Nintedanib Ameliorates Lipopolysaccharide-Induced Pulmonary Fibrosis after Acute Respiratory Distress Syndrome through Suppression of VEGF/PDGF/FGF


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Abstract

Pulmonary fibrosis after acute respiratory distress syndrome (ARDS) is a fatal disease. This study aimed to investigate the effect of nintedanib treatment on lipopolysaccharide (LPS)-induced mouse lung fibrosis after ARDS in vitro and in vivo. In vitro, nintedanib effectively inhibited the expression of both TGF-β1 and α-SMA after the LPS treatment in mouse lung fibroblasts. In vivo, nintedanib administrations at both early and late stages after LPS challenged protected the mice from lung fibrosis. First, nintedanib treatment resulted in a dramatic decrease in inflammatory cells in BAL fluid. Second, compared to the untreated mice with server lung fibrosis after LPS-induced ARDS, nintedanib treatment significantly reduced the levels of both lung tissue damage and collagen deposition. Third, nintedanib administration, both early and late stages, caused a decrease in Hyp contents and reduced α-SMA and I type collagen expression in the lung of C57BL/6 mice. These results suggest that nintedanib inhibits the transformation of LPS-induced fibroblasts into myofibroblasts in vitro and it ameliorates lung fibrosis in mice after LPS-induced ARDS.

Keywords: Acute respiratory distress syndrome; Nintedanib; Fibroblast growth factor; Platelet-derived growth factor; Vascular endothelial growth factor

Introduction

Acute Respiratory Distress Syndrome (ARDS) is a rapidly progressive disease occurring in critically ill patients caused by a variety of reasons including sepsis, pneumonia, trauma, multiple blood transfusions, lung contusion, aspiration of stomach contents, and either drug abuse or overdose. The clinical symptoms may include shortness of breath, rapid breathing, and a low blood oxygen level [1]. The pathological features of ARDS within the first week of disease onset are acute inflammatory lung tissue damage followed by a fibroblast hyper-proliferation. It has been well accepted that lung fibrosis occurs in ARDS lungs at least three weeks after the disease onset. Recent studies, however, suggest that lung fibrosis initiates during the early stages of ARDS. While the survival rates of patients have been significantly improved during the early phase of ARDS with technical advancement, high mortality due to chronic pulmonary fibrosis remains a clinical challenge.

Nintedanib, also known as BIBF1120, is a triple receptor kinase inhibitor that suppresses the signaling transduction mediated by vascular endothelial growth factor receptor (VEGFR), the platelet-derived growth factor receptor (PDGFR) and the fibroblast growth factor (FGFR) [2]. FDA has approved the clinical use of nintedanib for the treatment of several types of tumors, such as non-small cell lung carcinoma, multiple myeloma, and ovarian and prostate cancer. While results from the clinical trial studies have shown promising outcomes for nintedanib in the treatment of idiopathic pulmonary fibrosis [3], the therapeutic efficacy of nintedanib in the treatment of lung fibrosis after infection has not been evaluated.

In the current study, we established a lipopolysaccharide-induced murine lung fibrosis model and elucidated the preclinical efficacy of nintedanib in prevention and treatment of LPS-induced lung fibrosis as well as the underlying cellular and molecular mechanisms.

Materials and Methods

Cell culture and reagents

Mouse lung fibroblasts CRL-6013TM (originally isolated from C57BL/6 mice) were purchased from American Type Culture Collection (ATCC). Cells were cultivated in DMEM (Dulbecco's Modified Eagle Medium) with 10% of fatale bovine serum (FBS), 100 μ/ml penicillin and 100 g/ml streptomycin in an incubator at 37°C and 5% CO₂. Cells were then stimulated with LPS (O55:B5) (Sigma-Aldrich Co, Missouri, USA) at 1 μg/ml [4] in the presence of nintedanib (Cayman Chemical, Michigan, USA) at different concentrations (0-3 μM) as indicated in each figure. Experimental reagents including TRIzol (Thermo Fisher Scientific, Massachusetts, USA), PCR kit (TaKaRa Bio Inc., Kusatsu, Japan), and ELISA kit (Boshide Inc., Wuhan, China) were used according to the instructions from manufacturers. PCR primers were purchased from Shanghai Biotech (Shanghai, China). All antibodies including anti-SMA, anti-collagen I and HRP-conjugated anti-rabbit IgG were purchased from Boshide Inc. (Wuhan, China). Hyp detecting kit was purchased from Jiancheng Biotech Inc. (Nanjing, China).

Mice

32 Male C57BL/6 mice at age of 6-8 weeks and average body weight 20 g were purchased from the Experimental Animal Center at the Third Military Medical University (Chongqing, China license number SCXK-2012-0011). All mice used in this study were maintained at the University mouse facility under pathogen-free conditions.
conditions according to institutional guidelines and animal study proposals approved by the Institutional Animal Care and Use Committee. The rooms were maintained at 21-24°C and 40-65% humidity, with a 12 h light/dark cycle. Mice had free access to food and water. All mice were anaesthetised with an intraperitoneal injection of sodium pentobarbital (20 mg/kg). All mice were divided into 4 groups averagely, control group(8), Model group(8), nintedanib early treatment group(8), nintedanib late treatment group(8). The mice were administered a single intratracheal dose of LPS from the membrane of *Escherichia coli* 0111:B4 (Sigma-Aldrich, USA) at 5 mg/kg in a total volume of 50 µl. The Control in Control group were injected intratracheally with 50 µl sterile saline. Body weight, food and water intake of mice were collected during the experimental protocol concerning. ELISA, BALF collection and cellularity analysis, Histological analysis, Lung tissue Hyp analysis, Western blotting were performed with each C57BL/6 mice group.

### RNA extraction and PCR

Total RNA was extracted using Trizol reagent (Thermo Fisher Scientific, Massachusetts, USA). 2 µg total RNA were subjected to reverse-transcription. The levels of TGF-β1, α-SMA, FGF-2, PDGF, VEGF and β-actin control were determined by PCR in a total reaction volume of 25 µl; 94°C, 3 min for 1 cycle; 94°C, 30 s, 52°C, 30 s and 72°C, 1 min for 30 cycles. Primers used in this study are listed (Table 1).

#### Table 1: The sequences of primers.

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<th>Primer</th>
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<th>Size/bp</th>
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<td>FGF2-R, 5´-CACTTCTCAGACAGACTGGA-3´</td>
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<td></td>
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<td>β-actin</td>
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<tr>
<td></td>
<td>β-actin-R, 5´-AGGATGCCTGCTGGAAGGAC-3´</td>
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</table>

**ELISA (Enzyme-Linked Immunosorbent Assay)**

The culture supernatants from the C57BL/6 lung fibroblasts were collected at 72 h after treatment by either LPS or with addition of nintedanib at each indicated concentration. An ELISA kit was used to measure the concentrations of TGF-β1 following the protocol provided by the manufacturers.

**BALF (bronchoalveolar lavage fluid) collection and cellularity analysis**

After euthanasia, PBS was injected into the lung through trachea injection, 0.5 ml each side and then collected. BALF collection was performed three times on each mouse to ensure the most cells were collected. Cells were then pelleted down by centrifugation and suspended in 300 µl PBS. Cell numbers were counted and a fraction of cells were used for Giemsa staining. Macrophages, lymphocytes and neutrophils were counted based on their distinct morphologies after staining. Their percentages and absolute numbers were calculated accordingly.

**Histological analysis**

Lung tissues were fixed in 4% paraformaldehyde for 24 h, and wax embedded. Tissue sections were processed and subjected to either H&E or Masson staining. Lung fibrosis and pathological evaluations of the tissue sections were scored following the Ashcroft criteria [5].

**Lung tissue Hyp analysis**

Lung tissues were homogenized and the levels of Hyp were determined by using a kit following the manufacturer's protocol.

**Western blotting**

Lung tissues were homogenized and lyzed with RIPA (Radioimmunoprecipitation Assay) lysis buffer. Protein concentrations were determined and an equal amount of proteins were subjected to SDS-PAGE and transferred to nitrocellulose membrane. After blocking with 5% (w/v) skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T), the membrane was incubated overnight at 4°C with the indicated primary antibodies (anti-SMA or anti-Collagen I) followed by HRP-conjugated secondary antibody. Membranes were then washed and visualized with enhanced chemiluminescence (ECL). The bands identity was analyzed by NIH Image J software.

**Statistical Analysis**

The statistical analysis was performed using SPSS 17.0 software (IBM, New York, USA). Data are indicated as mean ± standard deviation (SD). Unpaired Student t test was used for the analysis and p<0.05 was considered as statistically significant.

**Results**

Nintedanib suppresses LPS-induced transcription of TGF-β1, α-SMA, FGF-2, PDGF and VEGF in mouse lung fibroblasts

As shown in Figure 1, stimulation of the mouse lung fibroblasts with 1 µg/ml LPS dramatically induced the mRNA transcription of TGF-β1, α-SMA, FGF-2, PDGF and VEGF. Importantly, further addition of nintedanib at each indicated concentration including 0.1, 0.3, 1 and 3 µM, totally abolished LPS-induced TGF-β1, α-SMA, FGF-2, PDGF and VEGF transcription in lung fibroblasts (Figure 1).
Total RNA was extracted from mouse lung fibroblasts either untreated or treated with LPS or LPS with nintedanib at each indicated concentration. The mRNA expression levels of TGF-β1, α-SMA, FGF-2, PDGF, VEGF and β-actin were determined by RT-PCR (A). Representative images from three independent experiments are shown. TGF-β1 (B), α-SMA (C), FGF-2(D), PDGF(E) and VEGF(F) mRNA levels as described (Figure 1) were quantified. Column 1: control group; 2: DMSO group; 3: LPS (1 µg/ml) challenged group; 4: LPS + nintedanib (0.1 µM); 5: LPS + nintedanib (0.3 µM); 6: LPS + nintedanib (1 µM); 7: LPS + nintedanib (3 µM). Error bars represent data from three independent experiments. Student t test was used for the statistical analysis. LPS group compared with Ctrl and DMSO group, *indicates for p<0.05. LPS+nintedanib (0.3, 1, 3μM) group compared with LPS group, *indicates for p<0.05.

Nintedanib inhibits LPS-induced TGF-β1 production by mouse lung fibroblasts

As indicated in Figure 2, LPS stimulation resulted in a dramatic (about 2 fold) induction of TGF-β1 production in the supernatants of the mouse lung fibroblasts. Interestingly, further addition of nintedanib inhibited the LPS-induced TGF-β1 production in a dose-dependent manner. Together with the results from our RT-PCR analysis, our studies demonstrated that nintedanib inhibits mouse lung fibrosis induced by LPS in vitro through suppressing TGF-β1 and α-SMA expression.

Mouse lung fibroblasts were cultivated with 1 µg/ml LPS with or without addition of nintedanib at each indicated concentration. 72 hours after treatment, TGF-β1 levels in the culture supernatants were determined by ELISA. Column 1: control group; 2: DMSO group; 3: LPS (1 µg/ml) challenged group; 4: LPS + nintedanib (0.1 µM); 5: LPS + nintedanib (0.3 µM); 6: LPS + nintedanib (1 µM).

Error bars represent data from three independent experiments. Student t test was used for the statistical analysis. LPS group compared with Ctrl and DMSO group, *indicates for p<0.05. LPS+nintedanib (0.3, 1, 3 µM) group compared with LPS group, *indicates for p<0.05.

Cellularity analysis in the BALF from mice with LPS-induced lung fibrosis

Two mice died in M group, one mice died in LT group, and no mice died both in Ctrl group and ET group. In contrast to the basal level of immune cells in the BALF from normal mice, there are about five-fold increase in total immune cells in the BALF from mice with LPS-induced lung fibrosis.

Further characterization detected a dramatic increase in BALF macrophages, lymphocytes and neutrophils (Table 2 and Figure 3). Notably, the early nintedanib treatment from 7 days after LPS-challenging resulted in an average of 30% and statistic significant reduction in the BALF immune cells and a similar result was obtained from the late-treatment group.

Therefore, nintedanib showed great therapeutic potential in the treatment of LPS-induced lung fibrosis in mice.

Cell differentiation (%)

<table>
<thead>
<tr>
<th>Group</th>
<th>Total cells (x10⁴)</th>
<th>Macrophages</th>
<th>Lymphocytes</th>
<th>Neutrophils</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>2.25 ± 0.43</td>
<td>1.59 ± 0.31</td>
<td>0.47 ± 0.06</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>Model</td>
<td>10.4 ± 0.86</td>
<td>6.04 ± 0.62</td>
<td>1.85 ± 0.20</td>
<td>2.17 ± 0.20</td>
</tr>
<tr>
<td>Early Treatment</td>
<td>7.3 ± 0.48</td>
<td>4.37 ± 0.24</td>
<td>1.39 ± 0.26</td>
<td>1.36 ± 0.23</td>
</tr>
<tr>
<td>Late Treatment</td>
<td>7.95 ± 0.82</td>
<td>4.84 ± 0.36</td>
<td>1.5 ± 0.27</td>
<td>1.22 ± 0.12</td>
</tr>
</tbody>
</table>

Table 2: Analysis of bronchoalveolar lavage.

Mice were euthanized 28 days after LPS challenging and BALF were collected. The total numbers and each type of immune cells were analyzed. Error bars represent data from 8 mice per group. Student t test was used for the statistical analysis. *indicates for p<0.05. NS indicates for non-significant (Figure 3).
Figure 3: Effect of nintedanib on LPS-induced fibrosis.

C57BL/6 mice received an intratracheal instillation of control normal saline or 5 mg/kg LPS. Nintedanib was administered each day by gavage at 50 mg/kg from day 7 to day 21 in ET group. Nintedanib was administered each day by gavage at 50 mg/kg from day 14 to day 28 in LT group. The total numbers of each type of BAL immune cells per mouse lungs were expressed. Error bars represent data from 8 mice per group. Ctrl: control group; M: LPS challenged model group; ET: early nintedanib treated group; LT: late nintedanib treated group. *indicates for p<0.05.

Histopathological analysis of lung fibrosis by H&E staining

H&E staining of the lung tissue sections from LPS challenged mice revealed extensive immune cell infiltration, thickening alveolar septa and lung tissue damages (Figure 4). In contrast, while still with modest inflammatory response in the lung from nintedanib treated mice, the overall lung histology was dramatically improved, suggesting that nintedanib has a therapeutic potential in treating LPS-induced lung inflammation (Figure 4).

Figure 4: Histological analysis of lung fibrosis by H&E staining (100X).

C57BL6 mice were challenged with LPS. 7 (early treatment) or 14 (late treatment) days after LPS challenging, mice were treated with nintedanib. All mice from each group were sacrificed and the lung tissue samples were processed at the endpoint of day 28 after LPS challenging. Lung tissue samples were analyzed by H&E staining. Representative images from (A) untreated control, (B) LPS challenging alone, (C) nintedanib early treatment and (D) late treatment are shown.

Histopathological analysis of lung fibrosis by Masson staining

Masson staining is an approach that can detect collagen deposition during fibrosis. As shown in Figure 5, in addition to the tissue damage and immune cell infiltration as detected by H&E staining, Masson staining detected a high level of collagen deposition in the lung tissue sections from mice 28 days after LPS challenging, indicating LPS challenges induce lung fibrosis in mice. Interestingly, nintedanib treatment, both in early and late treatment groups, largely diminished the collagen deposition in the lung from LPS-challenged mice (Figure 5), indicating that nintedanib protect mice from LPS-induced pulmonary fibrosis (Figure 5).

Figure 5: Lung collagen depositions were detected by Masson staining (200X).

Tissue sections as described in Figure 3 were subjected to Masson staining. Representative images from (A) untreated control, (B) LPS challenging alone, (C) nintedanib early treatment and (D) late treatment are shown. (E) The levels of collagen deposition were scored and the average Ashcroft scores from 8 mice in each group are indicated. Error bars represent data from 8 mice per group. Ctrl: control group; M: LPS challenged model group; ET: early nintedanib treated group; LT: late nintedanib treated group. Student t test was used for the statistical analysis. *indicates for p<0.05.

Effects of nintedanib on Hyp production during lung fibrosis

Consistent to our histological analysis, the elevated Hyp expression in lung tissues from LPS-challenged mice was detected by comparing to the control groups, and this Hyp increase was largely abolished by nintedanib treatment. Importantly, early nintedanib treatment achieved a more efficient inhibition of Hyp production than that of the late nintedanib treatment (Figure 6).

Figure 6: Effects of nintedanib on Hyp production during lung fibrosis.
Lung tissues from mice as described in Figure 3 were homogenized. The Hyp levels were determined by hydroxyproline test kit. Error bars represent data from 8 mice per group. Ctrl: control group; M: LPS challenged model group; ET: early nintedanib treated group; LT: late nintedanib treated group. Error bars represent data from 8 mice per group. Student t test was used for the statistical analysis. *indicates for p<0.05.

Effects of nintedanib on α-SMA and collagen I expression during lung fibrosis

The expression levels of both α-SMA and collagen I in the lung tissues were determined by western blotting. As expected, significant increases in both α-SMA and collagen I in the lung tissues from LPS-challenged mice were detected (Figure 7).

Lung tissues from mice as described in Figure 3 were homogenized. (A) The protein expression levels of collagen I (top panel), α-SMA (middle panel) and control β-actin (bottom panel) were determined by western blotting. Representative images are shown (B & C) the band intensities in (A) were determined; the relative levels to β-actin are shown. Error bars represent data from 8 mice per group. Ctrl: untreated control group, M: LPS challenged model group; ET: early nintedanib treated group; LT: late nintedanib treated group. Student t test was used for the statistical analysis.*indicates for p<0.05.

Discussion

Despite significant improvement in the treatment of ARDS during the early inflammatory phase during the last decades, the treatment of lung fibrosis remains as a clinical challenge. Accumulated evidence suggests that lung fibrosis initiates during the early stages of ARDS [6], suggesting that a treatment of lung fibrosis from the early stage of ARDS is needed. Our study has demonstrated that nintedanib as great therapeutic potential in treatment of LPS-induced lung fibrosis in mice.

Inflammatory stimulation in particular by cytokines promotes fibroblast proliferation, migration and differentiation into myofibroblasts, which produce α-SMA in the lung [7]. It has been demonstrated that myofibroblasts play a major role in lung tissue damage and fibrosis due to its strong contraction [8], production of extracellular matrix [9] and induction of cell apoptosis [10]. We show here that LPS stimulation indeed significantly induced α-SMA gene transcription in the lung fibroblasts in vitro. In the present study, C57BL/6 mice have been used to investigate pathomechanisms of LPS-induced pulmonary fibrosis. In addition, the C57BL/6 mice lung fibroblasts were chosen as subject in our previous cell experiments. Therefore, we choose C57BL/6 mice as animal model. Consistent with our observations, He et al. [11] reported that LPS induces myofibroblasts differentiation from fibroblasts. In addition, it has been shown that LPS challenge of mice can induce acute inflammatory response in their lungs, which peaks at day 7 after LPS challenging [12]. In the current study, we detected severe lung fibrosis in the lung tissues 28 days after LPS challenging, demonstrating that LPS-induced lung fibrosis is a useful model for studying the prevention and treatment of pulmonary fibrosis.

Nintedanib is a triple receptor kinase inhibitor that suppresses the signaling transduction mediated by vascular endothelial growth factor receptor (VEGFR), the platelet-derived growth factor receptor (PDGFR) and the fibroblast growth factor receptor (FGFR). VEGF is originally known as vascular permeability factor composed with two subunits that promote the vascular endothelial cell growth and new blood vascular formation. While elevated VEGF has been detected during fibrosis and it promotes lung fibrosis [13], the underlying cellular and molecular mechanisms remain undefined. Similar to VEGF, PDGF has potent activity in promoting fibroblast proliferation. In addition, PDGF stimulates fibroblast production of collagenase for tissue damage during inflammation [14]. Therefore, it has been suggested that PDGF play an important role during lung fibrosis [15]. Therefore, the PDGF receptor antagonist Nilotinib has been shown to protect mice from the bleomycin-induced acute lung injury and, more importantly pulmonary fibrosis [16]. A recent study demonstrated that PDGF promotes collagen I and α-SMA expression by activating the mitogen-activated protein kinase p38, indicating PDGF is a pathogenic factor in lung fibrosis induction [17]. Moreover, similar to VEGF and PDGF, Fibroblast growth factor 2 (FGF2) enhances the proliferation of...
endothelial cells, fibroblasts and astrocytes. FGF2 can function as a chemotactrant that induces the migration of endothelial cells, fibroblasts and astrocytes. Furthermore, TGF-β1 can induce fibroblast accumulation through FGF2 to promote lung fibrosis [18].

In this study, we show that LPS induces fibroblast differentiation into myofibroblasts in vitro as documented by the elevated α-SMA transcription. This LPS-induced α-SMA expression is largely diminished by nintedanib. In addition, nintedanib significantly suppresses lung fibroblast proliferation and the production of hydroxyproline (Hyp), a marker for lung fibrosis diagnosis [19]. Indeed, Hyp levels were largely inhibited by nintedanib treatment in mice with LPS-induced lung fibrosis. Histological analysis by both H&E and Masson staining demonstrated that nintedanib treatment has potent protective activity from LPS-induced lung injury and collagen deposition. Therefore, our both in vitro and in vivo studies demonstrate that nintedanib is a promising therapeutic drug in lung fibrosis treatment.

In conclusion, our study indicates that the triple receptor kinase inhibitor nintedanib is a potential drug to treat lung fibrosis. As nintedanib inhibits VEGF, PDGF and FGF receptor-mediated kinase activation in fibroblasts, our study suggests that suppression of VEGF, PDGF and FGF prevents and/or treats lung fibrosis during the late stages of ARDS. However, the cellular and molecular mechanisms underlying how VEGF, PDGF and FGF promote pulmonary fibrosis remain largely unknown. Further studies are needed to reveal the mechanisms in lung fibrosis pathogenesis as well as to develop new therapies to combat ARDS and lung fibrosis.

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References


