Paeoniflorin, the main active constituent of *Paeonia lactiflora* roots, attenuates bleomycin-induced pulmonary fibrosis in mice by suppressing the synthesis of type I collagen

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Introduction

• Pulmonary fibrosis is a progressive disorder characterized by the excessive proliferation of fibroblasts and deposition of extracellular matrix (ECM), which destroy normal tissue architecture and function.

• Transforming growth factor (TGF)-β1, a fibrogenic cytokine, plays a critical role in the production of collagen in PF.

• TGF-β1 is able to up-regulate mRNA expression of type I collagen, promote the proliferation of fibroblasts and drive the differentiation of fibroblasts to myofibroblasts. (Bataller et al, 2005)
- TGF-β1 functions mainly through the activation of Smads, which locate at the downstream of TGF-β receptors.

- Phosphorylated Smad2/3 can combine with Smad4, forming Smad2/3-Smad4 complexes, and transcription into the nucleus to regulate the mRNA expression of type I collagen.

- Smad7 inhibits the phosphorylations of Smad2/3.
• On contrary, IFN-γ, as a classical anti-fibrotic cytokine, can inhibit TGF-β1 expression, fibroblast proliferation, differentiation of fibroblasts to myofibroblasts, and collagen synthesis (Aggarwal et al., 2000).

• It prevents the signals of TGF-β through increasing the expressions of Smad7 via activating JAK1 and STAT1.
On the other hand, matrix metalloproteinases (MMPs) are key enzymes responsible for the degradation of ECM, and tissue inhibitors of metalloproteinases (TIMPs) can inhibit the activity of MMPs by forming 1:1 complexes with MMPs (Sundararajan et al., 2012).
• Paeonia lactiflora Pall root, a famous traditional Chinese medicine (TCM), has been used for more than 1200 years because of its anti-inflammatory and immune-regulatory properties.

• Paeoniflorin is the principal bioactive ingredient in *P. lactiflora*.
• Intratracheal instillation (IT) of bleomycin is a widely used experimental model for lung fibrosis.

• Bleomycin 是一種醣蛋白類抗生素，1962 年發現。

• Bleomycin 目前多用於癌症治療，如睾丸癌、淋巴瘤以及各個部位的鱗狀細胞癌: 頭頸部腫瘤、子宮頸癌、陰莖癌、皮膚癌、女陰癌，副作用造成肺部纖維化。

• Bleomycin 也可受到 Bleomycin hydrolase 的作用。Bleomycin hydrolase 可在腫瘤細胞、肝臟、和腎臟發現，但在肺臟和上皮組織則明顯地缺乏。因此，Bleomycin 對肺臟和皮膚就會產生獨一無二的毒性。
Materials and methods
• Hydroxyproline assay (560 nm wavelength)
The upper lobes of left lung tissues
• Histology (Hematoxylin-eosin and Masson’s trichrome)
The lower lobes of left lung tissues
• Western blot analysis
  (α-SMA, p-Smad2, p-Smad3, Smad2/3, Smad4, and Smad7)
The lower lobes of right lung tissues
• ELISA (Type I collagen, INF-γ, TGF-β1)
The lower lobes of right lung tissues
• RT-PCR analysis (MMP-1 and TIMP-1)
In middle lobes of right lung tissues
Hydroxyproline

Hydroxyproline (HYP) is a major component of the body's collagen, making up about 13.4% in normal collagen.

The tissue is hydrolyzed with hydrochloric acid to release free hydroxyproline, and then oxidized with chloramine T to hydroxyproline for colorimetric determination.

Hydroxyproline Assay Steps:
* 1. Each well add 10 µl of sample, then add Standard (2~3 strips).
* 2. Each well add 100 µl of Chloramine T reagent, then place in room temperature for 5 minutes.
* 3. Add 100 µl of DMAB reagent to each well, then place in an oven at 60°C for 90 minutes.
* 4. Measure at 560 nm wavelength.
Score grades

• The results of H&E stain were graded according to epithelial proliferation, alveolitis, edema, inflammatory cell infiltration and interstitial fibrosis, and the results of Masson's trichrome stain were graded according to the levels of ECM.

Criteria for grading were as follows:
• grade 0, normal
• grade 0.5, slight
• grade 1, mild
• grade 2, moderate
• grade 3, severe
Results
• (A) BLM_IT reduced the survival rates of mice to 50%.
• Paeoniflorin, at the dose of 50 mg/kg, significantly increased the survival rates of mice to 87.5%. However, prednisone did not affect the survival rates of BLM-treated mice.
• (B) Normal group were soft with white color. model group were hard and shrunken in size with pale color. Paeoniflorin (50 and 100 mg/kg) and prednisone (6 mg/kg) treatments resulted in the lungs of mice softer with pink color.
(C) Histopathological changes of the lower lobes of left lung tissues were examined by H&E stain (magnification 200x).

(D) The histological scores of all groups were calculated.

Data were expressed as means±S.D., n=6. ## P<0.01 vs. normal; *P<0.05, **P<0.01 vs. model.
• (A) Masson's trichrome stain (magnification 200x).
• (B) The histological scores of all groups were calculated.
• Data were expressed as means±S.D., n=6. # P<0.05 vs. normal; *P<0.05 vs. model.

• Paeoniflorin (50 and 100 mg/kg) and prednisone (6 mg/kg) treatments obviously attenuated the production of ECM in lung tissues.
(C) Paeoniflorin (50 and 100 mg/kg) and prednisone (6 mg/kg) treatments significantly decreased the contents of hydroxyproline, and the inhibitory percentages were 68.1%, 72.1% and 70.1%, respectively.

(D) The elevated content of type I collagen in lung tissues of BLM-induced mice were also decreased by paeoniflorin (50 and 100 mg/kg) and prednisone (6 mg/kg) treatments. Their inhibitory percentages were 39.2%, 43.7% and 53.0%, respectively.
• (A) In the lung tissues of mice induced by BLM, the levels of α-SMA were significantly elevated.

• (B) Paeoniflorin (100 mg/kg) and prednisone (6 mg/kg) produced marked inhibition of α-SMA levels in lung tissues of mice, and the inhibitory percentages were 72.6% and 91.8%, respectively.

• Data were expressed as means±S.D., n=6. ## P<0.01 vs. normal; *P<0.05 vs. model.
• (A) BLM instillation resulted in significant increase of TGF-β1 expression in lung tissues of mice. Paeoniflorin (50 and 100 mg/kg) and prednisone (6 mg/kg) treatments obviously decreased the levels of TGF-β1.
• (B) we investigated the effects of paeoniflorin on the phosphorylations of Smad2/3 and the expressions of Smad4 and Smad7 in mouse lung tissues induced by BLM. Data showed that BLM instillation resulted in increased phosphorylations of Smad2/3 and expression of Smad4, but decreased expression of Smad7. Paeoniflorin (100 mg/kg) and prednisone (6 mg/kg) treatments obviously down-regulated Smad2/3 phosphorylations and Smad4 expression, and conversely up-regulated Smad7 expression.
• Data were expressed as means±S.D., n=6. ## P<0.01 vs. normal; * P<0.05, ** P<0.01 vs. model.
• To recognize whether inhibition of paeoniflorin on the synthesis of type I collagen was associated with IFN-γ, the levels of IFN-γ in mouse lung tissues were detected by ELISA.
• BLM instillation led to significant decrease of IFN-γ levels in lung tissues, and paeoniflorin (50 and 100 mg/kg) and prednisone (6 mg/kg) treatments increased IFN-γ levels.
BLM instillation promoted the expressions of MMP-1 and TIMP-1 at mRNA levels, and paeoniflorin only showed slight inhibition of MMP-1 and TIMP-1 expressions. On contrast, prednisone (6 mg/kg) significantly decreased the mRNA expression of TIMP-1 but not MMP-1. It was suggested that paeoniflorin had little effect on the degradation of type I collagen.
Discussion
• Smad7 is an inhibitory Smad (I-Smad), can inhibit the activation of both TGF-β and bone morphogenetic protein (BMP) signaling pathways.

• It prevents the phosphorylation of R-Smad and the subsequent nucleus translocation of R-Smad/Smad4 hetero complexes.

• Furthermore, Smad7 competitively inhibited the binding of Smad3 to TGF-β-RI
Conclusion
• Paeoniflorin can significantly attenuate pulmonary fibrosis induced by BLM in mice.

• It acts mainly by suppressing ECM deposition in lung tissues through reducing the synthesis of type I collagen via down-regulating the expression of TGF-β1 and activation of related signal pathway.

• Paeoniflorin has a therapeutic potential for the treatment of pulmonary fibrosis.
Thanks For Your Listening
Q & A
• 初期MMP-2/9 ↑ (TIMP-1/2没有增加造成MMPs的活性提高)，造成内皮损伤基底膜瓦解，ECM降解。刺激肌纤维母细胞与Ⅱ型上皮细胞等细胞外基质生成细胞的活化

• 后期TIMP-1/2 ↑ 抑制了MMP-2/9活性造成活性下降，ECM降解减少导致肺泡与间质的ECM过度增生与异常沉积，Fibrosis ↑