysis or stored in liquid nitrogen for further analysis.

2.8. Histological examination of lung tissues

The lungs from each group fixed in 4% polymethylaldehyde for 24 h were dehydrated in ethyl alcohol and then embedded in paraffin according to a routine process. 5-μm-thick sections were used for hematoxylin and eosin (H&E) staining to evaluate the histopathological changes and Masson’s trichrome staining to identify the density of accumulated collagen fibers. Microscopic images were acquired with the 10× and 20× objectives of an Olympus fluorescence microscope.

2.9. Immunohistochemistry of lung tissues

5-μm-thick sections were treated with 0.3% H2O2 for 30 min to quench the endogenous peroxidase activity and then incubated with 1% BSA and 0.1% Tween-20 in PBS at 37 °C for 1 h to eliminate nonspecific binding. Following blocking, the sections were rinsed and incubated overnight at 4 °C with rabbit polyclonal IgG primary antibodies against α-SMA or Nrf2 at a 1:100 dilution and then incubated with a horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody for 1 h at room temperature. The antigen–antibody complexes were visualized by incubation with diaminobenzidine (DAB) substrate and counterstained with diluted hematoxylin. Images were taken by an Olympus fluorescence microscope.

2.10. Statistical analysis

All experiments were performed at least in triplicate. The results were expressed as the mean ± standard deviation (SD). The difference between multiple groups was assessed by a one-way analysis of variance (ANOVA) followed by Scheffé’s multiple range tests. All statistical analyses were performed using SPSS 19.0, and \( P < 0.05 \) was considered statistically significant in all cases.

3. Results

3.1. SalB inhibited TGF-β1-induced myofibroblast transdifferentiation in MRC-5 cells

In this study, MRC-5 human lung fibroblasts were treated with TGF-β1 in the presence or absence of SalB to evaluate the inhibitory effects of SalB on myofibroblast differentiation in vitro. α-SMA, vimentin and fibronectin were detected as biomarkers. The results of cell immunofluorescence staining assay (Fig. 1A) and Western blot analysis (Fig. 1B and C) demonstrated that these proteins were widely distributed in the cytoplasm of TGF-β1-stimulated cells, while SalB coinubation significantly decreased the expression of these marker proteins when compared with the TGF-β1 group. The cells treated with SalB alone exhibited an expression pattern similar to that of the control cells. It suggested that SalB could inhibit the myofibroblast transdifferentiation induced by TGF-β1 in MRC-5 cells.

3.2. SalB attenuated TGF-β1-induced oxidative stress in MRC-5 cells via activating Nrf2

MRC-5 cells were loaded with DHR-123 to detect the accumulation of intracellular ROS. A significant amount of ROS was

Fig. 1. SalB inhibited TGF-β1-induced myofibroblast differentiation in MRC-5 cells. MRC-5 cells treated with or without 40 μM SalB were stimulated by 10 ng/ml TGF-β1 or for 48 h (A): Immunofluorescence staining for α-SMA, vimentin and fibronectin. Scale bars: 50 μm, magnification: 20×. (B): Western blots for expression of α-SMA, vimentin fibronectin and GAPDH proteins in MRC-5 cells; (C): Band densitometry analysis of protein expression. Results are presented as mean ± SD, n = 3. *P < 0.05, **P < 0.01 vs. The control; #P < 0.05, ##P < 0.01 vs. TGF-β1 group.
generated in the cells treated with TGF-β1 for 24 h (Fig. 2A). The addition of SalB significantly decreased the accumulation of ROS induced by TGF-β1 from 1.75-fold to 1.43-fold, while SalB alone decreased the ROS basal level by over 20% when compared with control cells (Fig. 2B).

The levels of GSH and MDA in MRC-5 cells were also estimated to evaluate the protection of SalB against oxidative stress in Table 1. As expected, the level of GSH was considerably decreased in TGF-β1-stimulated cells compared to the control cells, while the level of MDA was significantly enhanced upon TGF-β1 treatment. Treatment with SalB increased the level of GSH and reduced the level of MDA compared to the fibrotic groups.

Cells can be protected from oxidative stress either by the direct scavenging of ROS or the fortification of the body’s antioxidant defenses. To identify the upstream events that mediate the anti-pulmonary fibrosis function of SalB and its suppression of oxidative stress in pulmonary fibrogenesis, we focused on Nrf2, an essential defender against oxidative stress and a modulator of TGF-β1 signaling. The expression of the Nrf2 protein in MRC-5 cells was first determined by a Western blot analysis after 24 h of treatment.

Fig. 2. SalB attenuated TGF-β1-induced oxidative stress in MRC-5 cells via activating Nrf2. ROS generation in TGF-β1-induced MRC-5 cells was detected by fluorescence microscope (A) and flow cytometer (B). Protein expression of Nrf2 (C and D) and mRNA expression of Nrf2 (E and F) in TGF-β1-induced MRC-5 cells were evaluated. All the experiments were repeated at least three times. Data are presented as mean ± SD, n = 3. *P < 0.05, **P < 0.01 vs. the control; *P < 0.05, **P < 0.01 vs. TGF-β group.
The results in Fig. 2C and D shows that SalB alone significantly increased both the total Nrf2 and nuclear Nrf2 levels, which were significantly decreased in TGF-β1-stimulated cells compared with the control cells. The PCR results exhibited a similar trend to the Western blot analysis, indicating that SalB could up-regulate Nrf2 not only at the protein level but also at the mRNA level to protect MRC-5 cells against oxidative stress during TGF-β1-induced fibrogenesis (Fig. 2E and F).

3.3. SalB ameliorated bleomycin-induced pulmonary fibrosis in rats via up-regulating the expression of Nrf2

Pulmonary fibrosis was induced in rats by BLM treatment to evaluate the anti-fibrotic activity of SalB in vivo. The histopathological changes in the lungs were detected by H&E staining (Fig. 3A) and Masson’s trichrome staining (Fig. 3B). The lungs of rats in the control group showed normal alveolar spaces and normal thickening of alveolar septa. BLM treatment led to significantly abnormal morphologies, including thickening of the alveolar/bronchial walls, collapse of the alveolar spaces and interstitial infiltration by inflammatory cells. However, a remarkable suppression of the BLM-induced inflammatory cellular infiltration was observed in rats treated with both BLM and SalB, as evidenced by the decrease in the cellular infiltrates and thin-lined alveolar septa. In addition, SalB treatment strongly inhibited the extent and intensity of collagen staining compared with the BLM-treated rats, which displayed a remarkable increase in lung collagen deposition. SalB treatment alone showed no significant effect on the lung tissues compared with the control rats.

Immunohistochemical staining of lung sections indicated a decreased amount of α-SMA expression in the lung tissues of rats in the BLM + SalB group compared with the BLM group (Fig. 3C). Western blotting similarly demonstrated that SalB strongly inhibits the increased α-SMA from 2.5-fold to 1.60-fold compared with the controls (Fig. 3D and E). The expression of α-SMA in the rats treated with SalB alone was similar to that of the control rats. The above results verified that SalB could ameliorate experimental pulmonary fibrosis in vivo.

The levels of GSH and MDA were estimated in animals with BLM-induced pulmonary fibrosis to additionally evaluate the oxidative stress and presented a similar result to the cell model (Table 2).

### Table 1

<table>
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<tr>
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<th>Control</th>
<th>SalB</th>
<th>TGF-β</th>
<th>TGF-β + SalB</th>
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<tr>
<td>GSH (μmol/g protein)</td>
<td>4.06 ± 0.38</td>
<td>4.31 ± 0.45</td>
<td>2.09 ± 0.32**, 3.34 ± 0.45***</td>
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<tr>
<td>MDA (μmol/g protein)</td>
<td>1.04 ± 0.11</td>
<td>0.92 ± 0.09</td>
<td>2.57 ± 0.19**, 1.49 ± 0.22**</td>
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Data are presented as mean ± SD, n = 3. *P < 0.05, **P < 0.01 vs. the control; **P < 0.01 vs. TGF-β group.

![Fig. 3](image-url)

**Fig. 3.** SalB ameliorated bleomycin-induced pulmonary fibrosis in rats. H&E (A, scale bars: 100 μm, magnification: 10 ×), Masson (B, scale bars: 50 μm, magnification: 20 ×), immunohistochemical staining for α-SMA proteins and Nrf2 proteins (C and F, scale bars: 80 μm, magnification: 40 ×) of lung tissues during BLM-induced pulmonary fibrosis. Protein expressions of α-SMA and Nrf2 proteins were also determined by western blotting (D and G). Band densitometry analysis of protein expression (E and H) is presented as mean ± SD, n = 3. *P < 0.05, **P < 0.01 vs. the control; #P < 0.05, ##P < 0.01 vs. BLM group.
A Western blot analysis was also performed on lung tissues to determine the role of SalB in the regulation of Nrf2 in vivo. In contrast to the cell model, although BLM-injection could lower the expression of the total Nrf2 and the addition of SalB could remarkably up-regulate the total Nrf2 expression in lung tissues compared with BLM treatment alone, the alteration of the total Nrf2 expression in vivo was not as dramatic as in MRC-5 cells (Fig. 3G and H). As lung tissue includes many cell types along with lung matrix, and Western blot analysis cannot differentiate between the cell-specific variability in the expression of Nrf2, in healthy and diseased lungs, immunohistochemistry was conducted as a subsequent analysis. The result in Fig. 3F shows that Nrf2 was mainly localized in the alveolar epithelium in the control group and the hyperplastic epithelium in the BLM group but was absent in the fibroblastic foci (FF) lesions that were observed in the BLM group. The addition of SalB increased the expression of Nrf2 in lung tissues, especially in FF areas.

4. Discussion

Although the precise pathological mechanisms of IPF are still poorly understood, recent evidence has strongly suggested that oxidative stress is an important mechanism that can result in an aberrant wound-healing response to sequential lung injury and lead to fibrogenesis. Therefore, antioxidant medicines have received attention as potential therapies for pulmonary fibrosis.

Salvianolic acid B has been found to be anti-inflammatory, antioxidative and anti-fibrotic, especially in experimental models of renal and liver fibrosis [7,8]. Previous research has demonstrated that SalB attenuated CCl4-induced hepatic fibrosis [9] and inhibited TGF-β1 activation of hepatic stellate cells [10]. There is also evidence that SalB prevents epithelial-mesenchymal transition (EMT) in HgCl2-induced rats [11] and TGF-β1-induced HK-2 cells [12]. In our previous study, SalB was screened to have a preventive effect on BLM-induced pulmonary fibrosis in rats [6]. In this study, we intended to investigate the protective effect of SalB on TGF-β1-induced myofibroblast transdifferentiation in MRC-5 cells and BLM-induced pulmonary fibrosis in rats. Our results showed that SalB significantly inhibited the production of myofibroblast biomarkers in TGF-β1-stimulated cells. Meanwhile, the histological analysis demonstrated that SalB could attenuate inflammation and collagen deposition in the lung tissues of BLM-treated rats. In addition, SalB also remarkably decreased α-SMA expression. The combined results revealed that SalB is strongly antifibrotic and could have potential applications in pulmonary fibrosis.

In the presence of ROS, DHR-123 is rapidly oxidized to the highly fluorescent rhodamine 123. By detecting the fluorescence intensity of rhodamine 123, the amount of ROS in MRC-5 cells was estimated. The result showed that SalB could reduce the production of ROS in TGF-β1-stimulated cells. MDA and GSH are common markers of oxidative stress in cells and tissues. In agreement with previous reports, SalB restored GSH and decreased MDA content in vitro and in vivo, demonstrating its protective effect against oxidative stress in experimental pulmonary fibrosis. To identify whether the antioxidative activity is due to the reduction of SalB itself or the regulation of the oxidative/antioxidative system, the upstream regulatory element Nrf2 was subsequently investigated.

Nrf2, which is a major transcription factor that responds to variations in redox state, plays an important role as a central regulator of antioxidants by activating antioxidant response elements (AREs). In response to oxidative stress, the Nrf2 protein is stabilized and translocated to the nucleus and binds to AREs in the promoters of many antioxidant genes. Previous reports have demonstrated the protective effects of Nrf2 in reducing oxidative stress in different kinds of pulmonary disorders, including IPF [13,14]. SalB has been reported to induce Nrf2 activation to protect against lung inflammation induced by cigarette smoke [15] and paraquat-induced pulmonary injury in mice [16].

Our study focused on Nrf2 as well. Total Nrf2 expression was significantly decreased no matter in TGF-β1-stimulated MRC-5 cells or lung tissues of BLM-injected rats, which was in contrast to the studies on inflammation induced by cigarette smoke and paraquat-induced pulmonary injury but was in accordance with previous findings that Nrf2 was inhibited in IPF fibroblasts and IPF lung tissues [13,17]. That may be due to the differences between their pathologic processes. The result of Nrf2 immunohistochemistry showed Nrf2 expression were highly localized to the hyperplastic epithelium and seldom in FF areas in the lung tissues of rats in the BLM group, which was also in accordance with Mazur’s report [13] and explained the less pronounced alteration of the total Nrf2 expression between the control group and the model group in vivo compared with in vitro. In TGF-β1-stimulated MRC-5 cells, Nrf2 expression was significantly decreased at both the protein level and mRNA level, and the translocation of the Nrf2 protein to the nucleus was remarkably inhibited, which may be the reason of the lower Nrf2 expression in FF areas. The addition of SalB in the experimental pulmonary fibrosis model was observed to restore Nrf2 expression and the oxidant/antioxidant balance, which may be the mechanism underlying the anti-fibrotic effects of SalB.

In conclusion, our results showed that the SalB treatment inhibited myofibroblast transdifferentiation and decreased oxidative stress via activating Nrf2 in experimental pulmonary fibrotic lung fibroblasts, which suggests that SalB treatment may be a potential therapy option for IPF.

Conflicts of interest

There is no conflict of interest.

Acknowledgments

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