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Long-term treatment with fasudil improves bleomycin-induced pulmonary fibrosis and pulmonary hypertension via inhibition of Smad2/3 phosphorylation

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ABSTRACT

Pulmonary hypertension (PH) associated with pulmonary fibrosis (PF) considerably worsens prognosis of interstitial lung diseases (ILD). RhoA/Rho-kinases (ROCK) pathway is implicated in high pulmonary vascular tone and pulmonary fibrosis but the effect of ROCK inhibitors on PH associated with PF is not known. We therefore aimed to determine whether long-term treatment with fasudil, a selective ROCK inhibitor, could attenuate PF and PH induced by bleomycin in mice. Male C57BL/6 mice received a single dose of intratracheal bleomycin (3.3 U/kg) to induce PF. Treatment with fasudil (30 mg kg^{-1} day^{-1}) was given intraperitoneally for 7, 14 or 21 days until mice underwent hemodynamic measurements. Right ventricular systolic pressure (RVSP) and RV/(LV + S) ratio were assessed. Lung inflammatory cells profiles, including macrophages, neutrophils, lymphocytes B and lymphocytes T were assessed by immunohistochemistry. Lung fibrosis was evaluated by histological and biochemical methods. Pulmonary arteriole muscularization and medial wall thickness (MWT) were evaluated by immunohistochemical staining for α-SMA. Bleomycin induced severe PF and PH in mice, associated with an increased RhoA/ROCK activity in the lung. Fasudil reduced lung inflammation and lung collagen content, and attenuated the increased RVSP, RV hypertrophy, and pulmonary vascular remodeling in bleomycin-intoxicated mice. Fasudil inhibited the increased activity of RhoA/ROCK pathway, and partly altered bleomycin-associated activation of TGF-β1/Smad pathway, via inhibition of Smad2/3 phosphorylation. The efficacy of long-term treatment with fasudil suggests that the blockade of RhoA/ROCK pathway may be a promising therapy for patients with ILD-associated PH.

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1. Introduction

Pulmonary hypertension (PH) associated with pulmonary fibrosis (PF) considerably worsens prognosis of chronic interstitial lung diseases (ILDs). The most common ILDs causing PF and PH include idiopathic pulmonary fibrosis (IPF), connective tissue disorders (CTDs) and sarcoidosis. To date, no specific treatment for PH associated with PF is available. On the other hand, high-quality clinical trials investigating current conventional therapies such as prostacyclins, endothelin-1 (ET-1) receptor antagonists and phosphodiesterase type 5 (PDE5) inhibitors are scarce in patients with ILD-associated PH [1], thus highlighting the need for finding new therapies for these patients.

There is increasing evidence to suggest that the RhoA/Rho-kinase (ROCK) pathway is involved in various vascular disorders in the systemic and the pulmonary circulations [2]. RhoA, one of the best-known members of Rho GTPases, and its effector proteins, ROCKs, are strongly implicated in a wide range of cell functions,

Abbreviations: PH, pulmonary hypertension; PF, pulmonary fibrosis; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; CTD, connective tissue disease; ROCK, Rho-kinase; BALF, bronchoalveolar lavage fluid; PVR, pulmonary vascular resistance; RVSP, right ventricular systolic pressure; MWT, medial wall thickness; BLM, bleomycin.

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such as migration, proliferation, apoptosis, differentiation and contraction [3]. RhoA/ROCK pathway plays a pivotal role in the pathogenesis of PH as its abnormal activation either causes or worsens endothelial dysfunction, sustained vasoconstriction, vascular remodeling and inflammation [4]. Fasudil, one of the inhibitors of ROCKs, has been extensively studied in animal models of PH over the past decade, notably in monocrotaline- or chronic hypoxia- induced PH [5–9]. A small number of clinical trials also reported effective effects of acute administration of fasudil either intravenously or by inhalation in patients with severe PH [10–13]. However, little is known about the effects of long-term treatment with fasudil in PH secondary to PF neither in animal models nor in humans.

Bleomycin, a chemotherapeutic antibiotic produced from *Streptomyces verticillus*, is extensively used in rodent models of PF. Intratracheal instillation of bleomycin causes oxidative stress and triggers sequential increased production of lung pro-inflammatory cytokines, activation of lung fibroblasts that eventually leads to PF [14]. Altering RhoA/ROCK signaling pathway, either by treatment with simvastatin or direct inhibition by the ROCK inhibitor, Y-27632, lowers inflammatory cell numbers in bronchoalveolar lavage fluid (BALF) and decreases lung hydroxyproline content (HPC) thereby attenuating bleomycin-induced PF in mice [15,16]. Intratracheal instillation of bleomycin also induces PH in rodents at late time-points [17–19] whereas a single intraperitoneal bolus of ROCK inhibitors (Y-27632 or fasudil) completely normalizes pulmonary vascular resistance (PVR) in bleomycin-induced PH in rats [20]. Furthermore, sildenafil treatment in bleomycin-intoxicated mice reduces pulmonary hypertension and right ventricular (RV) hypertrophy through inhibition of RhoA/ROCK pathway [17].

Although long-term treatment with fasudil has been shown to attenuate bleomycin-induced PF in mice [21], the effect of fasudil on the occurrence of both PH and PF in the same mice intoxicated by bleomycin has not yet been investigated nor the underlying mechanisms fully deciphered. Prior studies have suggested that TGF-β1/Smad pathway activation plays a crucial role in the initiation and the development of inflammatory and fibrotic responses in PF [22]. It has been recently demonstrated that the protective effect of simvastatin may be mediated by reduced inflammatory response and TGF-β1 production in animals with bleomycin-induced PF [16]. On the other hand, there is evidence to suggest possible interactions between RhoA/ROCK activation and Smad2/3 phosphorylation in TGF-β1 family signaling [23,24].

The aims of the present study were to determine whether long-term treatment with fasudil could reduce occurrence and/or severity of both PF and PH induced by bleomycin, and if so whether this could be attributable to inhibition of TGF-β1/Smad pathway.

2. Materials and methods

2.1. Animal model

Eight week-old male C57BL/6 mice (Harlan, Gannat, France) were used in the present study according to animals guidelines for biomedical research applied in our institution. All mice were housed in autoclaved ventilated cages with a 12 h day–night cycle and free access to water and food.

Mice were randomly divided into 3 groups for each time-point: 1) saline control ($n = 8$); 2) bleomycin ($n = 20$); 3) bleomycin + fasudil ($n = 20$). Mice were anesthetized with intraperitoneal ketamine (50 mg/kg) and xylazine (6 mg/kg), and then received a single intratracheal dose of either saline (50 μL) or bleomycin (3.3 U/kg, dissolved in 50 μL saline; bleomycin Bellon, Sanofi-Aventis, Paris, France) on day 0. Briefly, the trachea was exposed via a midline neck incision and a blunt dissection. After a 22-gauge cannula was inserted into the trachea, 50 μL saline or bleomycin was instilled into the trachea followed by 150 μL fresh air, which facilitated bleomycin delivery into distal air spaces. The cannula was then removed and the skin incision was sutured. In order to investigate the kinetics of bleomycin-induced pulmonary inflammation and fibrosis, mice were sacrificed at three time-points: day 7, 14 and 21. From the same day of bleomycin instillation, daily treatment with fasudil (30 mg kg^{-1} day^{-1}, dissolved in PBS with a dilution of 2 mg/mL, LC laboratories, Woburn, MA) or vehicle was given intraperitoneally until the day of sacrifice. Mice were weighed every day and fasudil doses were calculated according to their body weights.

On day 7, 14 and 21, right ventricular systolic pressure (RVSP) was measured *in vivo*. After hemodynamic evaluation, the thorax was opened. Heart samples were removed for RV hypertrophy measurement. Left lungs were perfused with 10% formalin for histopathological analysis. Right lungs were stored at −80 °C until use for collagen content quantification or protein extraction.

2.2. Hemodynamic measurement and RV hypertrophy

At the end of each time-point, RVSP was measured in all mice via a closed-chest technique as previously described [25]. Briefly, mice were anesthetized with intraperitoneal ketamine (50 mg/kg) and xylazine (6 mg/kg). A 25-gauge needle attached to a pressure transducer was introduced directly into the RV. Pressure curve was gained by a 4-channel data acquisition system (Lab-Trax-4, World Precision Instruments, UK) and recorded simultaneously by running the software Labscribe2.

After hemodynamic measurement, the thorax was opened and the heart was removed. The RV was dissected from the left ventricle (LV) plus septum (S). The ratio of RV/(LV + S) weight was calculated to assess RV hypertrophy.

2.3. Lung histopathology

Left lungs were perfused with 10% formalin, embedded in paraffin blocks, and cut into 5 μm-thick sections. Lung sections were stained with hematoxylin-eosin for routine examination or picro-sirius red for lung fibrosis evaluation. Slides were examined with a light microscope at a 100-fold magnification (Leica DC300, LeicaMicrosystems, Wetzlar, Germany).

2.4. Morphometric analysis of pulmonary vessels

To evaluate vascular structural alterations, immunohistochemical staining was carried out with a mouse monoclonal antibody against smooth muscle α-Actin (α-SMA) (Santa Cruz, sc-32251). Briefly, 5 μm-thick lung sections were deparaffinized in xylene and rehydrated with descending concentrations of ethanol. Endogenous peroxidase activity was blocked by 0.3% H_2O_2 in methanol for 30 min. Antigen retrieval was performed by protease for 30 min. Sections were washed and blocked by 2% bovine serum albumin (BSA) in PBS-Tween (0.05%) for 1 h and then incubated with mouse anti-α-SMA antibody (1:200 in 1% BSA) for 1 h. After washing with PBS-Tween (0.05%), sections were incubated with biotinylated horse anti-mouse IgG secondary antibody (1:200 in 1% BSA, Vector Laboratories Inc., Burlingame, CA) for 1 h. After incubation with avidin-biotinylated enzyme complex (Vectastain Elite ABC kit, Vector laboratories) for 30 min, staining was finally visualized by using diaminobenzidine (DAB). Sections were then counterstained by hematoxylin and dehydrated with increasing concentrations of ethanol.

Fully muscularization of pulmonary distal arterioles (outside diameter between 10 and 50 μm) was determined if >75% of the

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