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Biochemical and histological impact of direct renin inhibition by aliskiren on myofibroblasts activation and differentiation in bleomycin induced pulmonary fibrosis in adult mice

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ABSTRACT

Aliskiren is a drug classified as a direct renin inhibitor. The renin–angiotensin system plays an important role in pulmonary fibrogeneses. This study aimed to investigate the impact of aliskiren on pulmonary fibrosis induced by bleomycin. Forty adult mice were divided into group I (control), group II (aliskiren 25 mg/kg/day IP), group III (bleomycin 0.035 U/g intraperitoneally twice weekly for 4 weeks) and group IV (aliskiren + bleomycin). Plasma renin activity (PRA), lung content of hydroxyproline and transforming growth factor-β1 (TGF-β1) were assayed. Lung paraffin sections were prepared for histological study and immunohistochemical detection of alpha smooth muscle actin (αSMA) as a marker for myofibroblasts activation and differentiation.

Bleomycin induced a significant elevation of PRA with a significant increase in hydroxyproline and TGF-β1 in group III. Microscopically, pulmonary fibrosis was evident in the form of areas of collapsed alveoli, intense inflammatory cells infiltrations, excess accumulation of collagen, and excessively encountered αSMA positively immune-stained myofibroblasts, compared to a negative immune-reaction in groups I and II.

In group IV, aliskiren resulted in a significant decrease in PRA, TGF-β1 and hydroxyproline, with an attenuation of pulmonary fibrosis and a decrease in αSMA positively immune-stained myofibroblasts.

In conclusion, renin inhibition by aliskiren attenuated pulmonary fibrosis through decreasing TGF-β1 and myofibroblasts activation and differentiation.

1. Introduction

Pulmonary fibrosis is a fatal disease refractory to treatment. It includes a heterogeneous group of lung disorders characterized by progressive, irreversible destruction of lung architecture by fibrosis leading to organ malfunction, disruption of gas exchange, and death from respiratory failure (Selman et al., 2001). The clinical prognosis for the patients with this condition remains poor. Yet, there is no effective treatment for this disease except lung transplantation (Wynn, 2011).

It is important to understand biochemical and histological changes triggered in lung tissue in cases of pulmonary fibrosis to develop new strategies for treatment. Pulmonary fibrosis is characterized by inflammation, excessive proliferation of myofibroblasts, and abnormal deposition of collagen (Graves et al., 2010). There is growing evidence that both inflammation and fibrosis are mediated by cytokine activity (Bonniaud et al., 2005). Transforming growth factor-β1 (TGF-β1) is a key cytokine that links inflammation to fibrogenesis. It induces lung injury and contributes to pulmonary fibrosis, through its actions to induce collagen gene expression or by stimulation of myofibroblast trans-differentiation (Anscher, 2010; Sueblinvong et al., 2014). Myofibroblasts are TGF-β1 dependent cells that arise secondary to epithelial injury or inflammation (Hinz, 2010). They promote lung fibrosis as they are the primary source of type I collagen gene expression in active fibrotic sites (Phan, 2002).

Studies indicated that the renin–angiotensin system (RAS) plays an important role in pulmonary fibrogeneses through the action of

Abbreviations: PRA, plasma renin activity; TGF-β1, transforming growth factor-β1; αSMA, alpha smooth muscle actin; RAS, renin–angiotensin system; IP, intraperitoneal; RPM, revolutions per minute; U.S.A, United States of America; IHC, immunohistochemical; LAB, labeled avidin-biotin; PBS, phosphate buffered saline; DAB, 3,3′-diaminobenzidine; SD, standard deviation; SPSS, Statistical Package for Social Science; Fig, figure; H&E, haematoxylin and eosin; Ang1, angiotensin I; Ang II, angiotensin II; AT1 receptor, angiotensin type 1 receptor; ERK1/2, extracellular signal-regulated kinase.

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angiotensin II (Ang II) and angiotensin type 1 receptor (Antoniu, 2008; Marshall et al., 2004; Veerappan et al., 2013). Therefore, the use of RAS blockers may have a role in controlling the development of pulmonary fibrosis. Aliskiren is a drug classified as a direct renin inhibitor (Furukawa et al., 2013). In contrast to the conventional RAS blockers, aliskiren blocks renin–angiotensin system directly through the inhibition of plasma renin activity and the prevention of the formation of both angiotensin I and Ang II (Brown, 2008). Aliskiren has been used in the treatment of high blood pressure. However, many studies demonstrated the effectiveness of this drug in attenuating fibrosis in different pathological conditions, including cardiac fibrosis, liver fibrosis and renal glomerulosclerosis (Yamamoto et al., 2009; Lizakowski et al., 2012; Aihara et al., 2013).

Therefore, the aim of the present study was to investigate biochemical and histological effect of aliskiren on pulmonary fibrosis induced by bleomycin in adult mice.

2. Materials and methods

2.1. Drugs

Bleomycin (Blecocip, vial, 15 units, CIPLA LTD, India), aliskiren powder was purchased from Novartis Pharma, Germany. ***Kits***: Ready to use target retrieval solution (S1700, Dakocytomation), primary antibody Monoclonal Mouse Anti-Human Smooth Muscle Actin 1A4 (code M0851 from Dako, Glostrup, Denmark), ready to use antibody diluent with background reducing components (S3022, Dakocytomation) and universal detection kits (K 0673, Dakocytomation).

2.2. Experimental protocol

All procedures in this study were performed in accordance with the Medical Research Ethics Committee of Mansoura Faculty of Medicine, Egypt. Forty male Swiss mice (10 weeks old, 20–25 g body weight) were housed under conditions of controlled temperature and 12 h lighting cycle. They received standard diet and water ad libitum. The animals were randomized into four groups of 10 animals each. **Group I**: control mice receiving 200 μL saline intraperitoneally (IP). **Group II**: mice receiving aliskiren in a daily single dose of 25 mg/kg body weight dissolved in 200 μL saline IP for four weeks (Yamamoto et al., 2009). **Group III**: bleomycin treated mice. **Group IV**: mice receiving aliskiren 1 h before bleomycin in the same doses mentioned above.

2.3. Bleomycin-induced pulmonary fibrosis

Pulmonary fibrosis was induced in mice by IP injection of bleomycin at a dose of 0.035 U/g dissolved in 200 μL of a saline solution twice weekly for 4 weeks. The IP administration of this dose of bleomycin has been previously reported to induce murine pulmonary fibrosis in the subpleural regions in a way similar to that observed in human pulmonary fibrosis (Baran et al., 2007; Zhou et al., 2011).

2.4. Obtaining the samples

One week after the final injection of bleomycin, animals were anaesthetized by IP injection of 50 mg/kg pentobarbital (Chen et al., 2005). For the biochemical study, blood was collected from the heart in EDTA-pretreated tubes, and then the plasma was separated by centrifugation at 1000 RPM (79 × *g*) for 30 min and kept at −20 °C for the assay of the plasma renin activity (PRA). The right extra pulmonary bronchus was closed tightly by a Kocher and the right lung was excised, weighed, quickly frozen and stored at −80 °C till the time of TGF-β1 assay and collagen quantification through hydroxyproline assessment. A cannula was inserted into the trachea and fixed with a ligature. Lung was inflated via the cannula by gentle infusion of neutral buffered formalin solution at a constant fluid pressure of 25 cm for 5 min. The lung was filled slowly with the fixative until fully inflated. The suture was tightened surrounding the trachea to prevent backflow of fixative out of the lungs. The lung was placed in a glass vial containing the corresponding fixative overnight at a temperature of 4 °C (Braber et al., 2010). Samples were obtained from the same region (the upper part) of the left lung in all the animals to avoid regional heterogeneity.

2.5. Biochemical study

2.5.1. Plasma renin activity (PRA)

PRA was assessed using angiotensin I radioimmunoassay kits (Diasorin Laboratories, Stillwater, Minn., USA) according to the protocol provided by the manufacturer. Values were expressed as (ng Ang1/mL/h).

2.5.2. Quantification of collagen

Tissue was hydrolyzed with 6 mol/L HCl at 130 °C for 5 h following Woessner's method (1961). After neutralization with NaOH, the hydrolyzates were diluted with distilled water. Hydroxyproline in the hydrolyzates was assessed colorimetrically at 550 nm for the presence of p-dimethylaminobenzaldehyde. Results were expressed as μg hydroxyproline per mg wet lung weight using hydroxyproline standards (Sigma, St. Louis, MO, USA).

2.5.3. TGF-β1 assay

Lungs were thawed at 4 °C, homogenized on ice in 50 mM Tris–HCl buffer containing 180 mM KCl and 10 mM EDTA, final pH 7.4, and then centrifuged at 10,000 × *g*, 4 °C, for 30 min. The supernatants were collected and used for assay of TGF-β1 using Mouse TGF-β1 Platinum ELISA kit (BMS608/4, eBioscience, Austria) following the protocol provided by the manufacturer. Values are expressed as pg/mg proteins, the latter determined by the method of Lowry et al. (1951).

2.6. Histological study

Tissue samples were dehydrated in alcohols, cleared in xylol and embedded in paraplast. Serial tissue sections of 5 μm thickness were stained with: haematoxylin & eosin to study the histological changes, and Masson's trichrome to stain collagen in the tissue (Suvarna et al., 2013). Sections of 4 μm thickness were used for immunohistochemical localization of alpha smooth muscle actin (αSMA) as a marker specific for myofibroblast activation and differentiation (Achar et al., 2014).

2.7. Immunohistochemical (IHC) technique for localization of αSMA

The standard peroxidase immunohistochemistry technique was applied to slides of paraffin-embedded tissue. Sections were dewaxed in xylol for 20 min (two changes), and hydrated in descending grades of alcohol down to distilled water. They were immersed in a preheated target retrieval solution to 95–99 °C. Sections were rinsed three times with phosphate buffered saline (PBS). Excess liquid was tapped off the slides. Enough hydrogen peroxide was applied to cover the specimen for 5 min, then slides were rinsed gently with PBS and excess liquid was tapped off. Enough amount of primary antibody (dilution = 1:100 according to Thuy le et al., 2011) was applied to specimens, and was incubated for 2 h in humidity chamber at room temperature. Slides were rinsed in