Lysyl Hydroxylase Inhibition by Minoxidil Blocks Collagen Deposition and Prevents Pulmonary Fibrosis via TGF-β1/Smad3 Signaling Pathway

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Background: Idiopathic pulmonary fibrosis (IPF) is a deadly disease characterized by excessive collagen in the extracellular matrix (ECM) of the lungs. Collagen is the primary protein component of the ECM. However, the exact mechanisms underlying the formation and deposition of collagen in the ECM under normal and pathological conditions remain unclear. Previous studies showed that lysyl hydroxylase (LH) plays a crucial role in the formation of collagen. Minoxidil is an FDA-approved anti-hypertensive agent that inhibits LH that reduces fibrosis. In this study, we investigated the functional roles of LHs (LH1, LH2, and LH3) in pulmonary fibrosis and the anti-fibrotic effects of minoxidil.

Material/Methods: Patient serum samples were examined for their expression of procollagen-lysine, 2-oxoglutarate 5-dioxygenases (PLOD) 1-3, the genes encoding LH 1-3. Mice with bleomycin (BLM 2.5 mg/kg)-induced pulmonary fibrosis were administered a minoxidil solution (30 mg/kg) by oral gavage.

Results: The PLOD mRNA levels were significantly higher in the IPF patients than in the healthy control subjects. Minoxidil suppressed the BLM-induced pulmonary fibrosis in vivo. These effects were associated with blocking TGF-β1/Smad3 signal transduction and attenuating the expression and activity of LHs, resulting in decreased collagen formation, thus reducing the pulmonary fibrosis. The anti-fibrotic effects of minoxidil may be mediated through competitive inhibition of LHs activity, resulting in decreased pyridine cross-link formation and collagen production and deposition.

Conclusions: The results of this study suggest that LH represents a target to prevent or treat pulmonary fibrosis, and minoxidil may provide an effective agent to inhibit LHs.

MeSH Keywords: Idiopathic Pulmonary Fibrosis • Minoxidil • Procollagen-Lysine, 2-Oxoglutarate 5-Dioxygenase • Smad3 Protein • Transforming Growth Factor beta1

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**Background**

Idiopathic pulmonary fibrosis (IPF) is an invasive and frequently fatal disease. Although the precise mechanisms underlying the development of IPF are still being elucidated, excessive collagen accumulation has been suggested to play an important role in fibrotic processes [1]. An imbalance between the synthesis and degradation of matrix proteins causes fibrosis, leading to an excessive accumulation of collagen.

Collagen synthesis is a multi-step process involving several post-translational modifications that are essential for the stability of collagen. The assembly of collagen molecules into collagen fibrils is stabilized by covalent cross-links among collagen molecules [2]. Lysyl hydroxylase (LH), a member of the 2-oxoglutarate-dependent dioxygenase family, catalyzes the hydroxylation of -X-Lys-Gly- sequences in collagen and collagen-like proteins with Fe$^{2+}$, 2-oxoglutarate, O$_2$, and ascorbate. Because the lysyl hydroxylases modify and cross-link proteins by converting lysine to hydroxylysine, they make collagen more resistant to degradation [3,4]. The hydroxylation of lysine residues is required for the maturation of collagen (via glycosylation), and thus determines the chemical nature of the cross-links among collagen molecules [5].

Three LHs (LH1, LH2, and LH3) have been identified and characterized in vertebrates. These proteins are respectively encoded by procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (PLOD1), PLOD2, and PLOD3 [6,7]. Although the substrates of LH2 and LH3 are still under investigation, it has been demonstrated that LH1 catalyzes the formation of hydroxylysyl residues in collagen and other helical proteins with collagenous domains, and therefore fulfills an important role in collagen modification and cross-linking [8,9]. Excessive accumulation of collagen has been observed in various tissues, including fibrotic skin (keloids) and organs (lung, kidney, and liver). This was associated with increased crosslinks derived from hydroxylysine [10,11].

TGF-$\beta_1$ may also represent a potential therapeutic target for lung fibrosis because it is involved in the initiation and progression of fibrosis and the deposition of type I and type III collagens [12,13]. The role of TGF-$\beta_1$ in fibrosis has been suggested to be primarily mediated through the Smad-dependent signaling pathway [13].

Minoxidil is a U.S. Food and Drug Administration (FDA)-approved drug used for the treatment of alopecia and hypertension. Minoxidil reduces lysyl hydroxylase activity by decreasing the LH1 mRNA level [14]. It also limits the supply of hydroxylsines for hydroxylysyl cross-link formation, leading to anti-fibrotic effects [15]. It has also been suggested that minoxidil and its analogs may inhibit TGF beta signaling [16]. In the present study, we examined how minoxidil affects the gene and protein expression of the PLODs/LHs, other fibrosis-associated molecules, and TGF-$\beta_1$ signaling, and on the cross-linking of collagen, to further elucidate the pathogenesis of pulmonary fibrosis in vivo, and to determine whether minoxidil affects hydroxyallysine cross-link formation (and thereby fibrosis).

**Material and Methods**

**Patients and serum samples**

The study recruited 30 patients from Guizhou Provincial People’s Hospital in 2016. Fifteen of the patients had a relevant clinical history of IPF, while the remaining 15 patients were healthy and were being seen for other conditions, with no evidence or history of pulmonary or inflammatory disease. For each patient, 10 mL of venous blood was collected using red vacuum tubes containing a separating gel. The blood was clotted for 60 min at room temperature. After centrifugation at 3000 rpm for 10 min, the separated serum was transferred to an Eppendorf tube and stored at −20°C for later use. This study was approved by the Ethics Committee of Guizhou Provincial People’s Hospital (No. 2014015) and all of the patients provided written informed consent for the study. All procedures were performed in accordance with the guidelines of Guizhou Medical University.

**Animals and treatments**

Male C57Bl/6 (6 weeks old) mice were obtained from the Laboratory Animal Center of the Third Military Medical University, China [animal use permit No.: SCXK(yu) 2012-0005] and were maintained in the pathogen-free facility of the Animal Laboratory of Guizhou Medical University. All animal protocols complied with the ethical standards and were approved by the Ethics Committee of Guizhou Medical University (No. 1203109). All research involving animals followed the National Health and Medical Research Council of China’s Code for the Care and Use of Animals for Scientific Purposes. The mice were housed at 22±2°C with 50±5% humidity with a 12-h light/dark cycle and free access to water and food. All mice were allowed to adapt to the housing environment for at least 7 days before experiments were initiated. All efforts were made to minimize stress, and animals were sedated before surgical procedures via an intraperitoneal injection of chloral hydrate (4% chloral hydrate, 0.01 ml/g).

For the present study, the C57Bl/6 mice received 2.5 mg/kg of bleomycin (BLM) (NIPPON KAYAKU) by intratracheal instillation using a 10-G needle with a flow rate of 10 μl/s (n=20; BLM diluted in 100 μl saline buffer). The mice in the normal saline (NS) group (n=10) received the vehicle only (saline buffer), while the mice in the control group (n=3) did not receive any treatment.
undergo any intervention. The mice in the BLM group (n=10) received 30 mg/kg of minoxidil (Sigma Aldrich) via intragastric administration beginning the second day after BLM injection, and were treated with minoxidil daily until day 21, when animals were sacrificed by exsanguination under sodium chloral hydrate anesthesia (intraperitoneal injection of 4% chloral hydrate, 0.01 ml/g).

**Hydroxyproline (HYP) assay**

The deposition of collagen in the lungs was assessed by determining the HYP content of lung homogenates using a hydroxyproline assay kit (Jiancheng Bioengineering Company, Nanjing of China). The absorbance of each sample was read using a microplate reader at 550 nm. A standard curve was generated using samples with a known quantity of hydroxyproline (Molychem), and the HYP content in the lungs was calculated based on the standard curve (as the μg/g of wet lung tissue weight). The experiment was repeated 3 times for each mouse using different tissue specimens.

**Histological analyses**

**Hematoxylin-eosin and Masson staining**

Lung tissue specimens (3–5/mouse) were fixed in 10% formalin and embedded in paraffin. Sections were then stained with hematoxylin and eosin (HE). Masson’s trichrome staining was performed on lung sections to reveal fibrillary collagen. The stained tissue slides were examined under a light microscope and photographed.

**Immunohistochemical staining**

Tissue sections were dewaxed and hydrated, then immunohistochemical staining was performed using the Histostain-Plus Kit (PV-9000, ZSGB-BIO, China; 20—40 μm), followed by a DAB Kit (ZLI-9017, ZSGB-BIO, China; 1—10 min). The sections were incubated with an anti-PLOD1 antibody (LH1, 1: 100, Abcam, ab171140), anti-PLOD2 antibody (LH2, 1: 100, Abcam, ab90088), anti-PLOD3 antibody (LH3, 1: 100, Abcam, ab128698), or an anti-collagen I antibody (COLI, 1: 100, Abcam, ab72939), or an anti-collagen I antibody (COLI, 1: 100, Abcam, ab6308) overnight at 4°C (12–16 h), followed by incubation with fluorescence-conjugated secondary antibodies and staining with 4',6-diamidino-2-phenylindole (DAPI). Finally, the sections were observed using a Leica confocal microscope (Leica DM400B, Germany) and 5 regions of interest were randomly selected for each group and captured with a CCD camera.

**Enzyme-linked immunosorbent assay (ELISA)**

The protein expression of lysylpyridinoline (LP) and hydroxylysylpyridinoline (HP) in the lung tissue specimens was detected using commercial mouse ELISA kits (Jianglai, Shanghai, China) according to the manufacturer’s instructions. After the reaction had been allowed to proceed, the optical density was measured at 450 nm using a plate reader (ELX-800, BIOTEK, USA). The HP levels are expressed as ng/ml of lung homogenate and the LP levels are expressed as nmol/L of lung homogenate.

**RNA extraction and quantitative real-time RT-PCR (qRT-PCR)**

Total RNA was isolated from the lung tissue specimens using Trizol reagent (Thermo, USA). The RNA was then reverse-transcribed into cDNA using reverse transcription kits (TIANGEN, Beijing, China) and the cDNA was subjected to qRT-PCR amplification. The qRT-PCR amplification of cDNA sequences was performed for PLOD1, PLOD2, PLOD3, collagen I (COLIa1), fibronectin (FN), matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of metalloproteinases (TIMP)-1, and TIMP-2 (additional molecules involved in fibrosis). Each cDNA was amplified using the specific primers shown in Table 1 in a total reaction volume of 20 μl containing 10 μl 2×SuperReal Premix Plus, 2 μl cDNA, 500 nM of each primer, and 6 μl RNase-free ddH2O. PCR was performed in a Bio-Rad sequence detection system consisting of a 15-min interval at 95°C, followed by 40 cycles of 95°C for 10 s, 58°C for 20 s, and 72°C for 30 s. Data were then analyzed using Sequence Detector version 1.7 software.

**Western blot analysis**

Lung tissue specimens were washed twice with PBS, and homogenates were lysed with RIPA lysis buffer (Beiyotime P0013B, China) containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail (both purchased from KangChen, Shanghai, China). The protein concentration was determined using a BCA Protein Assay Reagent Kit (Beyotime P0012, China). The protein samples were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were then blocked with 5% skim milk in TBS-T

bovine serum albumin (BSA) diluted in PBS. The sections were subsequently incubated with an anti-PLOD2 antibody (LH2, 1: 100, Abcam, ab72939), anti-PLOD3 antibody (LH3, 1: 100, Abcam, ab89263), or anti-collagen I antibody (COLI, 1: 100, Abcam, ab6308) overnight at 4°C (12–16 h), followed by incubation with fluorescence-conjugated secondary antibodies and staining with 4',6-diamidino-2-phenylindole (DAPI). Finally, the sections were observed using a Leica confocal microscope (Leica DM400B, Germany) and 5 regions of interest were randomly selected for each group and captured with a CCD camera.
for 1 h at room temperature and incubated with anti-β-actin (1: 4000, Lianke China, ab008-100), anti-α-SMA (1: 300, Abcam, ab7817), anti-collagen-I (1: 500, Abcam, ab6308), anti-collagen-IV (1: 500, Sigma, SAB4200500), anti-TGF-β1 (1: 400, Santa Cruz, sc-52893), anti-Smad3 (1: 1000, Cell Signaling Technology, 9513), anti-phospho-Smad3 (1: 1000, Cell Signaling Technology, 9520), anti-PLOD1 (LH1, 1: 500, Abcam, ab171140), anti-PLOD2 (LH2, 1: 500, Abcam, ab90088), and anti-PLOD3 (LH3, 1: 500, Abcam, ab128698) antibodies overnight at 4°C (12–16 h).

The secondary antibody used for the anti-TGF-β1, anti-Smad3, anti-phospho-Smad3, anti-PLOD1, anti-PLOD2, and anti-PLOD3 antibodies was goat-anti-rabbit HRP (1: 5000) (Boshide, Wuhan, China). The secondary antibody used for the anti-β-actin (1: 4000, Lianke China, ab008-100), anti-α-SMA (1: 300, Abcam, ab7817), anti-collagen-I (1: 500, Abcam, ab6308), and anti-collagen-IV (1: 500, Sigma, SAB4200500) antibodies was goat-anti-mouse HRP (1: 5000) (Boshide, Wuhan, China). The reaction was developed using ECL reagents (Clarity Western ECL Substrate, Bio-Rad, 170-5061) plus a Western blot detection system (ChemiDoc™Touch Imaging System, Bio-Rad, USA). The signals for the Western blots signals were quantified by measuring the intensity of the specific bands with the Quantity One v. 4.6 image analysis program. The expression levels of the proteins were calculated after normalizing their expression to that of β-actin.

**Statistical analysis**

All experiments were repeated at least 3 times. The values are presented as the means ± standard deviation (SD), and the significance of differences between means was calculated with a one-way ANOVA followed by the Student-Newman-Keuls test using the SPSS 17.0 software (IBM, New York, NY, USA). A value of P<0.05 was considered to be statistically significant.

**Results**

**PLOD expression in human serum**

We used qRT-PCR to detect PLOD expression in the serum obtained from patients with IPF and healthy controls. The PLOD mRNA levels were significantly higher in the IPF patients than in the healthy control subjects (Figure 1).

![Table 1. Characteristics of the primers used for quantitative real-time PCR.](image)

**hydroxyproline content decreased (Figure 2A).**
Histological changes in the lungs were examined using HE staining. The lung tissues from mice in the NS group showed normal alveolar walls and no inflammatory cell infiltration in the alveolar septum. In contrast, there was severe inflammatory cell infiltration and obvious alveolar wall thickening in the BLM group. Minoxidil attenuated these histological changes induced by BLM (Figure 2B). Masson’s staining showed the presence of collagen (blue color) on day 21 following BLM exposure. BLM – bleomycin.

The lysylpyridinoline and hydroxylysylpyridinoline expression levels in the lung tissues of mice

The protein expression levels of HP and LP were significantly increased in the lung tissues from mice with pulmonary fibrosis. However, in the mice treated with minoxidil, the HP and LP levels were similar to those of the controls (Figure 3).

COL1, LH1, LH2, and LH3 expression in the lung tissues of mice

Immunohistochemical staining showed that the expression levels of COL1 (Figure 4A) and the LH1, LH2, and LH3 proteins (Figure 4B–4D, respectively) were increased in the BLM group, and these protein expression changes were substantially prevented in the BLM + Mi group.
Immunofluorescent staining was performed to confirm the immunohistochemical findings, and showed that the LH2 (Figure 5A, green), LH3 (Figure 5B, green), and COL1 (Figure 5C, red) expression levels were increased in the BLM group, which had an increase in endochylema. The LH1 levels could not be confirmed because no antibodies are available. In the mice treated with minoxidil, the expression of the three proteins was closer to that of the control group.

**Figure 4.** Immunohistochemical staining for COL1 and the LH proteins. The expression of the COL1 (A), LH1 (B), LH2 (C), and LH3 (D) proteins. Representative images are shown. All images are 200× magnification.
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Figure 5. Immunofluorescence staining for COL1 and the LH proteins. The expression and localization of the LH2 (A; green), LH3 (B; green), and COL1 (C; red) proteins was confirmed by immunofluorescence staining. Representative images are shown. All images are 100× magnification.

Figure 6. Minoxidil decreased the PLOD1, PLOD2, PLOD3, α-SMA, FN, Col1a1, MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNA levels in the lung tissues of BLM-treated mice (n=6, * P<0.05 vs. the NS group, ** P<0.01 vs. the NS group; # P<0.05 vs. the BLM group; ns P>0.05 vs. the BLM group).

**Bleomycin induced the expression of PLODs and ECM genes**

The qRT-PCR analysis demonstrated that the bleomycin-exposed mice had significantly higher expression of PLODs (PLOD1, PLOD2, PLOD3) mRNA compared to the control group. The mRNA expression levels of α-SMA, COL1a1, FN, TIMP-1, TIMP-2, MMP-2, and MMP-9 were also upregulated in the BLM group compared to the control group. The expression levels of these genes in the minoxidil-treated group were not significantly increased (Figure 6).

**Minoxidil treatment inhibits the induction of fibrosis marker proteins**

We next examined the effects of minoxidil treatment on the expression levels of protein markers of fibrosis. We found that the LH1, LH2, LH3, α-SMA, collagen I, and collagen IV levels were all significantly higher in the BLM group. These increases in protein expression were largely abrogated by minoxidil treatment (Figure 7).
Minoxidil suppressed bleomycin-induced TGF-β1/Smad3 signaling

To further evaluate the effects of minoxidil, we examined whether the TGF-β1/Smad3 signaling pathway was related to the BLM-induced pulmonary fibrosis in mice, and whether minoxidil could inhibit this pathway. The mice with BLM-induced fibrosis showed high levels of TGF-β1 and p-Smad3 protein expression in their lung tissue specimens (Figure 8). Treatment with minoxidil prevented these increases, and there was reduced bleomycin-induced pulmonary fibrosis in the minoxidil-treated mice (Figure 8).

Discussion

Fibrosis is characterized by the excessive deposition of collagen, which may result from an imbalance between collagen synthesis and degradation [17]. The collagen accumulated in fibrotic lesions shows an increase in hydroxylysine-derived cross-links, which likely increases its accumulation by conferring increased resistance to proteolytic degradation [8].

It was previously discovered that minoxidil could inhibit lysyl hydroxylase gene expression, and it was postulated that the drug might exert anti-fibrotic properties by reducing the total number of hydroxylysine residues in the collagen molecule, thus reducing the formation of hydroxyllysine cross-links [7]. However, the ability of minoxidil to prevent pulmonary fibrosis,
and the mechanism(s) of action underlying the anti-fibrotic effects of the drug, have been unclear. The present study was undertaken to obtain a better understanding of the pathogenesis of lung fibrosis, the anti-fibrotic potential of minoxidil, and the effects of minoxidil on the expression levels of the individual lysyl hydroxylases and fibrosis-related molecules.

Although treatment with minoxidil repressed the expression of the PLOD1, PLOD2, and PLOD3 genes and their encoded LH proteins, it exerted the most profound effects on PLOD1/LH1, the lysyl hydroxylase involved in the hydroxylation of triple helical lysine [17]. LH may preferentially hydroxylate lysine residues at cross-linking positions [7]. Therefore, further studies, including in vitro studies, are warranted to investigate the mechanisms by which lysyl hydroxylase(s) is/are involved in collagen deposition during pulmonary fibrosis and how minoxidil may affect collagen cross-linking.

Our data also indicated that these effects of minoxidil were at least partly mediated via inhibition of the MMPs and TGF-β1/Smad3 signaling. Therefore, additional studies are needed to determine how minoxidil exerts its effects on the MMPs and TGF-β, and to confirm whether the agent can be used therapeutically or prophylactically for pulmonary fibrosis.

Our findings from human serum samples also suggested that the PLODs are upregulated in human patients with IPF. Therefore, although these results are very preliminary (N=30), the effects of minoxidil observed in the model mice with upregulated PLODs/LHs expression may also occur in human patients. Further studies are needed to determine whether minoxidil decreases LHs expression in humans, and whether this impacts the development or severity of IPF.

Conclusions

Our present results show that there is increased LH expression in the lung tissues of mice with BLM-induced fibrosis and of humans with diagnosed IPF. Minoxidil, an FDA-approved drug already in use for several indications, reduced the lung fibrosis in these mice. The drug also downregulated the expression of the PLOD genes and their encoded LH proteins to prevent BLM-induced pulmonary fibrosis in mice. The effects of minoxidil appear to be mediated at least partly through the TGF-β1/Smad3 and the MMPs/TIMPs pathways, resulting in reduced levels of lysylpyridinoline and hydroxlysylpyridinoline. These reductions in turn led to a decrease in collagen biosynthesis, thus decreasing the development of pulmonary fibrosis. Although confirmation of the present findings will be necessary, our results suggest that minoxidil exerts potent anti-fibrotic effects via downregulation of the LHs, MMPs, and TGF-β, suggesting that it may be a useful therapeutic or preventive agent for pulmonary fibrosis.

Conflict of interest

None.

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