

Article

Neotuberostemonine inhibits the differentiation of lung fibroblasts into myofibroblasts in mice by regulating HIF-1 α signaling

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Abstract

Pulmonary fibrosis may be partially the result of deregulated tissue repair in response to chronic hypoxia. In this study we explored the effects of hypoxia on lung fibroblasts and the effects of neotuberostemonine (NTS), a natural alkaloid isolated from *Stemona tuberosa*, on activation of fibroblasts *in vitro* and *in vivo*. PLFs (primary mouse lung fibroblasts) were activated and differentiated after exposure to 1% O₂ or treatment with CoCl₂ (100 μ mol/L), evidenced by markedly increased protein or mRNA expression of HIF-1 α , TGF- β , FGF2, α -SMA and Col-1 α /3 α , which was blocked after silencing HIF-1 α , suggesting that the activation of fibroblasts was HIF-1 α -dependent. NTS (0.1–10 μ mol/L) dose-dependently suppressed hypoxia-induced activation and differentiation of PLFs, whereas the inhibitory effect of NTS was abolished by co-treatment with MG132, a proteasome inhibitor. Since prolyl hydroxylation is a critical step in initiation of HIF-1 α degradation, we further showed that NTS treatment reversed hypoxia- or CoCl₂-induced reduction in expression of prolyl hydroxylated-HIF-1 α . With hypoxyprobe immunofluorescence staining, we showed that NTS treatment directly reversed the lower oxygen tension in hypoxia-exposed PLFs. In a mouse model of lung fibrosis, oral administration of NTS (30 mg/kg⁻¹·d⁻¹, for 1 or 2 weeks) effectively attenuated bleomycin-induced pulmonary fibrosis by inhibiting the levels of HIF-1 α and its downstream profibrotic factors (TGF- β , FGF2 and α -SMA). Taken together, these results demonstrate that NTS inhibits the protein expression of HIF-1 α and its downstream factors TGF- β , FGF2 and α -SMA both in hypoxia-exposed fibroblasts and in lung tissues of BLM-treated mice. NTS with anti-HIF-1 α activity may be a promising pharmacological agent for the treatment of pulmonary fibrosis.

Keywords: pulmonary fibrosis; neotuberostemonine; hypoxia; fibroblasts; HIF-1 α

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Introduction

Fibrosis is a common pathway to organ injury and failure. Like other organ fibrosis, pulmonary fibrosis is characterized by exaggerated, persistent, and usually irreversible accumulation of extracellular matrix (ECM)^[1]. Aberrant accumulation of ECM replaces normally functioning parenchyma and distorts the architecture of adjacent tissue^[2]. This fibrotic process often leads to rapid and severe abnormalities in lung mechanics and gas exchange properties^[3]. Pulmonary fibrosis is the end stage of almost all advanced interstitial lung diseases with diverse etiologies and has attracted increasing attention in recent years due to its increasing occurrence and poor therapy^[4, 5]. Idiopathic

pathic pulmonary fibrosis (IPF), clinically characterized by progressive dyspnea, is the most common type of idiopathic interstitial pneumonia, has a poor prognosis and is unresponsiveness to traditional therapies^[6]. Although nintedanib and pirfenidone are available to treat IPF, neither of them appears to be able to completely arrest disease progression, and long-term treatment is accompanied by serious adverse effects^[7, 8]. Therefore, it is necessary to provide more therapeutic options for patients.

The cellular and molecular mechanisms of IPF are still not well understood. However, it is well known that the key hallmark of pulmonary fibrosis is the excessive differentiation of fibroblasts into myofibroblasts (marked by α -smooth muscle actin, α -SMA) and the excessive deposition of ECM (eg, collagen). Under pathological conditions, abnormal wound healing will augment the transformation of fibroblasts into myofibroblasts and prolong the survival of these ECM-producing cells^[9]. Transforming growth factor- β 1 (TGF- β 1) is a major

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profibrotic mediator that converts fibroblasts to myofibroblasts and regulates the turnover of collagen^[10]. In addition, increasing evidence has revealed that hypoxia-inducible factor-1 α (HIF-1 α) is required for TGF- β 1-stimulated collagen production in pulmonary or other fibrotic diseases^[11–14].

HIF-1 is a master regulator of hypoxic signaling by activating gene transcription to mediate cellular adaption to hypoxia^[15, 16]. It is a heterodimeric transcription factor consisting of a constitutive β -subunit and an oxygen-sensitive α -subunit. Under normoxic conditions, HIF-1 α is subject to ubiquitination and proteosomal degradation. However, under hypoxic conditions, HIF-1 α is stabilized and translocates into the nucleus, where it dimerizes with HIF-1 β and cofactors to drive the transcription of various target genes in response to hypoxia^[17, 18]. Chronic hypoxia has been considered a critical event during the development of renal, lung and hepatic fibrosis^[19–22]. Hypoxia could induce the differentiation of fibroblasts into myofibroblasts^[23–25]. Therefore, targeting HIF-1 α may be a new therapeutic strategy for the treatment of fibrotic diseases^[26].

The root of *Stemona tuberosa* Lour, known as Baibu in Chinese, is one of traditional Chinese medicines with good anti-tussive, expectorant and anti-asthmatic effects^[27, 28]. Recent studies showed that the extract of *S. tuberosa* could inhibit subacute lung inflammation, attenuate alveolar airspace enlargement and bronchiole epithelial hyperplasia in a cigarette smoke-induced mouse model^[29]. Neotuberostemonine (NTS) is one of the main alkaloids isolated from *S. tuberosa* with significant antitussive activity that is comparable to codeine but does not involve opioid receptors^[28]. Our previous study showed that NTS effectively ameliorated bleomycin-induced lung fibrosis by suppressing the recruitment and activation of macrophages^[30].

However, fibroblast activation, which may be related to the polarization of M2 macrophages^[31], is a key step in the development of organ fibrosis. As mentioned above, chronic hypoxia is a critical event for fibrosis development. Therefore, in the present study, we aimed to investigate the role of hypoxia in the activation of lung fibroblasts and the effect of NTS on hypoxia-exposed fibroblasts.

Materials and methods

Reagents

Neotuberostemonine (C₂₂H₃₃NO₄, M_w 375.24) (Figure 1A) was isolated from the root of *S. tuberosa* in our lab^[32] with a purity above 98% by HPLC analysis. Bleomycin hydrochloride (BLM) was purchased from Nippon Kayaku (Tokyo, Japan). Prednisone acetate (PDN) and digoxin (Dig) were provided by Jiangsu Pingguang Pharmaceutical Co Ltd (Xuzhou, China) and Tianjin Shilan Technology Co Ltd (Tianjin, China), respectively. CoCl₂ was obtained from Chengdu Kelong Chemical Reagent Factory (Chengdu, China), and MG132 was purchased from APEXBio (Houston, TX, USA). The above drugs were dissolved in dimethylsulfoxide (DMSO) as a stock solution, and the final working concentration of DMSO was less than 0.1% (*v/v*). All other reagents, including DMSO, were of analytical purity and were commercially available.

Animals

Six- to eight-week-old ICR male mice weighing 20–25 g were purchased from the Comparative Medicine Center of Yangzhou University (Yangzhou, China). Mice were housed at a constant temperature of 25°C with alternatively 12 h light and dark cycles and kept on a standard mouse chow and water *ad libitum*. All animal experiments were conducted in accordance with the National Institutes of Health Guide. Care and use of animals complied with the Provisions and General Recommendation of the Chinese Experimental Animals Administration Legislation. Mice were allowed to acclimate for one week before any experiment and fasted on the day prior to experiments being conducted.

Cell experiments

Isolation, cultivation and identification of fibroblasts

Primary lung fibroblasts (PLFs) were isolated from male ICR mice by combining trypsin digestion and tissue adherent methods^[33, 34] with modifications. Briefly, mouse lungs were excised and removed of tracheas and bronchials. After heavily washing with phosphate-buffered saline (PBS), lungs were dissected into 1–2 cm²-sized pieces and digested with 2.5 mg/mL of trypsin (Biosharp, Hefei, China) in PBS at 25°C for 15 min. Digested lung pieces were suspended in Dulbecco's minimum essential (Eagle's) medium (DMEM) (Gibco, NY, USA) supplemented with 10% (*v/v*) fetal bovine serum (FBS) (Bioind, Israel), 100 μ g/mL streptomycin (Biosharp, Hefei, China) and 100 U/mL penicillin (Biosharp, Hefei, China) at 37°C in 5% CO₂ for 8 h before being placed on the bottom of the cell culture flask. The lung pieces placed in the flasks were cultured in the same medium (changed 3 times a week) to allow the PLFs to migrate and divide continuously to reach a confluence of 70%–80%. Isolated PLFs were cultured and purified for three passages in DMEM supplemented with 10% FBS and streptomycin/penicillin at 37°C in a 5% CO₂ atmosphere. PLFs were identified using vimentin immunohistochemical staining. PLFs of passages 3–5 with a purity greater than 95% (checked by the trypan-blue exclusion assay) were used for the experiments.

Treatment of PLFs

PLFs were seeded at an initial density of 1 \times 10⁵ cells/mL in 6-well plates for 24 h. After 12 h of starvation, the cells were treated with drugs at the indicated concentrations for 30 min and then exposed to hypoxia (1% O₂, 94% N₂, 5% CO₂) or treated with 100 μ mol/L CoCl₂ under normoxia. After 24 h of incubation, cells were harvested for the detection of relevant indicators.

Immunofluorescent assay

HypoxyprobeTM-1 (Hypoxyprobe Inc, Burlington, USA) is a substituted 2-nitroimidazole named pimonidazole. It will bind to cells if the *p*O₂ levels are less than 10 mmHg, so it is often used as a probe for detecting cell hypoxia. PLFs were cultured to 80% confluence. After 12 h of starvation, PLFs were incubated with 200 μ mol/L hypoxyprobe and 10 μ mol/L

NTS under normoxia, hypoxia (1% O₂) or 100 μmol/L CoCl₂ treatment at 37 °C for 12 h. PLFs were fixed and blocked with 3% bovine serum albumin (BSA), then treated with anti-hypoxypore and DAPI. The blank control was treated in the same manner. All the treated PLFs were examined under confocal scanning microscopy.

Transfection with HIF-1α siRNA

NIH-3T3 cells (Passage 9, Cell Bank of the Chinese Academy of Sciences, Shanghai, China) grown to 70%–80% confluence were transfected with mouse HIF-1α siRNA (Santa Cruz, CA, USA) according to the manufacturer's instructions. After 8 h of transfection, the cells were cultured in fresh medium for 48 h and exposed to hypoxia (1% O₂, 94% N₂, 5% CO₂) or treated with 100 μmol/L CoCl₂ under normoxia for 24 h and then collected for the indicated experiments.

Animal experiments

BLM-induced pulmonary fibrosis and drug treatment design

ICR male mice were randomly divided into 5 groups (*n*=20), *ie*, the sham, model, NTS, PND and Dig groups. After 12 h of fasting, mice were intratracheally injected with BLM (3.5 U/kg in 0.9% NaCl) or 0.9% NaCl (sham group) after anesthesia with 4% chloral hydrate (10 mL/kg). After 7 d of model formation, BLM-treated mice were orally administered NTS (30 mg/kg), PDN (6.5 mg/kg) and an equivalent volume of the same menstruum (sham and model group) or intraperitoneally injected with Dig (1 mg/kg) once a day for 7 or 14 consecutive days. The lungs were excised on d 15 and 22 after BLM treatment.

Histopathological examination

Lung specimens fixed in 4% paraformaldehyde and embedded in paraffin were cut into 5-μm-thick sections and stained with hematoxylin & eosin (H&E) or Masson trichrome. The slides were examined under an Olympus BX53TR microscope and photographed with a DP72 digital color camera (Olympus, Tokyo, Japan). The contents of absolute collagen were semi-quantified by determining the integrated optical density (IOD) of at least five sections each in 10 microscopic fields using Image-Pro Plus image analysis software (version 6.0, Media Cybernetics, USA)^[35, 36].

Immunohistochemical analysis

For immunohistochemical analysis, 5-μm-thick sections as mentioned above were dewaxed and rehydrated, quenched with 3% hydrogen peroxide, incubated in 0.1% citric buffer for

antigen retrieval and blocked with 3% BSA. After pretreatment, sections were incubated with rabbit anti-fibronectin (BS1644, Bioworld, St Louis, MO, USA) antibodies using reagents from an Elivision™ super HRP IHC Kit (Maixin Biotech, Fuzhou, China) and developed with a DAB Kit (Maixin Biotech, Fuzhou, China).

Quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA of PLFs or lung tissue was extracted using TRIzol Reagent (Kangweishiji, Beijing, China) and reverse-transcribed to cDNA templates using an RT-MasterMix Kit (Applied Biological Materials Inc, Vancouver, Canada). Quantitative RT-PCR was performed using SYBR Green Supermix (BIO-RAD, CA, USA). Samples were measured in at least triplicate with a CFX96 real-time system (BIO-RAD, CA, USA). The specific gene primers for Col (collagen)-1α, Col-3α, LOX (lysyl oxidase), HIF-1α and β-actin were designed using Primer5 software by PRIMER-E Ltd (Plymouth, Devon, UK) (Table 1). Mouse β-actin was used as an internal control. All sample data were quantified using the comparative Ct method and presented as the mean ratio to β-actin.

Western blot analysis

Immunoblot analysis of PLFs or lung tissue was performed on total lysates as previously described^[30] using primary rabbit anti-α-SMA (smooth muscle actin) (YH090708D, Epitomics, Burlingame, USA), rabbit anti-HIF-1α (ab51608, Abcam, Cambridge, UK), rabbit anti-TGF-β (transforming growth factor-β) (#3711, Cell Signaling Technology, Beverly, USA), rabbit anti-FGF-2 (fibroblast growth factor-2) (ab8880, Abcam, Cambridge, UK), rabbit anti-FGFR-2 (fibroblast growth factor receptor-2) (ab109372, Abcam, Cambridge, UK), rabbit anti-hydroxy-HIF-1α (Pro564) (#3434, Cell Signaling Technology, Beverly, USA) or mouse anti-β-actin (BS6007M, Bioworld Technology, St Louis, MO, USA) antibodies and then incubated with corresponding secondary antibodies. Membranes were developed with enhanced chemiluminescence (ECL) reagent (Beyotime, Shanghai, China). The band intensity was quantitatively analyzed with Image-Pro Plus (IPP) 6.0 software and presented as the mean ratio to β-actin.

Statistical analysis

Data were expressed as the mean±SD (standard deviation) of at least three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison *post hoc* tests (GraphPad Prism 5,

Table 1. Sequences of real-time PCR primers.

Gene	Forward primer	Reverse primer
Col-1α1	5'-AATGGTGCTCCTGGTATTGC-3'	5'-ACTGTTACCCCTGGGACCTG-3'
Col-3α1	5'-CAAGATCTCGGCGAAGCAA-3'	5'-GGTGAGCCTCATAACAGAAGCTTT-3'
LOX	5'-CTCAATGGCTCACCAGGAC-3'	5'-CACCAGGACTGCCGTTATTC-3'
HIF-1α	5'-CAAGATCTCGGCGAAGCAA-3'	5'-GGTGAGCCTCATAACAGAAGCTTT-3'
β-Actin	5'-ACGGATGTCAACGTCACACT-3'	5'-CACTATTGGCAACGAGCGGTTCCG-3'

GraphPad Software, USA). *P*-values less than 0.05 were considered statistically significant.

Results

NTS suppresses the activation of hypoxia-exposed lung fibroblasts

To determine the effect of hypoxia on lung fibroblasts and the effect of NTS (Figure 1A) on hypoxia-exposed lung fibroblasts, PLFs were exposed to 1% O₂ or 100 μmol/L CoCl₂ (a model of chemical hypoxia) and treated with or without NTS and digoxin (positive control). Both 1% O₂ and CoCl₂ treatments significantly increased the expression of α-SMA, a marker of myofibroblasts, indicating that the PLFs had been activated (Figure 1B, 1C). NTS treatment significantly reduced the 1% O₂-induced α-SMA levels at concentrations of 1.0 and 10 μmol/L (Figure 1B) and obviously reduced the CoCl₂-induced α-SMA levels at concentrations of 0.1, 1.0 and 10 μmol/L (Figure 1C). Correspondingly, NTS decreased the hypoxia-induced mRNA expression of Col-1α, Col-3α and LOX (a critical enzyme in the covalent cross-linkages of collagen and elastin) at 1.0 and 10 μmol/L (Figure 1D). Expectedly, digoxin, a HIF-1α inhibitor, effectively reduced the protein expression of α-SMA and the mRNA expression of Col-1α, Col-3α and LOX after hypoxia/chemical hypoxia exposure. Taken together, these results demonstrated that hypoxia can directly activate

fibroblasts and differentiate them into myofibroblasts that produce ECM, while NTS inhibits collagen synthesis in hypoxia-exposed PLFs by suppressing the activation and differentiation of fibroblasts.

NTS decreases HIF-1α expression in hypoxia-induced lung fibroblasts

HIF-1α is a master regulator that mediates cellular adaption to hypoxia. For the two models mentioned above, 1% O₂ induces HIF-1α accumulation by lowering local oxygen tension, while CoCl₂ stabilizes HIF-1α by inhibiting the activity of HIF-1 prolyl hydroxylases (not true hypoxia). Therefore, we first detected whether hypoxia was present in the cells of the above two models. PLFs were incubated with hypoxypromote (pimonidazole) and stained using an anti-hypoxypromote antibody. As expected, pimonidazole adducts were clearly observed in cells exposed to 1% O₂ but were rarely found in cells treated with CoCl₂ (Figure 2A). Surprisingly, a reduction of adducts was observed with NTS (10 μmol/L) treatment, indicating that NTS can protect cells from hypoxia via an unknown mechanism. Next, we examined the expression of HIF-1α by Western blot analysis. The HIF-1α level was significantly increased in cells exposed to 1% O₂ or CoCl₂ (100 μmol/L) compared with the control, but NTS reduced the expression of HIF-1α in a dose-dependent manner in both models (Figure 2B, 2C).

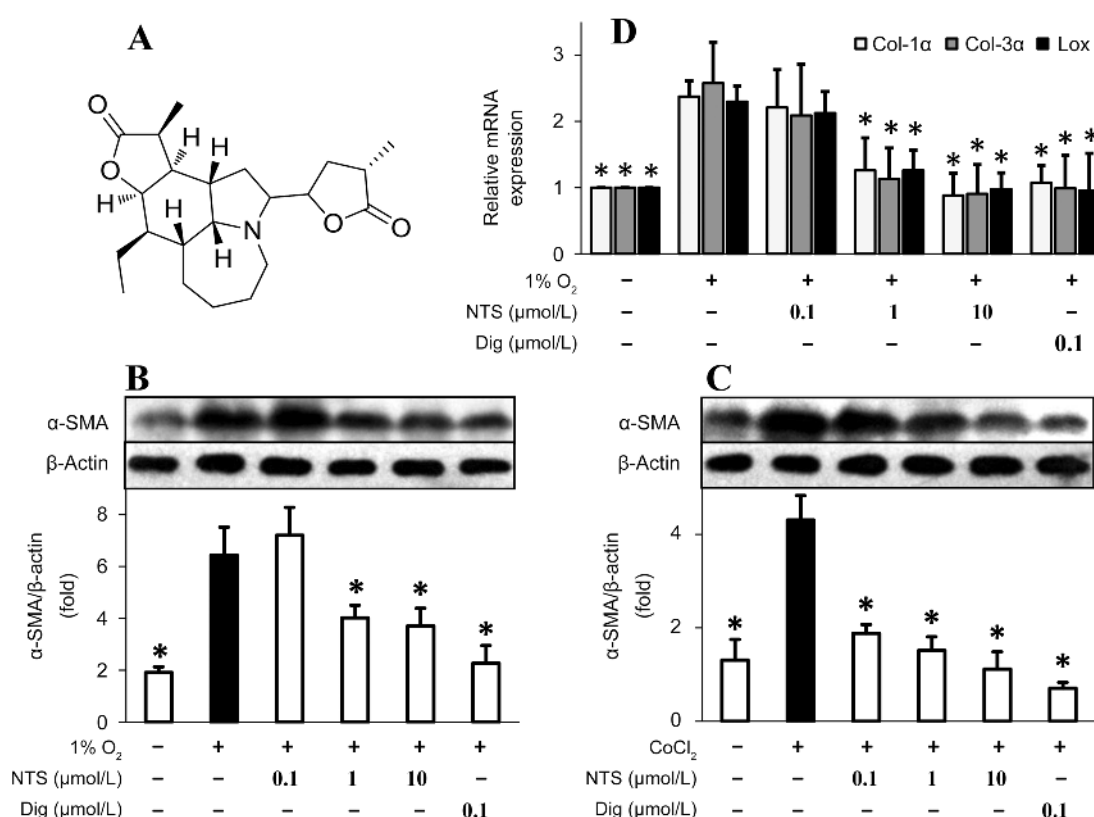


Figure 1. NTS suppressed the activation of PLFs under hypoxia. (A) Structure of NTS; (B–D) PLFs were treated with NTS or digoxin (Dig) at indicated concentrations and then incubated under 1% O₂ or with 100 μmol/L CoCl₂ under normoxia for 24 h; (B, C) the protein expression of α-SMA was determined by Western blot (*n*=3); and (D) the mRNA expression of Col-1α, Col-3α and Lox (lysyl oxidase) was assayed by RT-PCR (*n*=4). Data were expressed as the mean±SD. **P*<0.05 vs the 1% O₂ or CoCl₂ treatment.

Digoxin, a HIF-1 α inhibitor, also displayed positive effects in both models (Figure 2B, 2C). The above results showed that NTS prevents the activation of hypoxia-exposed lung fibroblasts by improving hypoxic microenvironments and promoting the decomposition of HIF-1 α .

NTS decreases the expression of TGF- β and FGF2/FGFR2 in hypoxia-exposed lung fibroblasts

TGF- β , FGF2 and FGFR2 are involved in the activation of fibroblasts^[37, 38]; thus, we examined the activity of TGF- β and FGF2/FGFR2 in hypoxia-exposed PLFs and the effect of NTS on them. In fibroblasts challenged with 1% O₂ (Figure 3A–3D), the protein expression of TGF- β , FGF2 and FGFR2

was increased significantly. However, NTS treatment effectively decreased the expression of TGF- β (at 1–10 μ mol/L), FGF2 (at 0.1–10 μ mol/L) and FGFR2 (at 1–10 μ mol/L). Similarly, in fibroblasts stimulated with CoCl₂ (Figure 3E–3H), the expression of TGF- β , FGF2 and FGFR2 was also significantly elevated, and this elevated expression was effectively inhibited by NTS at 10 μ mol/L (for TGF- β) or 1–10 μ mol/L (for both FGF2 and FGFR2). The above results demonstrated that hypoxia or chemical hypoxia induced increased expression of TGF- β , FGF2 and FGFR2 but that NTS could reverse the elevations in expression to suppress fibroblast activation. As an inhibitor of HIF-1 α , digoxin also blunted 1% O₂- or CoCl₂-exposed changes in TGF- β , FGF2 and FGFR2 activity (Figure

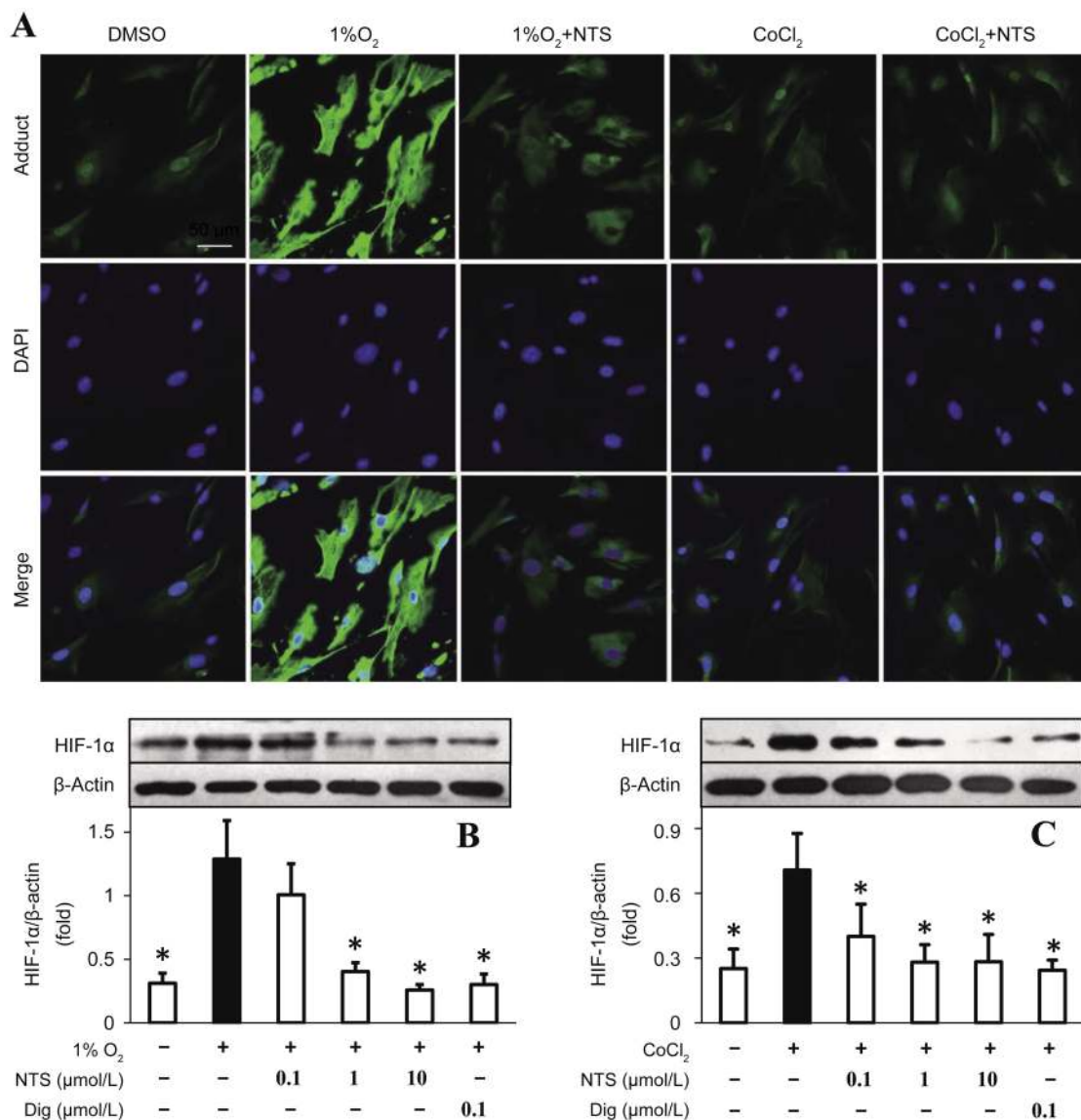


Figure 2. NTS decreased HIF-1 α levels by improving hypoxic microenvironments and promoting decomposition in hypoxia-exposed PLFs. (A) PLFs were treated with hypoxypromoter (200 μ mol/L) and NTS (10 μ mol/L), incubated under 1% O₂ or with 100 μ mol/L CoCl₂ under normoxia for 12 h, then treated with anti-hypoxypromoter antibody and DAPI, observed under confocal scanning microscopy ($\times 200$). (B, C) PLFs were treated with NTS or digoxin (Dig) at the indicated concentrations and incubated under the same hypoxic conditions mentioned above for 24 h. The protein expression of HIF-1 α was determined by Western blot ($n=3$). Data were expressed as the mean \pm SD. * $P<0.05$ vs the 1% O₂ or CoCl₂ treatment.

3), indicating that the up-regulation of TGF- β , FGF2 and FGFR2 might be dependent, at least in part, on the accumulation of HIF-1 α .

HIF-1 α is essential for the induction of TGF- β and FGF2 under hypoxia

To further dissect the role of HIF-1 α on the protein expression of TGF- β , FGF2 and α -SMA under hypoxia, NIH-3T3 cells, a mouse embryo fibroblast cell line, were transfected with HIF-1 α siRNA plasmid to silence endogenous HIF-1 α . HIF-1 α induction responding to 1% O₂ or CoCl₂ was successfully counteracted by specific HIF-1 α siRNA transfection in NIH-3T3 cells (Figure 4A, 4B). Correspondingly, the expression of α -SMA, TGF- β and FGF2 in cells transfected with HIF-1 α siRNA plasmids (Figure 4A, 4C–4F) was not increased after exposure to 1% O₂ or CoCl₂, although their expression was significantly increased in cells transfected with scramble siRNA plasmids. These results demonstrated that HIF-1 α is essential

for the induction of TGF- β and FGF2 to activate and differentiate fibroblasts into myofibroblasts (marked by α -SMA).

NTS inhibits the activation of lung fibroblasts by promoting the degradation of HIF-1 α

Given that HIF-1 α is essential for fibroblast activation and NTS inhibited the induction of HIF-1 α , TGF- β and FGF2 under hypoxia, we hypothesized that NTS inhibits the activation of lung fibroblasts in a HIF-1 α -dependent manner. Therefore, the PLFs exposed to 1% O₂ or CoCl₂ were treated with or without NTS and MG132 (a proteasome inhibitor blocking the degradation of HIF-1 α), and the protein expression of HIF-1 α , α -SMA, TGF- β and FGF2 was analyzed by Western blot. As expected, the inhibitory effect of NTS (10 μ mol/L) on HIF-1 α accumulation (Figure 5A, 5B, 5F, 5G) was absolutely abolished in both hypoxic models when MG132 (10 μ mol/L) was used. Accordingly, the inhibitory effect of NTS on the expression of α -SMA, TGF- β and FGF2 (Figure 5A, 5C–5E, 5F, 5H–5J) was

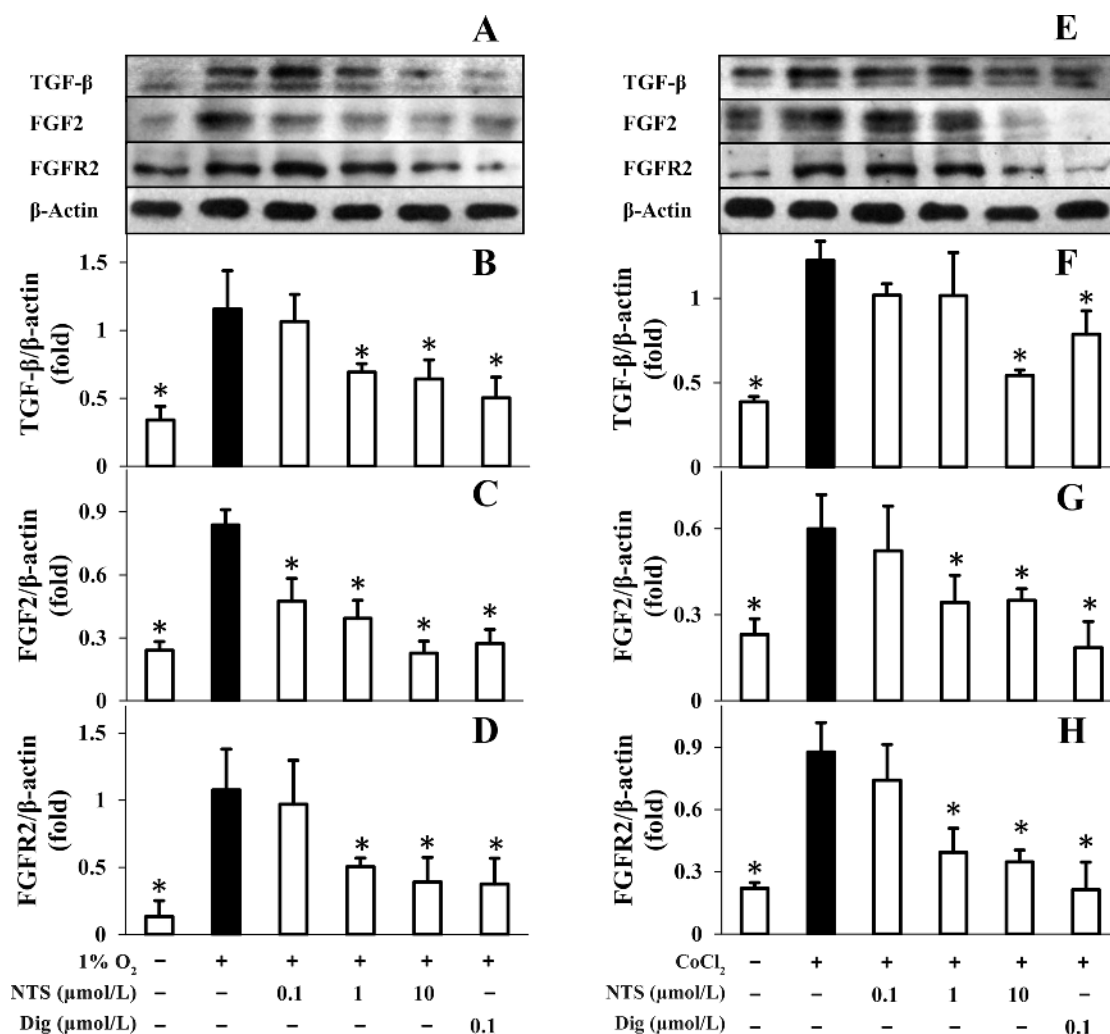


Figure 3. NTS decreased the expression of TGF- β , FGF2 and FGFR2 in hypoxia-exposed PLFs. PLFs were treated with NTS or digoxin (Dig) at the indicated concentrations, incubated under 1% O₂ or with 100 μ mol/L CoCl₂ under normoxia for 24 h. The protein expression of TGF- β , FGF2 and FGFR2 in cells with (A–D) 1% O₂ or (E–H) CoCl₂ treatment was determined by Western blot ($n=3$). Data were expressed as the mean \pm SD. * $P<0.05$ vs the 1% O₂ or CoCl₂ treatment.

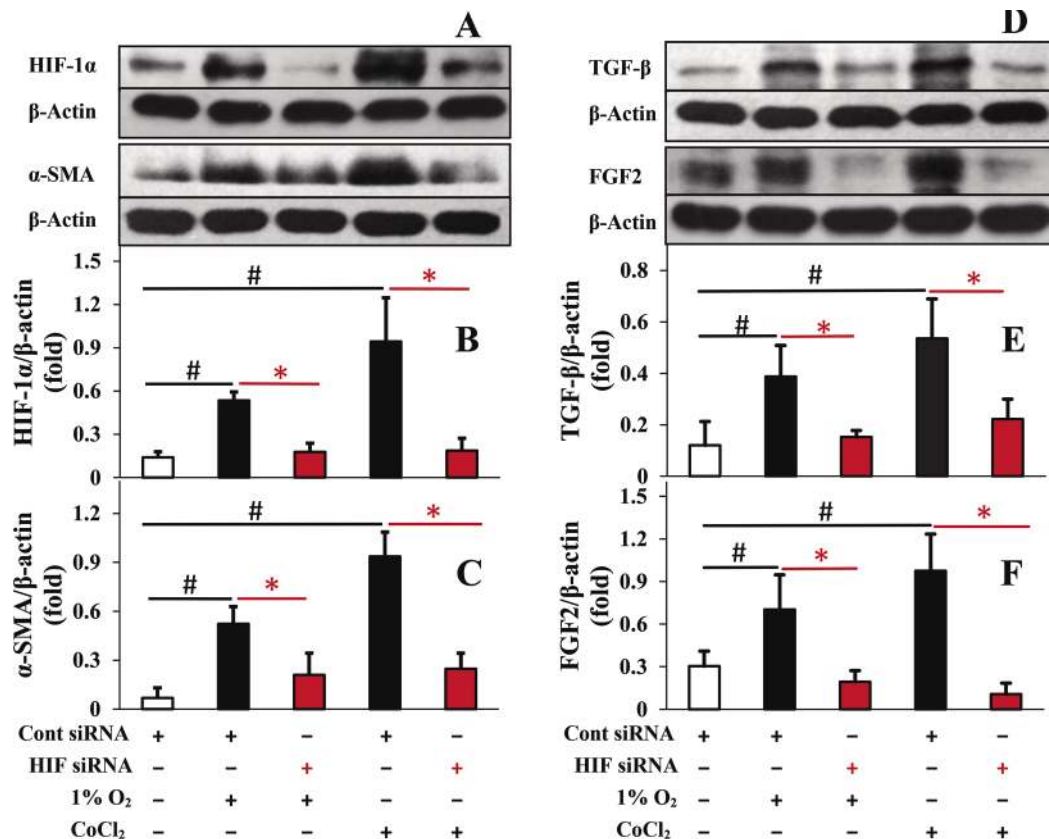


Figure 4. HIF-1 α was essential for the induction of TGF- β and FGF2 under hypoxic conditions. NIH-3T3 cells were transfected with HIF-1 α or control scrambled siRNAs and then incubated under 1% O₂ or with 100 μ mol/L CoCl₂ under normoxia for 24 h. (A–F) The protein expression of HIF-1 α , α -SMA, TGF- β and FGF2 was determined by Western blot ($n=3$). Data were expressed as the mean \pm SD. # $P<0.05$ or * $P<0.05$ vs the indicated treatment.

also completely reversed by co-treatment with MG132 in both hypoxic models. This suggested that NTS suppresses the activation of fibroblasts under hypoxia in a HIF-1 α -dependent manner.

Moreover, it is well known that prolyl hydroxylation is a critical step in the initiation of HIF-1 α degradation. Thus, the protein expression of PHD (prolyl hydroxylated)-HIF-1 α in lung fibroblasts exposed to 1% O₂ or CoCl₂ was also examined. The level of PHD-HIF-1 α decreased markedly under 1% O₂ and CoCl₂ conditions, but this decrease was effectively reversed by NTS (10 μ mol/L) (Figure 5K). This result indicated that NTS promotes degradation of HIF-1 α partially due to its improvement on HIF-1 α prolyl hydroxylation.

NTS inhibits HIF-1 α , TGF- β and FGF2 expression in the lungs of BLM-induced mice

IPF patients universally exhibit hypoxemia in association with increased levels of HIF-1 α ^[16]. To determine whether hypoxia was present in BLM-induced mice, the protein and mRNA expression of HIF-1 α in lung tissue was detected. Compared with sham mouse lungs (Figure 6A, 6B), both HIF-1 α protein and mRNA were significantly increased in BLM-induced mouse lungs, especially mRNA expression after 14 days of bleomycin administration. Our previous study found that NTS had a weak inhibitory effect at 15 mg/kg and a strong

inhibitory effect at 30 or 60 mg/kg on mouse lung fibrosis; however, the mortality of high-dose-treated animals was higher. Therefore, the medium dose (30 mg/kg) was chosen as the experimental dose. After 7 days of model formation, BLM-induced mice were administered with NTS and positive drugs for 7 or 14 consecutive days. After 7 and 14 days of treatment, NTS effectively reduced the protein and mRNA expression of HIF-1 α (Figure 6A, 6B). Although PDN and digoxin also reduced the levels of HIF-1 α , PDN destroyed the normal structure of lung tissue. Further, the changes in TGF- β and FGF2 in BLM-induced mouse lungs before and after drug administration were also analyzed by Western blotting. Compared to the control, the increased protein expression of FGF2 was significantly inhibited by NTS in both the 7- and 14-day treatments (Figure 6C). However, NTS reduced the expression of TGF- β only after a long treatment period (21 days = 7 days model formation + 14 days treatment) (Figure 6D).

NTS inhibits ECM accumulation in the lungs of BLM-induced mice

Our *in vitro* experiments demonstrated that NTS reduced the deposition of ECM by inhibiting the activation of fibroblasts, which needed to be confirmed by experiments *in vivo*. Thus, we investigated the effects of NTS on histopathological changes and ECM accumulation in the lungs of BLM-induced mice. Compared with the sham mouse lungs, BLM-injured

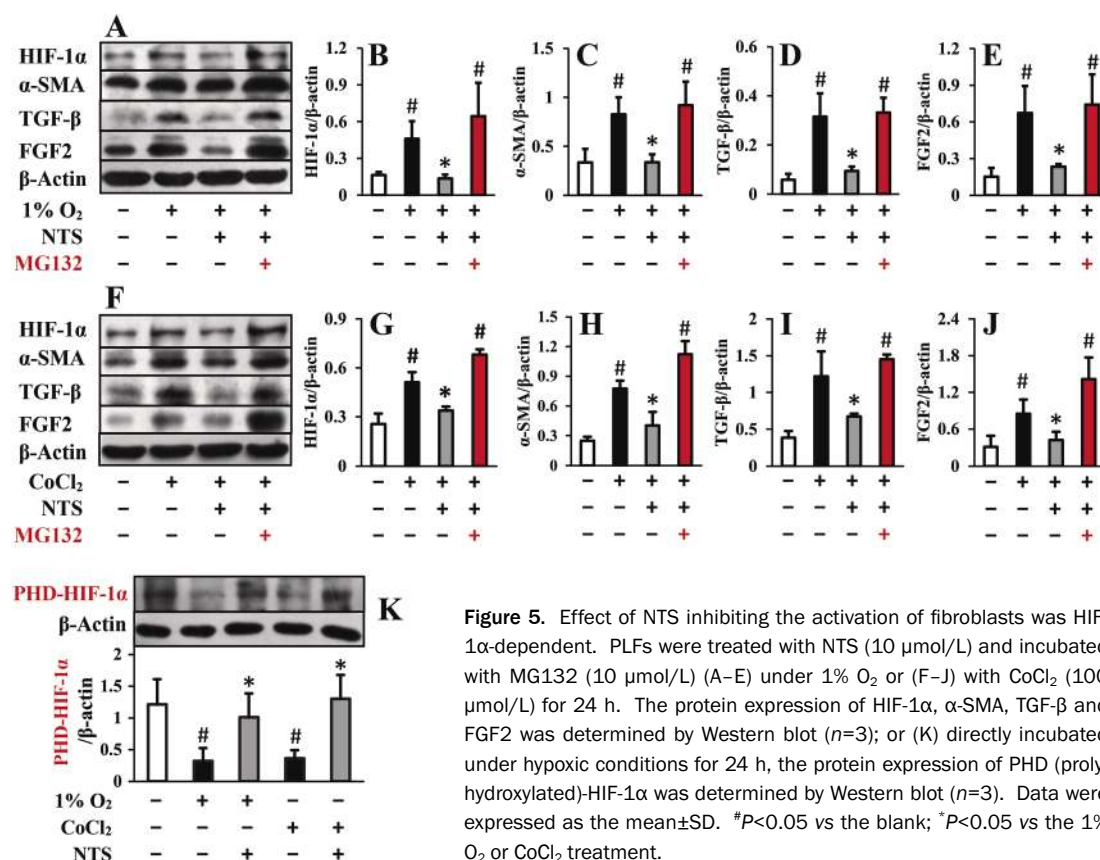


Figure 5. Effect of NTS inhibiting the activation of fibroblasts was HIF-1 α -dependent. PLFs were treated with NTS (10 μ mol/L) and incubated with MG132 (10 μ mol/L) (A–E) under 1% O₂ or (F–J) with CoCl₂ (100 μ mol/L) for 24 h. The protein expression of HIF-1 α , α -SMA, TGF- β and FGF2 was determined by Western blot ($n=3$); or (K) directly incubated under hypoxic conditions for 24 h, the protein expression of PHD (prolyl hydroxylated)-HIF-1 α was determined by Western blot ($n=3$). Data were expressed as the mean \pm SD. [#] $P<0.05$ vs the blank; ^{*} $P<0.05$ vs the 1% O₂ or CoCl₂ treatment.

lungs showed enhanced infiltration of inflammatory cells, the loss of normal alveolar structure, extensive thickening of the interalveolar septa, and extremely high Ashcroft scores (Figure 7A, 7C). NTS treatment for 7 or 14 days could effectively reduce BLM-induced histopathological changes and lower the Ashcroft scores in the lungs (Figure 7A, 7C). Masson trichrome staining and IOD values showed that BLM challenge led to substantial interstitial fibrosis in lung tissue with increased collagen deposition (indicated by blue staining) but treatment with NTS for 7 or 14 days significantly attenuated the collagen deposition in the lungs (Figure 7B, 7D). Moreover, the protein expression of α -SMA (Figure 7E) and the mRNA expression of Col-1 α , Col-3 α and LOX (Figure 7F, 7G) were also markedly elevated in the lungs of BLM-induced mice, and the administration of NTS for 7 or 14 days effectively reduced the levels of these factors, except that Col-1 α was reduced only after a long (14-day) treatment.

Fibronectin, a high-molecular-weight glycoprotein of the extracellular matrix, is often secreted from fibroblasts. It assembles extracellular matrix components such as collagen, fibrin, and heparan sulfate proteoglycan. Thus, the protein expression of fibronectin in BLM-induced mice was detected by immunofluorescence assay. The green-colored fibronectin was strongly increased in the lungs of BLM-induced mice, while the over-expression of fibronectin was obviously inhibited by NTS treatment for 7 and 14 days (Figure 7H).

Taken together, the above results showed that NTS can effectively inhibit fibroblast activation and prevent ECM depo-

sition *in vivo*.

Discussion

Although pirfenidone and nintedanib can slow lung function decline in IPF, lung transplantation remains the only curative treatment for this devastating disease. It is imperative to discover and develop more effective therapies and to provide more therapeutic options for patients. Our previous studies have demonstrated that NTS, an alkaloid isolated from *S. tuberosa*, effectively ameliorated BLM-induced lung fibrosis in mice^[30]. The present study investigated the effect and underlying mechanism of NTS on the activation of lung fibroblasts under hypoxia. *In vitro* experiments demonstrated that lung fibroblasts were activated and differentiated into myofibroblasts under hypoxic or chemical hypoxic conditions, whereas NTS blocked this activation and differentiation in a HIF-1 α -dependent manner. Accordingly, *in vivo* experiments confirmed that NTS attenuates BLM-induced pulmonary fibrosis by inhibiting the levels of HIF-1 α and related downstream cytokines such as TGF- β and FGF2.

As discussed earlier, fibrosis is increasingly regarded as the result of deregulated tissue repair in response to chronic hypoxia^[19–22], and severe chronic hypoxia is overt in involved tissues of patients with fibrotic disease^[26]. Significant expression of HIF-1 α was observed in the lung samples of IPF patients, especially of those with pulmonary hypertension^[16, 39]. It was also reported that hypoxia robustly stimulated the proliferation and differentiation of IPF fibroblasts^[40, 41]. Our experiment

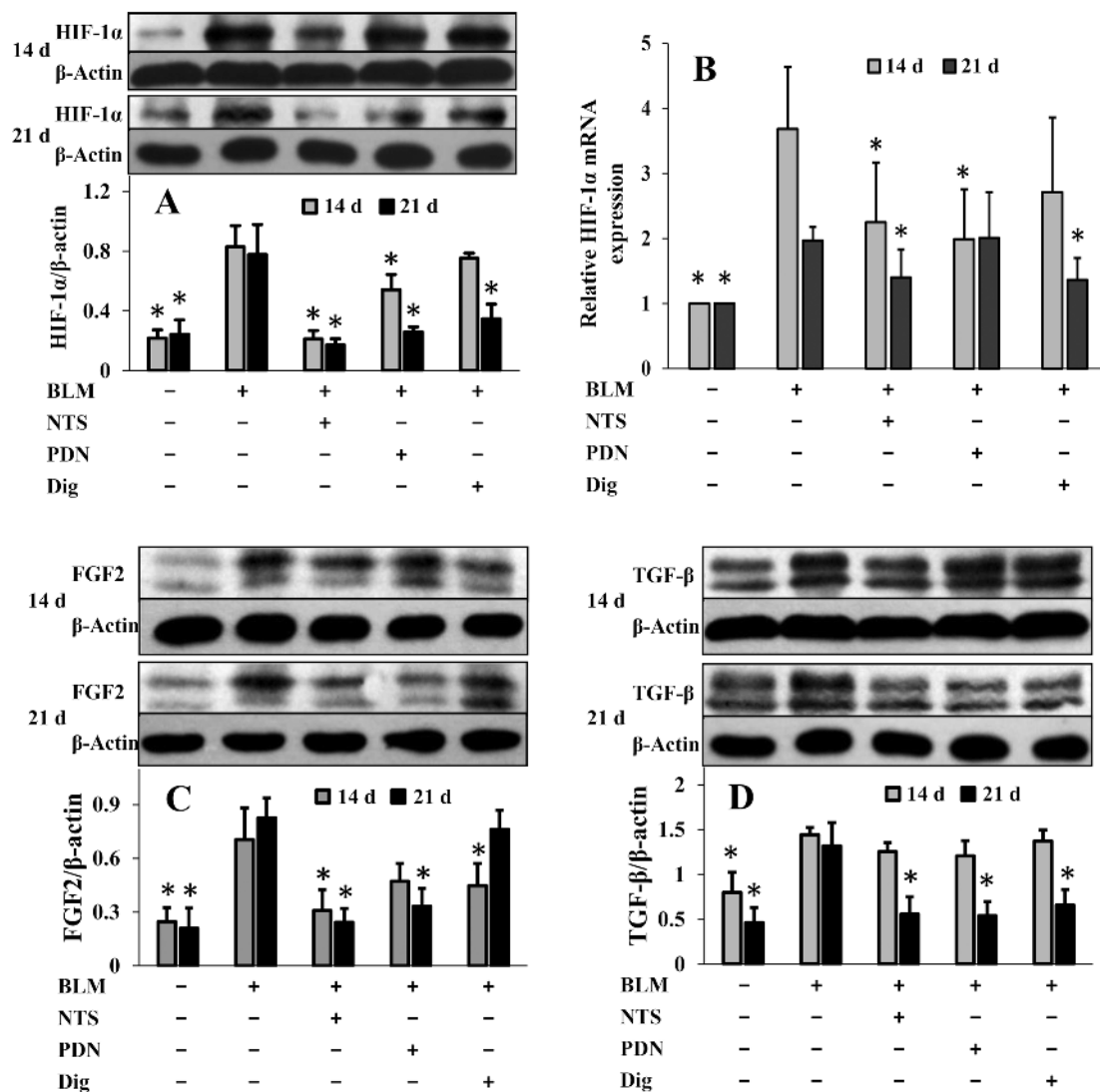


Figure 6. NTS inhibited HIF-1 α , TGF- β and FGF2 expression in BLM-induced pulmonary fibrosis mice. Mice were administered with NTS (30 mg/kg, orally) per day from days 8 to 14 or to 21 after BLM intratracheally administration (3.5 U/kg), taking PDN (prednisone, 6.5 mg/kg, orally) and Dig (digoxin, 1 mg/kg, ip) as the positive controls. (A, C, D) The protein expression of HIF-1 α , FGF2 and TGF- β in lungs was determined by Western blot ($n=3-4$), and (B) the mRNA expression of HIF-1 α was measured by RT-PCR ($n=4$). Data were expressed as the mean \pm SD. * $P<0.05$ vs the model.

demonstrated that NTS could effectively alleviate the hypoxic conditions in pulmonary fibroblast cells.

Inhibition of the pathways regulated by pro-fibrotic cytokines, such as TGF- β , FGF, VEGF and PDGF, is thought to be an effective approach to the treatment of pulmonary fibrosis^[42]. As a potent pro-fibrotic growth factor, TGF- β directly affects the proliferation and differentiation of fibroblasts^[43], but the underlying mechanism is still not completely established. TGF- β 1 can up-regulate HIF-1 α expression in MRC-5 cells (human fetal lung fibroblasts) at both the protein and mRNA levels^[41]. Our *in vitro* results showed that the stabilized HIF-1 α exposed to hypoxia or chemical hypoxia greatly up-regulated TGF- β proteins, supporting that HIF-1 α and TGF- β may form a positive feedback loop during the development of pulmonary fibrosis^[14].

FGF2 is released by activated fibroblasts. It stimulates the

proliferation of lung fibroblasts and the production of fibronectin^[38, 44]. Moreover, TGF- β 1-induced proliferation of fibroblasts is regulated through the release of extracellular FGF2^[38]. Therefore, blocking the FGF2/FGFR2 signaling pathway may be required for intervention in the proliferation and differentiation of fibroblasts. Our data showed that the high expression of HIF-1 α in hypoxia-exposed fibroblasts led to a great elevation of FGF2 and FGFR2 protein levels, together with significantly increased α -SMA, Col-1 α and Col-3 α expression.

However, all the elevation of TGF- β , FGF2 and α -SMA expression after exposure to 1% O₂ or CoCl₂ was reversed after the transfection of HIF-1 α , suggesting that hypoxia-induced HIF-1 α is the key upstream regulator of TGF- β and FGF2 signaling. Importantly, NTS exerts protective effects against experimental pulmonary fibrosis by regulating HIF-1 α signaling. NTS significantly inhibited the protein expression of

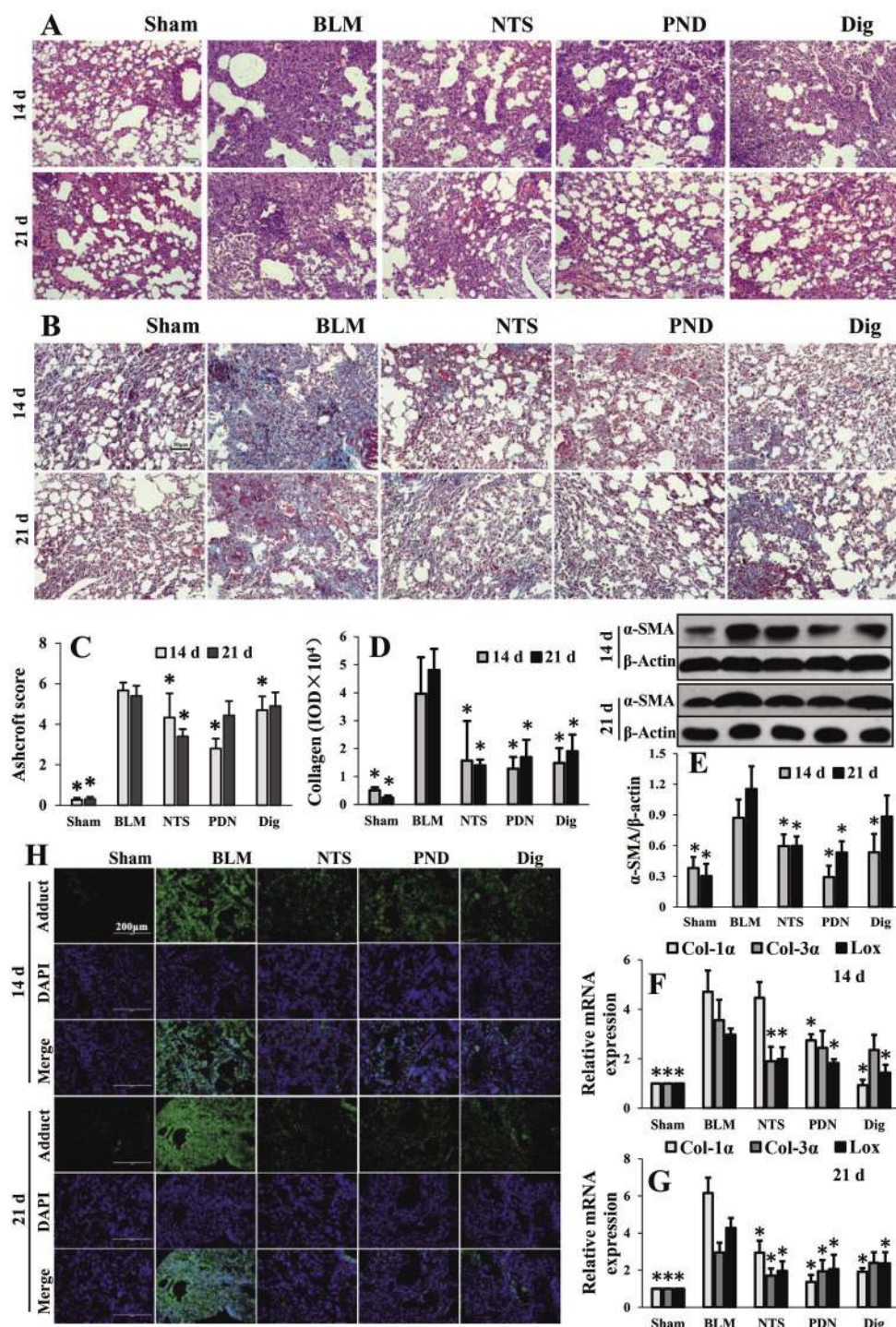


Figure 7. NTS inhibited ECM accumulation in BLM-induced pulmonary fibrosis. Mice were administered with NTS (30 mg/kg, orally) per day from days 8 to 14 or to 21 after BLM intratracheally administration (3.5 U/kg), taking PND (prednisone, 6.5 mg/kg, orally) and Dig (digoxin, 1 mg/kg, ip) as the positive controls. (A, B) Lung tissue was stained with HE or Masson trichrome (collagen in blue color) and (C, D) semiquantitatively assessed by Ashcroft score for histopathological change ($n=3$) and integrated optical density (IOD) for collagen deposition ($n=6$). (E) The protein expression of α -SMA and (F-G) the mRNA expression of Col-1 α , Col-3 α and LOX (lysyl oxidase) were determined by Western blot ($n=3$) or qPCR ($n=4$). (H) The expression of fibronectin in lung tissue was examined by immunofluorescence staining. Data were expressed as the mean \pm SD. * $P<0.05$, *** $P<0.001$ vs the model.

HIF-1 α , TGF- β and FGF2 both in hypoxia-exposed lung fibroblasts and in BLM-induced lung tissue. The inhibitory effect of NTS on HIF-1 α , TGF- β and FGF2 led to an inhibitory effect

on ECM deposition. These results suggested that NTS ameliorates pulmonary fibrosis by at least partially targeting HIF-1 α .

Molecular oxygen is a cofactor for prolyl hydroxylases

that hydroxylate HIF-1 α protein at positions P564 and P402. Hydroxylated HIF-1 α protein is recognized by the VHL-E3 ligase, which transports HIF-1 α to the proteasome for degradation^[45-47]. Low oxygen levels inhibit the prolyl hydroxylation at positions P564 and P402 and allow HIF-1 α to escape degradation and enter the nucleus to drive the expression of target genes^[48]. Consistent with the above, PHD (prolyl hydroxylated)-HIF-1 α was obviously inhibited under hypoxic/chemical hypoxic conditions, while NTS effectively elevated the PHD-HIF-1 α levels. Proteasomes are the executor to degrade the protein of HIF-1 α . It was reported that HIF-1 α can be degraded by proteasomes through ubiquitin-dependent or ubiquitin-independent pathways^[49, 50]. Our experiments showed that the inhibitory effects of NTS on hypoxia-exposed HIF-1 α could be abolished by co-treatment with proteasome inhibitors. Thus, we speculated that the effect of NTS on the promotion of HIF-1 α degradation may be achieved by increasing the activity of both prolyl hydroxylases and proteasomes. This speculation requires further studies to draw conclusions.

Our results highlight that the activation of lung fibroblasts under hypoxia is HIF-1 α dependent and HIF-1 α will stimulate downstream profibrotic cytokines such as TGF- β and FGF2/FGFR2. NTS, a natural compound from *S. tuberosa*, effectively inhibited the activation of lung fibroblasts and ameliorated mouse pulmonary fibrosis by alleviating hypoxic conditions and inhibiting HIF-1 α signaling. This finding suggested that NTS or related small molecules, which can improve the cell hypoxic milieu and promote HIF-1 α degradation, possibly representing a promising novel therapeutic approach for pulmonary fibrosis treatment^[26]. However, the therapeutic mechanism of NTS for pulmonary fibrosis may not be limited to HIF-1 α signaling. Moreover, the upstream factors of HIF-1 α in BLM-induced mice and the manner in which HIF-1 α induces profibrotic cytokines, especially TGF- β , require further investigation.

In conclusion, hypoxia, which stabilizes HIF-1 α , is an early event for the activation of lung fibroblasts, and NTS, or related drugs with activities that improve hypoxic conditions and promote HIF-1 α degradation, may be a novel pharmacological approach in the treatment of pulmonary fibrosis. However, there is still an unmet need for selective, effective and safe anti-lung fibrosis drugs.

Abbreviations

α -SMA, α -smooth muscle actin; BLM, bleomycin; BSA, bovine serum albumin; Col, collagen; Dig, digoxin; DMEM, Dulbecco's minimum essential medium; DMSO, dimethylsulfoxide; ECM, extracellular matrix; FBS, fetal bovine serum; FGF2, fibroblast growth factor 2; FGFR2, fibroblast growth factor receptor 2; HIF-1 α , hypoxia inducible factor-1 α ; IOD, integrated optical density; IPF, idiopathic pulmonary fibrosis; NTS, neotuberostemonine; PDN, prednisone acetate; PLFs, primary mouse lung fibroblasts; RT-PCR, real-time polymerase chain reaction; TGF- β 1, transforming growth factor- β 1; VEGF, vascular endothelial growth factor.

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Author contribution

Ming-dan LI, Bao-lin LIU and Mian ZHANG designed the study; Kang LIU, Chao-feng ZHANG and Xiang-hong XU participated in the experimental design; Xin-miao LV and Ming-dan LI conducted the experiments; Si CHENG participated in the animal experiments and analyzed experimental data; Chao-feng ZHANG and Xiang-hong XU conducted data analysis; Xin-miao LV and Ming-dan LI summarized the experimental data and drafted the paper; Mian ZHANG, Bao-lin LIU and Kang LIU revised the paper.

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