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Original article

The antifibrotic effects of alveolar macrophages 5-HT2C receptors blockade on bleomycin-induced pulmonary fibrosis in rats

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ABSTRACT

Background: The most widespread chronic fibrosing lung disease is idiopathic pulmonary fibrosis. Lung serotonin (5-HT) content is increased during pulmonary fibrosis with the implication of 5-HT2 receptors in the pathogenesis. Serotonin plays important roles in alveolar macrophages function through 5-HT2C receptors activation. Numerous studies described the important role of 5-HT2A/B receptor blockers in suppressing different types of fibrosis as idiopathic pulmonary fibrosis. The current study pointed to examine the antifibrotic effects of RS-102221 and/or terguride through *in vivo* model of pulmonary fibrosis.
Methods: Induction of pulmonary fibrosis was through a single dose of intra-tracheal bleomycin instillation (5 mg/kg dissolved in phosphate buffer saline) in adult male albino Wistar rats. Next day of bleomycin instillation, intraperitoneal injection of RS-102221 (0.5 mg/kg/d) and/or terguride (1.2 mg/kg/d) were administered and continued for 14 days.
Results: Noticeable increase in 5-HT2C receptors expression was observed in fibrotic lung tissues with marked allocation belonging to alveolar macrophages. Either RS-102221 or terguride reduced the increments in lung water contents, grading of lung fibrosis, lung tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta1 (TGF-$_{\beta 1}$) and vascular endothelial growth factor (VEGF) levels in lung injury and fibrosis-induced by bleomycin. Moreover, collagen content and myofibroblasts-alpha smooth muscle actin (α – SMA) were significantly decreased. Additionally, the simultaneous administration of RS-102221 with terguride had a synergistic outcome compared to that obtained by individual monotherapy.
Conclusion: These findings suggested the potential use of 5-HT2A/B/C antagonists as anti-fibrotic agents in lung fibrosis.

Introduction

Idiopathic pulmonary fibrosis is a chronic progressively fibrosing idiopathic interstitial pneumonia [1]. Initial injury resulting in disorganized tissue healing through activation of the alveolar epithelial cells and enrolled circulated macrophages are involved in idiopathic pulmonary fibrosis through the release of different cytokines and potent growth factors [2,3].

Tumor necrosis factor-alpha (TNF-α) is considered one of the inflammatory initiators that motivate further inflammatory cytokines with promoted adhesive molecules expression in endothelial cells [3,4]. Transforming growth factor-beta1 (TGF-$_{\beta 1}$) is a major pro-fibrogenic cytokine, which stimulates differentiation of fibroblasts to alpha-smooth muscle actin (α-SMA) expressing myofibroblasts and accumulation of extracellular matrix protein including collagen and finally fibrosis [5,6]. Vascular endothelial growth factor (VEGF) is a pro-angiogenic factor, involved in vascular remodeling, endothelial cell proliferation and inflammation with angiogenesis in a variety of inflammatory lesions [7].

Improvement the mortality is the therapeutic target in idiopathic pulmonary fibrosis, which is not approached even with recent emphasis in the pathogenesis [5].

Serotonin (5-hydroxytryptamine, 5-HT) concentrations in lung increased significantly over the time course of bleomycin-induced fibrosis, with a maximum at day seven [8]. The serotonin receptor family consists of 13 genes. 5-HT2 receptors include three subtypes 2A, 2B and 2C [9,10].

In the lung, the expression of 5-HT2A and 5-HT2B receptors increased after bleomycin treatment [8]. 5-HT2A and 5-HT2B

Abbreviations: α-SMA, Alpha-smooth muscle actin; ELISA, Enzyme linked immunoassay; PBS, Phosphate buffered saline; 5-HT, Serotonin; TGF-$_{\beta}$1, Transforming growth factor-beta1; TNF-α, Tumor necrosis factor-alpha; VEGF, Vascular endothelial growth factor.

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receptors were highly confined to fibroblasts and lung epithelium respectively. In lung samples derived from patients with idiopathic pulmonary fibrosis, fibroblasts with an intense expression of 5-HT2B receptors were obvious in the fibroblastic foci [8,9].

Serotonin, by 5-HT2C receptors, is a modulator of alveolar macrophages function through initiating intracellular calcium elevation, secretion of cytokines with transcriptional modulation [10].

In bleomycin-induced lung fibrosis, blockage of 5-HT2A and 5-HT2B receptors improved lung function, the histopathological changes and decreasing collagen content introducing a hopeful therapy for idiopathic pulmonary fibrosis [8,11].

However, through *in vivo* induction of pulmonary fibrosis by bleomycin, the antifibrotic effects of a blockade of 5-HT2C receptors alone or in combination with blockade of 5-HT2A and 5-HT2B receptors have not fully investigated. Therefore, this study was directed to clarify the possible antifibrotic effects of 5-HT2C receptors antagonist (RS-102221) alone and in combination with terguride, a 5-HT2A/B receptors antagonist, on bleomycin-induced rat pulmonary fibrosis.

Material and methods

Experimental animals

In this study, ninety (n = 90) adult male albino Wistar rats (220–260 g) were used. Rats were allowed free access to food and water and were maintained under standard conditions (normal light/dark cycle, temperature 25 ± 3 °C). Animals were left for acclimatization for one week before the start of the experiment.

Drugs and chemicals

Bleomycin hydrochloride was provided in vials (15 mg each) from Nippon Kayaku, Tokyo, Japan. RS-102221 hydrochloride hydrate was provided as a white powder from Sigma-Aldrich Company, St. Louis., USA. Terguride was supplied as a white powder from Abcam Company, Cambridge, UK. Further chemicals were obtained from Sigma-Aldrich Company, St. Louis., USA.

Experimental protocol

Rats were divided into nine groups of ten animals each. Daily, our animals have received the solvents or drugs twice intraperitoneally the day after either phosphate buffer saline (PBS) or bleomycin administration and continued for 14 days.

Control untreated group

In this group, rats were not given any medications.

PBS control group

Rats were received a single dose intratracheal instillation of PBS, and then received intraperitoneally 100 mM dimethyl sulfoxide (DMSO; 1 ml/kg) twice daily.

Bleomycin control group

Pulmonary fibrosis was induced through a single dose of intratracheal instillation of bleomycin 5 mg/kg dissolved in PBS [6].

Terguride control group

Rats were given terguride 1.2 mg/kg/day, dissolved in 100 mM DMSO intraperitoneally (twice daily, 1 ml/kg) [11,12].

RS-102221 control group

Rats were received RS-102221 at a dose of 0.5 mg/kg/day, dissolved in 100 mM DMSO (twice daily, 1 ml/kg) [13].

Terguride and RS-102221 control group

Rats were received both terguride (1.2 mg/kg/day) and RS-102221 (0.5 mg/kg/day).

Terguride treated group

Rats were received intratracheal instillation of bleomycin as a single dose, and then received terguride 1.2 mg/kg/day.

RS-102221 treated group

Rats were received intratracheal instillation of bleomycin as a single dose, and then received RS-102221 at a dose of 0.5 mg/kg/day.

Terguride and RS-102221 treated group

Rats were received intratracheal instillation of bleomycin as a single dose and then received both terguride at a dose of 1.2 mg/kg/day and RS-102221 at a dose of 0.5 mg/kg/day.

In all groups, all rats were sacrificed on the 15th day of treatment. After opening the thorax of each rat, the hilum of each lung was ligated and dissected free from its bronchi, blood vessels, and hilar nodes. Wet weight for each lung sample was weighed before being perfused free of blood with ice-cold saline. Right lungs were frozen in liquid nitrogen, freeze-dried (on −80 °C) for the measurement of total lung collagen, TNF-α, TGF-$_{\beta 1}$, and VEGF. Left lungs were blotted dry and weighed (dry weight), fixed using 10% phosphate-buffered paraformaldehyde solution and then inserted into paraffin. Then, tissues were segmented at 4 μm thicknesses and left to dry at 37 °C overnight. Then, sections were deparaffinized for histological or immunohistochemical staining for 5-HT2C receptors and α-SMA.

Calculations of fluid contents of lungs

Each left lung sample was processed for calculation of water content (wet weight-dry weight) to dry weight ratio, which is a pointer to lung inflammation and edema [6,14].

Lung tissue homogenate preparation

Each right lung was weighed and homogenized in PBS, using Teflon pestle homogenizer (Glas-Col homogenizer system, Vernon hills, USA). Each lung homogenate suspension was centrifuged at 14000 × *g* for 10 min at 4 °C and the supernatant will be kept at −80 °C until analysis for total lung collagen, TGF-$_{\beta 1}$, TNF- α and VEGF [6,15].

Total lung collagen measurements

A Sircol™ collagen assay kit (Biocolor, Carrickfergus, Northern Ireland, UK) was used to measure the total amount of soluble collagen as previously described. Frozen lung was homogenized in 1 ml of ice-cold sample buffered solution (10 mM Tris-HCl- (pH 7.4) containing 2 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and 0.01% Tween 20), shaken at 4 °C for 24 h, then centrifuged for 60 min at 12000 × *g* . The clear supernatant fluid was used regarding the manufacturer's instructions with optical density readings at 550 nm [16].

Total lung TNF-α, TGF-$_{\beta 1}$ and VEGF measurements

Total TNF-α, TGF-$_{\beta 1}$ and VEGF lung contents were measured in lung homogenates by an automated enzyme-linked immunosorbent assay (ELISA) reader (Metertech, M960).

The concentrations TGF-β1 was measured using rat ELISA kit (BioVendor Laboratory medicine, USA), while TNF-α and VEGF were measured using rat ELISA kits (Ray Biotech Inc.®, Norcross,