ACE2 activator associated with physical exercise potentiates the reduction of pulmonary fibrosis

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Abstract

The interstitial lung diseases are poorly understood and there are currently no studies evaluating the association of physical exercise with an ACE2 activator (DIZE) as a possible treatment for this group of diseases. We evaluate the effects of pharmacological treatment with an angiotensin-converting enzyme 2 activator drug, associated with exercise, on the pulmonary lesions induced by bleomycin. From the 96 male Balb/c mice used in the experiment, only 49 received 8 U/kg of bleomycin (BLM, intratracheally). The mice were divided into control (C) and bleomycin (BLM) groups, sedentary and trained (C-SED, C-EXE, BLM-SED, BLM-EXE), control and bleomycin and also sedentary and trained treated with diminazene (C-SED/E, C-EXE/E, BLM-SED/E, BLM-EXE/E). The animals were trained five days/week, 1 h/day with 60% of the maximum load obtained in a functional capacity test, for four weeks. Diminazene groups were treated (1 mg/kg, by gavage) daily until the end of the experiment. The lungs were collected 48 h after the training program, set in buffered formalin and investigated by Gomori's trichrome, immunohistochemistry of collagen type I, TGF- β_1 , beta-prolyl-4-hydroxylase, MMP-1 and -2. The BLM-EXE/E group obtained a significant increase in functional capacity, reduced amount of fibrosis and type I collagen, decreased expression of TGF- β_1 and beta-prolyl-4-hydroxylase and an increase of metalloproteinase -1, -2 when compared with the other groups. The present research shows, for the first time, that exercise training associated with the activation of ACE2 potentially reduces pulmonary fibrosis.

Keywords: ACE2 activation, physical exercise, TGF- β , MMPs, beta-prolyl-4-hydroxylase, type I collagen

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Introduction

The interstitial lung disease (ILD) is difficult to understand due to its pathogenic mechanisms that are poorly understood and, at the same time, heterogeneous.¹ The ILD can range from acute inflammation to progressive and irreversible fibrosis. Idiopathic pulmonary fibrosis (IPF) is a type of ILD whose causative agent is unknown and it is characterized by the sudden onset of lung parenchyma, with thickening of the alveolar septa, hyperplasia of type II pneumocytes (PII), and myofibroblasts, causing narrowing of airways.^{2,3} The estimated prevalence of IPF is around 30 cases per 100,000 people, reaching more than 100 individuals per 100,000 people aged 75 years or more.⁴

Studies have shown that in both human and experimental pulmonary fibrosis, the PII are capable of producing the transforming growth factor- β (TGF- β), which increases

expression and induces the proliferation of fibroblasts, their change into myofibroblasts, production of type I collagen and inhibits their degradation.^{5,6} Therefore, it contributes to a decreased lung compliance, reduce of gas exchange, and other changes in pulmonary functions. Collagen is the main protein of mammals' connective tissue, its synthesis occurs due to the action of the beta-prolyl-4-hydroxylase enzyme which catalyzes the hydroxylation of the proline residue, producing hydroxyproline.⁷

The complex mechanism of lung remodeling occurs through the digestion of the extracellular matrix by metalloproteinases (MMPs) and phagocytosis.⁸ The expression of the MMPs can be up regulated or down regulated due to integrin-derived stimulus, alteration of cell architecture, extracellular matrix proteins, and mechanical stress.⁹ The generation and function of MMPs are also regulated by

the transcription, activation, and inhibition of pro-enzymes. Probable modulators of MMP expression are TGF- β , fibroblast growth factor, vascular endothelium, tissue inhibitors of metalloproteinases (TIMPs), and physical stress.¹⁰ The MMPs are quickly blocked when released, once the increase of its activity can destroy the body's tissues. In physiological condition, there is a low expression of MMP-1 and it could be undetectable in the lungs.¹¹ The MMP-1 (collagenase-1) is synthesized by alveolar epithelial cells, fibroblasts, and it degrades type I, II, and III collagens. The degradation resulting fragments are digested by MMP-2 and MMP-9 (gelatinases).¹²

Several animal models have been established to study the pathophysiology of IPF. The Bleomycin (BLM) is a chemotherapeutic that is used in the treatment of several neoplasias. The most used and described method to cause pulmonary fibrosis in rodents¹³ is the intratracheal administration of this drug. This chemotherapy can cause some lung lesions such as parenchyma inflammation, lesion of the alveolar epithelial cells with reactive hyperplasia, activation and fibroblast to myofibroblast differentiation,¹⁴ pulmonary fibrosis, hypersensitive pneumonitis and pulmonary nodule.¹⁵ Patients with IPF have limited functional capacity due to ventilatory impairment and dyspnea, which is the main symptom of these patients and is worse during physical activities.¹⁶ Currently, the IPF patients have been studied by many authors.¹⁷⁻¹⁹ These studies show the importance of the pulmonary rehabilitation program to patients with ILD. The pulmonary rehabilitation program, which includes physical exercise, has positive results in endurance capacity^{20,21} and improves the symptoms and life quality of these patients.²²

Exercise physiology has used laboratory animals to simulate conditions of physical stress observed in humans and its purpose is to better monitor the systemic, cellular, and molecular changes resulted from physical activity.²³

Some proteins involved in the regulation of the renin-angiotensin system (RAS) may have therapeutic potential for the treatment of IPF. The RAS, in addition to being related to cardiovascular and renal diseases such as hypertension, heart failure, and chronic renal failure, may also be involved in lung diseases. Several searches have shown the involvement of a ACE/AngII/AT1 physiopathological pathway hyperactivity among several diseases such as pulmonary hypertension and the development of fibrosis.²⁴⁻³¹ Therefore, drugs that increase the activity of the ACE₂/Ang-(1-7)/Mas pathway may contribute to the treatment of IPF, once this pathway promotes counterbalance of the SRA.³¹

Recently, an antitripanosomal drug called diminazene (DIZE) has been shown to have activating effects on the angiotensin-converting enzyme 2 (ACE2) and reducing effects on pulmonary fibrosis induced by bleomycin.³² Studies in humans have also shown the efficacy of pulmonary rehabilitation on the deleterious effects of pulmonary fibrosis, improving the endurance exercise capacity.^{19,21} In our previous study, a mice experimental model of bleomycin-induced pulmonary fibrosis was used. The swimming protocol used in this study achieved physical performance improvement, which was detected in animals using an

endurance exercise capacity test. Additionally, the protocol reduced the pulmonary fibrosis and the quantity of type I collagen.³³ Furthermore, the expression level of TGF- β ₁, beta-prolyl-4-hydroxylase, MMP-1 and MMP-2, and their relationship with the lung lesions and the exercise are not known. There are no studies about the association between the physical training and the use of an ACE2 stimulator drug, thus the aim of this study is to evaluate the effects of ACE2 activator associated with physical exercise on pulmonary lesions experimentally induced by bleomycin.

Material and methods

Animals

Ninety-six, 10-week-old, male Balb/c mice were bred in an accredited animal facility within the Federal University of Minas Gerais (UFMG). The mice were divided into the following groups: sedentary control (C-SED, $n = 11$), sedentary control treated with an ACE2 stimulator (C-SED/E, $n = 12$), exercise control (C-EXE, $n = 11$), exercise control treated with ACE-2 stimulator (C-EXE/E, $n = 13$), sedentary bleomycin (BLM-SED, $n = 11$), sedentary bleomycin treated with ACE2 stimulator (BLM-SED/E, $n = 13$), exercise bleomycin (BLM-EXE, $n = 12$), and exercise bleomycin treated with ACE-2 stimulator (BLM-EXE/E, $n = 13$). The mice were housed in group cages, at an ambient temperature of around 23°C, a regular 12:12 h light cycle, receiving water ad libitum, and handled in accordance to the guidelines in the Ethical Committee in Animal Experimentation.

Pulmonary fibrosis induction

After anesthesia (5% ketamine, 200 mg/kg and 2% xylazine, 10 mg/kg dissolved in 2 ml of 0.9% saline, ip), 8 U/kg of bleomycin (bleomycin sulfate, Meizle Biopharma) was intratracheally instilled. The control group received the corresponding volume of saline solution. After nine days of intratracheal instillation, all mice were adapted to the pool water with a temperature of $31 \pm 1^\circ\text{C}$ and no overload as described by Prata *et al.*³³

Treatment with the drug that activates the ACE 2

On the 14 day of the experiment, the animals (C-SED/E, C-EXE/E, BLM-SED/E and BLM-EXE/E) were daily treated with 1 mg/kg of DIZE acetate, by gavage, until the end of the experiment.³⁴

Exercise endurance capacity evaluation

The assessment of functional capacity was done 14 days after the intratracheal instillation and 48 h after the last day of training, at the end of the experiment. The mice were tested individually at the pool with a progressive load attached to their tail, until the moment of exhaustion. The final load of the first test (maximum load) was used to calculate the adaptation load used in the animals' exercise and training.³³

Exercise adaptation and physical training

As shown in our previous protocol, exercise adaptation was held in a pool with 60% of the maximum load.³³ The animals in the exercise group (C-EXE, C-EXE/E, BLM-EXE, BLM-EXE/E) swam for five days/week during four weeks. After adaptation, the animals were trained for five days/week for another four weeks.

Necropsy and histopathological lung analysis

The animals were anesthetized with ketamine-solution-10% 2% xylazine (60 and 6 mg/kg, respectively, i.p.) and a laparotomy was performed to the section of the abdominal aorta. Then, an incision in the diaphragm and a sternal thoracotomy was performed to remove the lungs. Slices of the left lung were fixed in 10% formalin, processed and included in paraffin. Sections of approximately 4 μ m were obtained and stained with Gomori's trichrome for quantitative analysis of fibrosis. Other sections were used to perform immunohistochemical reactions for TGF- β_1 , type I collagen, MMP-1, MMP-2, and beta-prolyl-4-hydroxylase.

Morphometric analysis of the pulmonary fibrosis

Histological sections stained with Gomori's trichrome and the sections submitted to immunohistochemical reactions were viewed with a 40 $\times$ objective and 20 random images scanned with a JVC TK-1270/RGB (Tokyo, Japan) micro camera, comprising an area of $1.06 \times 10^6 \mu\text{m}^2$ of lung analyzed in each type of immunohistochemical markers. The area of fibrosis and immunohistochemical markers was calculated using a KS300 software of the Carl Zeiss image analyzer (Oberkochen, Germany). In each image, all green (Gomori's trichrome) or brown (positive immunohistochemical staining) hued pixels were selected to create a binary image, to process it digitally, and to calculate the area.

Immunohistochemical reactions for TGF- β_1 , type I collagen, MMP-1, MMP-2, and beta-prolyl-4-hydroxylase

The sections of lung fragments were incubated with monoclonal antibody TGF- β_1 , MMP-1, MMP-2, type I collagen (Santa Cruz Biotechnology, Santa Cruz, USA, Catalog numbers: sc-52893, sc-21731, sc-13595, sc-25974) and beta-prolyl-4-hydroxylase (ACRIS, Herford, Germany, Catalog number: AF0910-1) for 16 to 18 h; incubated with both biotinylated IgG (Bethyl Laboratories Inc., Montgomery, USA) and peroxidase-conjugated streptavidin (Zymed Laboratories Inc., San Francisco, USA) diluted 1:50 for 1 h each. The marking was revealed using a 0.05% diaminobenzidine solution in H_2O_2 -40vv-0.2%. For the negative control, primary antibody was replaced by PBS. The sections were counterstained with Harris hematoxylin and mounted in Entelan.

Statistical analysis

The results are given as means $\pm$ SEM. Comparisons among groups were executed using one-way ANOVA followed by the Tukey test. Pearson's *r* or Spearman *r* was used to correlate the different variables. Statistical analyses were done with the GraphPad Prism 5 software (GraphPad Software,

Inc., San Diego, CA, USA). Statistical significance was set at $P < 0.05$.

Results

Evaluation of the endurance exercise capacity

Animals that died during the experiment were not included in the analyses (C-SED $n=1$, C-SED/E $n=4$, C-EXE/E $n=3$, BLM-SED $n=3$, BLM-SED/E $n=3$, BLM-EXE $n=2$, BLM-EXE/E $n=3$).

The evaluation was performed by a progressive load test to compare the functional capacity of animals between groups. The physical training proposed by the study increased the maximum physical conditioning of the C-EXE group (19.2 ± 0.8923 min, $P < 0.05$, one-way ANOVA followed by the Tukey test) when compared to the C-SED, BLM-SED, C-SED/E, BLM-SED/E, BLM-EXE, and BLM-EXE/E. By associating the physical training with ACE2 stimulation in the C-EXE/E group (20.29 ± 0.8843 min, $P < 0.05$, one-way ANOVA followed by the Tukey test), it was observed an increased functional capacity compared to the other groups (Figure 1).

The BLM-SED group (7.95 ± 0.3545 min, $P < 0.05$, one-way ANOVA followed by the Tukey test) had a lower functional capacity when compared to the C-SED group (9.61 ± 0.3465 min) demonstrating the effectiveness of the bleomycin dose (8 U/kg) in reducing the BLM-SED physical conditioning. The ACE2 stimulation reduced the bleomycin deleterious effects on the functional capacity of the BLM-SED/E group (11.42 ± 0.3826 min, $P < 0.05$, one-way ANOVA followed by the Tukey test) if compared to the BLM-SED group. Furthermore, the functional capacity results of the BLM-SED/E group were similar to the results obtained by the C-SED and C-SED/E groups (11.14 ± 0.3265 min).

The BLM-EXE/E group (16.48 ± 0.5874 min, $P < 0.05$, one-way ANOVA followed by the Tukey test) had a better physical conditioning when compared to the BLM-EXE

Figure 1 Endurance time of the different control groups (C-SED, C-SED/E and C-EXE, C-EXE/E) and the bleomycin groups (BLM-SED, BLM-SED/E and BLM-EXE, BLM-EXE/E) at the end of the experiment. ^a $P < 0.05$ (BLM-SED) compared to the other groups; ^c $P < 0.05$ (C-EXE and C-EXE/E) compared to the other groups; ^d $P < 0.05$ (BLM-EXE) compared to a (C-SED, C-SED/E and BLM-SED/E), and b (BLM-SED); ^e $P < 0.05$ (BLM-EXE/E) in relation to a (C-SED, C-SED/E, and BLM-SED/E), b (BLM-SED) and d (BLM-EXE). Data were analyzed by the one-way ANOVA followed by the Tukey test.

group (14.13 ± 0.3867 min, $P < 0.05$, one-way ANOVA followed by the Tukey test). This result shows, for the first time, that the improvement in functional capacity was enhanced by the combination of both DIZE pharmacological treatment and physical training (Figure 1).

Histopathology and morphometry of the lung parenchyma

The animals of the C-SED, C-SED/E, C-EXE and C-EXE/E groups showed parenchymal architecture compatible with controls (Figure 2(a)).

The BLM-SED group presented pulmonary parenchyma alterations characterized by an extensive and diffuse fibrosis with different intensities in the alveolar septa, perivascular, and peribronchiol interstices (Figure 2(b)). It was also observed in this group an intense fibrocellular thickening of the septum, causing the narrowing of the alveolar and bronchiolar airways (Figure 2(c)).

The BLM-SED/E group (Figure 2(d)) showed an apparent fibrosis reduction when compared to the BLM-SED group. The BLM-EXE and BLM-EXE/E groups (Figure 2(e) and (f)) had a decrease in fibrosis when compared to the BLM-SED and BLM-SED/E groups. Moreover, there was restoration of some airways in the pulmonary parenchyma of the BLM-EXE/E group when compared to the BLM-EXE group.

The BLM-SED group ($16,380 \pm 838.4 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) presented a significant increase of the measured fibrous connective tissue area, in comparison with the other groups (Figure 2(h)). The DIZE pharmacological treatment performed on BLM-SED/E group ($4599 \pm 106.1 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) was able to significantly reduce the fibrosis area when compared to the BLM-SED group. The physical training in a swimming pool reduced even more the fibrosis of the BLM-EXE group ($1662 \pm 82.13 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) in comparison with the BLM-SED and BLM-SED/E groups. By associating pharmacological treatment with regular physical exercise, it was also noticed a significant reduction of fibrous connective tissue in mice of the BLM-EXE/E group ($263.3 \pm 26.35 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) when compared to the animals that underwent the individually proposed treatments in the BLM-SED/E and BLM-EXE groups. There was no significant difference in the pulmonary fibrous connective tissue area between the C-SED ($154.8 \pm 18.16 \mu\text{m}^2$), C-SED/E ($154.9 \pm 11.85 \mu\text{m}^2$), C-EXE ($135.3 \pm 12.43 \mu\text{m}^2$), and C-EXE/E ($130.0 \pm 8.22 \mu\text{m}^2$) groups (Figure 2(g)).

The positive markers in immunohistochemistry were observed as brown precipitated zones in the lung parenchyma. The removal of the primary antibody produced a negative reaction in all markers. Mice of all control groups showed small areas of positive staining for type I collagen, TGF- β_1 , beta-prolyl-4-hydroxylase, MMP-1 and MMP-2. The BLM-SED group had extensive positive staining area for type I collagen, characterized by the presence of precipitates stained in shades of brown in the alveolar interstitial, peribronchial, perivascular, interlobular, and subpleural

spaces (Figure 3(b) and (c)). Mice of the BLM-SED/E group (Figure 3(d)) showed a reduction of type I collagen when compared to the BLM-SED group. In the BLM-EXE group (Figure 3(e)), it was observed a decrease of this marking in comparison with the BLM-SED and BLM-SED/E groups. In BLM-EXE/E group (Figure 3(f)), the exercise associated with DIZE reduced the expression of collagen I and lung changes more than just exercise in BLM-EXE group and more than just pharmacotherapy in BLM-SED/E group.

The results in Figure 3(g) and (h) were similar to the fibrous connective tissue area. There was no significant difference of positive staining area for type I collagen among the groups C-SED ($128.1 \pm 18.30 \mu\text{m}^2$), C-SED/E ($115.7 \pm 16.97 \mu\text{m}^2$), C-EXE ($106.4 \pm 6.63 \mu\text{m}^2$) and C-EXE/E ($103.7 \pm 9.04 \mu\text{m}^2$) (Figure 3(g)). However, in the BLM-SED group ($10,330 \pm 480.9 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test), an increase of type I collagen when compared to the other groups was observed. DIZE administration in the BLM-SED/E group ($2683 \pm 161.1 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) significantly reduced type I collagen when compared to the BLM-SED group. The BLM-EXE group ($1108 \pm 58.48 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) showed a decrease in type I collagen when compared to the BLM-SED and BLM-SED/E groups. The association of physical training with the activating ACE2 drug treatment reduced the positive staining area for type I collagen in the BLM-EXE/E group ($203.5 \pm 17.22 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) in comparison with the BLM-SED, BLM-SED/E, and BLM-EXE groups.

The BLM-SED group had large areas of positive staining for TGF- β_1 and beta-prolyl-4-hydroxylase (Figures 4 and 5, respectively). The BLM-EXE/E group was the one with the smallest positive reaction regions for these two markers. The BLM SED/E group showed larger marking areas in comparison with the BLM-EXE/E group, but smaller marking areas when compared to the BLM-SED group.

The TGF- β_1 expression area showed no significant difference between the C-SED ($80.74 \pm 8.76 \mu\text{m}^2$), C-SED/E ($77.24 \pm 3.79 \mu\text{m}^2$), C-EXE ($71.67 \pm 5.32 \mu\text{m}^2$), and C-EXE/E ($69.68 \pm 6.86 \mu\text{m}^2$) groups (Figure 4(h)). The BLM-SED group ($17,350 \pm 902.9 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) had a TGF- β_1 expression significantly higher than the other groups.

Animals treated with DIZE in the BLM-SED/E group ($3842 \pm 133.3 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) showed a TGF- β_1 area smaller than the BLM-SED group. Regular swimming practice of the BLM-EXE group ($1462 \pm 98.89 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) reduced the TGF- β_1 expression when compared to the BLM-SED and the BLM-SED/E groups. The BLM EXE/E group ($767.3 \pm 51.40 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) also showed, for the first time in the literature, that the combination of the proposed treatments further reduced the TGF- β_1 positive staining area when compared to the BLM-SED, BLM-SED/E, and BLM-EXE groups (Figure 4(h)).

Figure 2 Pulmonary parenchyma of mice in the sedentary control group, representing the other control groups, and the mice with bleomycin-induced pulmonary fibrosis, sedentary or submitted to swimming, with or without the ACE2 stimulation. (a) C-SED group: aerated alveoli (a), interalveolar septum with ducts and alveolar sacs with thin thickness (arrowheads), bar = 100 μm ; (b) BLM-SED group: intense fibrocellular septal thickening caused by the accumulation of cells and richly vascularized connective tissue (*), Lumen alveolar (a), bar = 100 μm ; (c) BLM-SED group: in more detail, intense deposition of fibrous connective tissue in the interalveolar septum (*), Lumen alveolar (a), bar = 50 μm ; (d) BLM-SED/E group: presence of still thickened septum (arrows) along with others with regular thickness (arrowheads), aerated respiratory alveoli (a), bar = 50 μm . (e) BLM-EXE group: observe septal thickness reduction when compared to the previous figure (arrowheads), aerated respiratory alveoli (a), bar = 50 μm . (f) BLM-EXE/E group: visible decrease in fibrosis with restoration of the airways when compared to all the other BLM groups (arrowheads) and aerated respiratory alveoli (a), bar = 50 μm ; Gomori's trichrome staining. (g) Pulmonary fibrous connective tissue area (μm^2) of the animals of the control group, sedentary and trained, with or without pharmacological treatment; (h) Pulmonary fibrous connective tissue area (μm^2) of the animals of the bleomycin group, sedentary and trained, with or without pharmacological treatment; ^a $P < 0.05$ compared to the other groups; ^b $P < 0.05$ compared to the previous figure; ^c $P < 0.05$ compared to the a, b, and d; ^d $P < 0.05$ compared to the other groups. Data were analyzed by the one-way ANOVA followed by the Tukey test. (A color version of this figure is available in the online journal.)

Figure 3 Immunohistochemical reaction for type I collagen in lung parenchyma of mice with bleomycin-induced pulmonary fibrosis, sedentary or submitted to swimming, with or without ACE2 activating drug. The brown staining in the alveolar interstice showing positive reaction. (a) Negative control of immunohistochemical reaction for type I collagen; (b) BLM-SED group: intense area of type I collagen in the thickened interalveolar interstice (*); (c) Higher view of the anterior figure showing positive reaction for type I collagen (*); (d) BLM-SED/E group: positive stain for type I collagen, in the thickened interalveolar interstice (*). The insert shows details of the figure (d); (e) BLM-EXE group: type I collagen immunoreactive areas (arrowheads) showing smaller area. The insert shows details of the figure (e); (f) BLM-EXE/E group: reduction of the immunostaining area for type I collagen (arrowheads). The insert shows details of the figure (f). Counterstaining with Harris hematoxylin. Bar = 50 μm. (g) Positive staining area for type I collagen (μm²) of the animals of the control group, sedentary and trained, with or without pharmacological treatment; (h) Positive staining area for type I collagen (μm²) of the animals of the bleomycin group, sedentary and trained, with or without pharmacological treatment; ^a $P < 0.05$ compared to the other groups; ^b $P < 0.05$ compared to a, c, and d; ^c $P < 0.05$ compared to the a, b, and d; ^d $P < 0.05$ compared to the other groups. Data were analyzed by the one-way ANOVA followed by the Tukey test. (A color version of this figure is available in the online journal.)

Figure 4 Immunohistochemical reaction for TGF- β_1 in lung parenchyma of mice with bleomycin-induced pulmonary fibrosis, sedentary or submitted to swimming, with or without the ACE2 activating drug. The brown staining in the alveolar interstice showing positive reaction. (a) Negative control of immunohistochemical reaction for TGF- β_1 (b) BLM-SED group: extensive TGF- β_1^{+} areas in the thickened interalveolar interstice (*); (c) Higher view of the anterior figure showing positive reaction for TGF- β_1 (*); (d) BLM-SED/E group: positive stain for TGF- β_1 in the thickened interalveolar interstice (arrowheads). The insert shows details of the figure (d); (e) BLM-EXE group: TGF- β_1 reduced immunoreactive areas (arrowheads). The insert shows details of the figure (e); (f) BLM-EXE/E group: reduction of TGF- β_1 immunohistochemical marking regions (arrowheads). The insert shows details of the figure (f). Counterstaining with Harris hematoxylin. Bar = 50 μm . (g) Positive staining area for TGF- β_1 (μm^2) of the animals of the control group, sedentary, and trained, with or without pharmacological treatment; (h) Positive staining area for TGF- β_1 (μm^2) of the animals of the bleomycin group, sedentary and trained, with or without pharmacological treatment; ^a $P < 0.05$ compared to the other groups; ^b $P < 0.05$ compared to a, c and d; ^c $P < 0.05$ compared to the a, b and d; ^d $P < 0.05$ relative to the other groups. Data were analyzed by the one-way ANOVA followed by the Tukey test. (A color version of this figure is available in the online journal.)

Figure 5 Immunohistochemical reaction for beta-prolyl-4-hydroxylase in lung parenchyma of mice with bleomycin-induced pulmonary fibrosis, sedentary or submitted to swimming, with or without the ACE2 activating drug. The brown staining in the alveolar interstice showing positive reaction. (a) Negative control of immunohistochemical reaction for beta-prolyl-4-hydroxylase; (b) BLM-SED group: beta-prolyl-4-hydroxylase⁺ areas in the thickened interalveolar interstice (*); (c) BLM-SED group: higher view of the anterior figure showing positive reaction for beta-prolyl-4-hydroxylase (*); (d) BLM-SED/E group: beta-prolyl-4-hydroxylase⁺ immunolabel regions in the thickened interalveolar space (arrowheads). The insert shows details of the figure (d); (e) BLM-EXE group: beta-prolyl-4-hydroxylase reduced immunoreactive areas (arrowheads). The insert shows details of the figure (e); (f) BLM-EXE/E group: Reduction of beta-prolyl-4-hydroxylase⁺ areas compared to the previous figures (arrowheads). The insert shows details of the figure (f). The regions of positive markers for beta-prolyl-4-hydroxylase in the different groups showed the same pattern observed with the TGF- β_1 . Counterstaining with Harris hematoxylin. Bar = 50 μ m. (g) Positive staining area for beta-prolyl-4-hydroxylase (μ m²) of the animals of the control group, sedentary and trained, with or without pharmacological treatment; (H) Positive staining area for beta-prolyl-4-hydroxylase (μ m²) of the animals of the bleomycin group, sedentary, and trained, with or without pharmacological treatment; $P < 0.05$ compared to the other groups; $^bP < 0.05$ compared to a, c, and d; $^cP < 0.05$ compared to the a, b, and d; $^dP < 0.05$ compared to the other groups. Data were analyzed by the one-way ANOVA followed by the Tukey test. (A color version of this figure is available in the online journal.)

The quantitative results of the beta-prolyl-4-hydroxylase enzyme expression in the lungs of the animals are shown in the Figure 5(g) and (h). There was no significant difference between the C-SED ($88.88 \pm 11.93 \mu\text{m}^2$), C-SED/E ($91.70 \pm 5.3 \mu\text{m}^2$), C-EXE ($96.41 \pm 8.27 \mu\text{m}^2$), and C-EXE/E ($83.08 \pm 5.01 \mu\text{m}^2$) groups (Figure 6(g)). The expression of this enzyme was significantly higher in the BLM-SED ($12,830 \pm 803.3 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) when compared to the other groups. Furthermore, the administration of DIZE produced a more significantly reduced expression of this enzyme in the BLM-SED/E group ($8761 \pm 183.4 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) than in the BLM-SED group. Animals in the BLM-EXE group ($5559 \pm 402.0 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) showed a decrease in this enzyme expression in comparison with the BLM-SED/E and exercise associated with DIZE was potentially able to significantly reduce the beta-prolyl-4-hydroxylase expression in the BLM-EXE/E group ($3636 \pm 122.0 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test).

The MMP-1 and MMP-2 expression had opposite results in comparison to the TGF- β_1 and beta-prolyl-4-hydroxylase markers in all bleomycin groups. The BLM-EXE and BLM-EXE/E groups had large areas of positive staining for MMP-1 and MMP-2 (Figures 6 and 7, respectively). In the BLM-SED group, smaller marking regions were observed.

The positive area for MMP-1 of the C-SED ($88.09 \pm 8.86 \mu\text{m}^2$), C-SED/E ($94.05 \pm 3.99 \mu\text{m}^2$), C-EXE ($112 \pm 6.37 \mu\text{m}^2$), and C-EXE/E ($109 \pm 6.99 \mu\text{m}^2$) groups showed no difference between groups (Figure 6(g)). The expression of MMP-1 in the BLM-SED group ($2298 \pm 326.8 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) was lower than in the other bleomycin groups. There was an increase of MMP-1 in the BLM-SED/E group ($7261 \pm 490.7 \mu\text{m}^2$) compared with the BLM-SED group. The BLM-EXE group ($8741 \pm 423.5 \mu\text{m}^2$) showed a larger positive area for MMP-1 than the BLM-SED/E. The MMP-1 area of the BLM-EXE/E group ($12,060 \pm 389.3 \mu\text{m}^2$) was significantly higher than the other groups used in the study. However, the BLM-SED, BLM-SED/E, BLM-EXE, and BLM-EXE/E groups showed larger MMP-1 positive areas than the control groups (Figure 6(h)).

As in the MMP-1 positive staining results, the C-SED ($49.21 \pm 4.36 \mu\text{m}^2$), C-SED/E ($50.74 \pm 3.94 \mu\text{m}^2$), C-EXE ($56.7 \pm 4.19 \mu\text{m}^2$), and C-EXE/E ($57.32 \pm 7.03 \mu\text{m}^2$) groups did not differ among themselves for the MMP-2 expression (Figure 7(g)). The BLM-SED group ($4711 \pm 332.3 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) had a significantly smaller area of positive staining of this enzyme when compared to all the other bleomycin groups. DIZE was able to increase the MMP-2 area of the BLM-SED/E group ($7422 \pm 602.9 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) when compared to the BLM-SED. The exercise promoted an increase of the MMP-2 area in the BLM-EXE group ($312.3 \pm 10.340 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) in comparison with the BLM-SED and BLM-SED/E. Moreover, DIZE administration associated with the

proposed physical training caused the increase of MMP-2 in the BLM-EXE/E group ($14,700 \pm 569.6 \mu\text{m}^2$, $P < 0.05$, one-way ANOVA followed by the Tukey test) when compared to the other bleomycin groups (Figure 7(h)).

In the correlations made between the items evaluated in the study, a significant negative correlation was found between the fibrous connective tissue area and the functional capacity of the animals in the bleomycin group ($r = -0.8422$, $P < 0.0001$; Pearson's r test, Figure 8(a)). The fibrous connective tissue also had a strong positive correlation with the TGF- β_1 area ($r = 0.9339$, $P < 0.0001$; Pearson's r test, Figure 8(b)) and with the Beta-prolyl-4-hydroxylase expression area ($r = 0.8921$, $P < 0.0001$; Spearman r test Figure 7(c)). In relation to the MMP-1 ($r = -0.8796$, $P < 0.0001$; Pearson's r test, Figure 8(d)) and MMP-2 ($r = -0.7871$, $P < 0.0001$; Spearman r test, Figure 8(e)) areas, a negative correlation was observed.

Discussion

This study showed for the first time that the association between the aerobic exercise and the activation of the ACE 2 potentiated the reduction of bleomycin-induced pulmonary fibrosis. The reduction of lung lesions, such as fibrosis and the narrowing of the bronchiolar and alveolar airways was detected by the reduction of the area occupied by fibrous connective tissue, especially of the type I collagen.

Among the factors involved in such reduction, the reduction of TGF- β_1 expression and of the beta-prolyl-4-hydroxylase, and the increase in MMP-1 and MMP-2 were shown. Thus, the reduction of the extracellular matrix synthesis mechanisms and the increase of its degradation were involved in the reduction of lung fibrosis. In addition, the concomitant use of two kinds of treatment was able to significantly increase the fitness, which reflects an improvement in cardiac function.

According to our previous studies, pool training was able to increase the physical conditioning of both healthy and bleomycin-induced pulmonary fibrosis mice. It was also showed that the exercise was able to reduce both the intensity of pulmonary lesions and the area of type I collagen.³³ As expected, the reduction of the fibrous connective tissue area was also observed along with an improvement of physical conditioning. In other words, physical training is able to alleviate lung injury and to increase the maximum swimming time as well as the animal's ability to withstand greater loads during swimming. Given these results, we decided to investigate whether exercise training associated with some form of drug treatment could improve the effects of physical training. Evidence from a randomized study in individuals with ILD shows that the use of pulmonary recuperation programs results in an improved exercise capacity, reduction of symptoms, and better life quality of patients in this group of diseases. However, the exercise protocol for patients with PID is the same used in pulmonary rehabilitation for patients with chronic obstructive pulmonary disease (COPD).²²

Some studies show evidence that components of the RAS can function as therapeutics for the treatment of fibrosing

Figure 6 Immunohistochemical reaction for MMP-1 in lung parenchyma of mice with bleomycin-induced pulmonary fibrosis, sedentary, or submitted to swimming, with or without the ACE2 activating drug. The brown staining in the alveolar interstice shows positive reaction. (a) Negative control of immunohistochemical reaction for MMP-1; (b) BLM-SED group: slight positive staining for MMP-1 in alveolar interstitium (arrowheads); (c) BLM-SED group: higher view of the anterior figure showing positive reaction for MMP-1 (arrowheads); (d) BLM-SED/E group: positive staining for MMP-1 in the pulmonary interstitial septum (arrowheads) more intense than figure (b). The insert shows details of the figure (d); (e) BLM-EXE group: marking area similar to the one in Figure (d) (arrowheads). The insert shows details of the figure (e); (f) BLM-EXE/E group: increased marking area for MMP-1 compared to the previous figures (arrowheads). The insert shows details of the figure (f). Counterstaining with Harris hematoxylin. Bar = 50 μm. (g) Positive staining area for MMP-1 (μm²) of the animals of the control group, sedentary, and trained, with or without pharmacological treatment; (h) Positive staining area for MMP-1 (μm²) of the animals of the bleomycin group, sedentary, and trained, with or without pharmacological treatment; $P < 0.05$ compared to the other groups; ^a $P < 0.05$ compared to the other groups; ^b $P < 0.05$ compared to a and c; ^c $P < 0.05$ compared to a and b. Data were analyzed by the one-way ANOVA followed by the Tukey test. (A color version of this figure is available in the online journal.)

Figure 7 Immunohistochemical reaction for MMP-2 in lung parenchyma of mice with bleomycin-induced pulmonary fibrosis, sedentary, or submitted to swimming, with or without the ACE2 activating drug. The brown staining in the alveolar interstitium shows positive reaction. (a) Negative control of immunohistochemical reaction for MMP-2; (b) BLM-SED group: discrete area of MMP-2 expression (arrowheads); (c) BLM-SED group: higher view of the anterior figure showing positive reaction for MMP-2 (arrowheads); (d) BLM-SED/E group: larger MMP-2 marking area in comparison to figure (b) (arrowheads). The insert shows details of the figure (d); (e) BLM-EXE group: increased expression of MMP-2 (arrowheads). The insert shows details of the figure (e); (f) BLM-EXE/E group: intense expression of interstitial MMP-2 in comparison to all BLM groups (arrowheads). The insert shows details of the figure (f). Counterstaining with Harris hematoxylin. Bar = 50 μm . (g) Positive staining area for MMP-2 (μm^2) of the animals of the control group, sedentary and trained, with or without pharmacological treatment; (h) Positive staining area for MMP-2 (μm^2) of the animals of the bleomycin group, sedentary, and trained, with or without pharmacological treatment; $P < 0.05$ compared to the other groups; $^aP < 0.05$ compared to the other groups; $^bP < 0.05$ compared to a, c, and d; $^cP < 0.05$ compared to the a, b, and d; $^dP < 0.05$ compared to the other groups. Data were analyzed by the one-way ANOVA followed by the Tukey test. (A color version of this figure is available in the online journal.)

Figure 8 Correlation between the fibrous connective tissue and the other results obtained in this study. (a) Strong negative correlation between pulmonary fibrosis tissue area (μm^2) and functional capacity (minutes); (b) Strong positive correlation between pulmonary fibrosis tissue area (μm^2) and TGF- β_1 area (μm^2); (c) Strong positive correlation between pulmonary fibrosis tissue area (μm^2) and the expression of beta-prolyl-4-hydroxylase (μm^2); (d) Strong negative correlation between pulmonary fibrosis tissue area (μm^2) and MMP-2 area (μm^2). Pearson's r or Spearman r was used to correlate the different variables

diseases.²⁴ The functional balance of RAS depends on the balance of the activity of the two pathways that make up this system (angiotensin-converting enzyme (ACE)/angiotensin(Ang)II/AT1 receptor and ACE2/Ang-(1-7)/Mas receptor). Different studies have shown that hyperactivity of the ACE/AngII/AT1 pathway is associated with the pathophysiology of various diseases, due to its inflammatory, fibrogenic, vasoconstrictor, proliferative, and oxidative stress-inducing actions.²⁴⁻³¹ Other authors also observed the association of this pathway in the development of pulmonary hypertension and fibrosis.^{24,31} In fact, the increase of AT1 receptor levels may contribute to the activation of fibroblasts, collagen synthesis, and its progress to the development of fibrosis.³⁵ This enzyme catalyzes the hydrolysis of the C-terminal residue of Ang II, producing the protective peptide Ang-(1-7), which is a specific ligand of the Mas receptor.³⁶⁻³⁸

The increase of the Ang-(1-7) expression, as well as the overexpression of ACE2, can reduce the excessive deposition of pulmonary collagen, the systolic pressure of the

right ventricle, the right ventricular fibrosis, and pulmonary vascular remodeling caused by the administration of bleomycin. However, the MAS receptor blockade inhibited the beneficial effects caused by the increase of Ang-(1-7) expression.^{24,39} Thus, this study also showed that the ACE2 stimulation by DIZE administration, significantly reduced type I collagen, as well as the expression of TGF- β_1 , beta-prolyl-4-hydroxylase and increased the endurance capacity, the MMP-1 and MMP-2 in mice with bleomycin-induced fibrosis.

We have also observed that in the group of sedentary animals treated with DIZE, there was a significant decrease of the fibrous connective tissue area. With this observation, coupled with what had already been studied by other authors, we demonstrated that the partial restoration of lung tissue integrity is correlated with the reduction in TGF- β and beta-prolyl-4-hydroxylase expressions, and with the increase of MMPs-1 and 2 markers. Thus, the raised activity of the ACE2/Ang-(1-7)/Mas pathway reduced the immunolabel of one of the major cytokines

involved in fibrosis development and also of an important enzyme involved in collagen synthesis. The activity of enzymes involved in the digestion of different elements of the extracellular matrix was also increased, including the collagen fibers.^{5,6} In addition to contributing to the initial understanding of the mechanisms by which DIZE contributes to the reduction of injuries, it was also shown that sedentary animals treated with DIZE have a significant improvement of physical conditioning.

This study also allowed us to further understand the effect of exercise on lung injury induced by bleomycin. Bleomycin animals that swam had a significant reduction in the expression of TGF- β_1 and beta-prolyl-4-hydroxylase. The decreased expression of this important fibrosing cytokine and of the enzyme was associated with the reduction of lesions observed with the DIZE treatment. An interesting finding is that the exercise was more effective in raising the MMP-2 marker than the treatment with DIZE. Also, the association of exercise with pharmacological treatment caused a further increase in the MMP-2 immunolabel. In relation to the MMP-1 immunolabel, no differences were found between the two therapeutic methods used separately. Also, the association between the two led to an increased MMP-1 immunolabel. Further studies are necessary to understand the differences in expression between treatments.

Our results show that exercise training associated with the use of DIZE was able to increase the exercise endurance level and significantly reduce lung lesions experimentally bleomycin-induced, presenting itself as a possible tool in the treatment of pulmonary fibrotic diseases such as the IPF.

Authors' contributions: All authors contributed in the design, analysis of the data and review of the manuscript. LOP, CRR, JMM, PCV, FMSO, AJF, MdGR-M, and MVC conducted the experiments; LOP, AJF, and MVC supplied critical reagents, animals and equipments; and LOP, MdGR-M, and MVC wrote the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

- Guerra M, Miranda JA, Leal F, Vouga L. Interstitial lung disease: diagnostic accuracy and safety of surgical lung biopsy. *Rev Port Pneumol* 2009;**XV**:433–42
- Rubin AS, Moreira JS, Porto NS, Irion KL, Moreira RF, Scheidt B. Pulmonary idiopathic fibrosis: clinical findings and survival in 132 histologically-proven patients. *J Pneumol* 2000;**26**:61–8
- Selman M, Pardo A. Idiopathic pulmonary fibrosis: an epithelial/fibroblastic cross-talk disorder. *Respir Res* 2001;**3**:1–8
- Coultais DB, Zumwalt RE, Black WC, Sobonya RE. The epidemiology of interstitial lung diseases. *Am J Respir Crit Care Med* 1994;**150**:967–72
- Chapman HA. Disorders of lung matrix remodeling. *J Clin Invest* 2004;**113**:148–57
- Gharaee-kermani M, Gyetko MR, Hu B, Phan SH. New insights into the pathogenesis and treatment of idiopathic pulmonary fibrosis: a potential role for stem cells in the lung parenchyma and implications for therapy. *Pharm Res* 2007;**24**:819–41
- McGee JO'D, Langness U, Udenfriend S. Immunological evidence for an inactive precursor of collagen proline hydroxylase in cultured fibroblasts. *Proc Natl Acad Sci U S A* 1971;**68**:1585–9
- Kjetil A, Martin GEM, Kolb M, Gaudie J. Targeting genes for treatment in idiopathic pulmonary fibrosis. *Am Thorac Soc* 2006;**3**:389–93
- Hasaneen NA, Zucker S, Cao J, Chiarelli C, Panettieri RA, Foda HD. Cyclic mechanical strain-induced proliferation and migration of human airway smooth muscle cells: role of EMMPRIN and MMPs. *FASEB J* 2005;**19**:1507–9
- Gueders MM, Foidart JM, Noel A, Cataldo DD. Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs in the respiratory tract: potential implications in asthma and other lung disease. *Eur J Pharmacol* 2006;**533**:133–44
- Pardo A, Selman M. Matrix metalloproteinases in aberrant fibrotic tissue remodeling. *Am Thorac Soc* 2006;**3**:383–8
- Corbel M, Boichot E, Lagente V. Role of gelatinases MMP-2 and MMP-9 in tissue remodeling following acute lung injury. *Braz J Med Biol Res* 2000;**33**:749–54
- Moeller A, Ask K, Warburton D, Gaudie J, Kolb M. The bleomycin animal model: a useful tool to investigate treatment options for idiopathic pulmonary fibrosis? *Int J Biochem Cell Biol* 2008;**40**:362–82
- Latta VD, Cecchetti A, Del Ry S, Morales MA. Bleomycin in the setting of lung fibrosis induction: from biological mechanism to counteractions. *Pharmacol Res* 2015;**97**:122–30
- Benítez SC. Bleomicina: un modelo de fibrosis pulmonar. *Rev Inst Nal Enf Resp Mex* 2006;**19**:53–61
- Spruit MA, Janssen DJA, Franssen FME, Wouters EFM. Rehabilitation and palliative care in lung fibrosis. *Respirology* 2009;**14**:781–7
- Garibaldi BT, Danoff SK. Symptom-based management of the idiopathic interstitial pneumonia. *Respirology* 2015. Epub ahead of print 9 October. DOI: 10.1111/resp.12649
- Dowman L, Hill CJ, Holland AE. Pulmonary rehabilitation for interstitial lung disease. *Cochrane Database Syst Rev* 2014;**10**:CD006322
- Vainshelboim B, Oliveira J, Yehoshua L, Weiss I, Fox BD, Fruchter O, Kramer MR. Exercise training-based pulmonary rehabilitation program is clinically beneficial for idiopathic pulmonary fibrosis. *Respiration* 2014;**88**:378–88
- Vainshelboim B, Oliveira J, Fox BD, Fruchter O, Kramer MR. Long-term effects of a 12-week exercise training program on clinical outcomes in idiopathic pulmonary fibrosis. *Lung* 2015;**193**:345–54
- Gaunaud IA, Gómez-Marín OW, Ramos CF, Sol CM, Cohen MI, Cahalin LP, Cardenas DD, Jackson RM. Physical activity and quality of life improvements of patients with idiopathic pulmonary fibrosis completing a pulmonary rehabilitation program. *Respir Care* 2014;**59**:1872–9
- Holland AE, Dowman LM, Hill CJ. Principles of rehabilitation and reactivation: Interstitial lung disease, sarcoidosis and rheumatoid disease with respiratory involvement. *Respiration* 2015;**89**:89–99
- Papoti M, Almeida PBL, Prada FJA, Eleno TG, Hermi HA, Gobatto CA, Mello MAR. Maximal lactate steady-state during swimming in rats recovered from protein malnutrition. *Motriz* 2003;**9**:103–10
- Ferreira AJ, Shenoy V, Yamazato Y, Sriramula S, Francis J, Yuan L, Castellano RK, Ostrov DA, Oh SP, Katovich MJ, Raizada MK. Evidence for angiotensin-converting enzyme 2 as a therapeutic target for the prevention of pulmonary hypertension. *Am J Respir Crit Care Med* 2009;**179**:1048–54

25. Ferreira AJ, Santos RAS, Almeida AP. Angiotensin-(1-7): cardioprotective effect in myocardial ischemia/reperfusion. *Hypertension* 2001;**38**:665-8
26. Ferrario CM. Angiotensin-converting enzyme 2 and angiotensin-(1-7): an evolving story in cardiovascular regulation. *Hypertension* 2006;**47**:515-21
27. Oudit GY, Kassiri Z, Patel MP, Chappell M, Butany J, Backx PH, Tsushima RG, Scholey JW, Khokha R, Penninger JM. Angiotensin II-mediated oxidative stress and inflammation mediate the age-dependent cardiomyopathy in ACE2 null mice. *Cardiovasc Res* 2007;**75**:29-39
28. Bader M, Ganten D. Update on tissue renin-angiotensin systems. *J Mol Med* 2008;**86**:615-21
29. Fraga-silva RA, Pinheiro SV, Gonçalves AC, Alenina N, Bader M, Santos RA. The antithrombotic effect of angiotensin-(1-7) involves mas-mediated NO release from platelets. *J Mol Med* 2008;**14**:28-35
30. Mercure C, Yogi A, Callera GE, Aranha AB, Bader M, Ferreira AJ, Santos RA, Walther T, Touyz RM, Reudelhuber TL. Angiotensin(1-7) blunts hypertensive cardiac remodeling by a direct effect on the heart. *Circ Res* 2008;**103**:1319-26
31. Shenoy V, Ferreira AJ, Qi Y, Fraga-silva RA, Díez-freire C, Dooies A, Jun JY, Sriramula S, Mariappan N, Pourang D, Venugopal CS, Francis J, Reudelhuber T, Santos RA, Patel JM, Raizada MK, Katovich MJ. The angiotensin-converting enzyme 2/angiogenesis-(1-7)/Mas axis confers cardiopulmonary protection against lung fibrosis and pulmonary hypertension. *Am J Respir Crit Care Med* 2010;**182**:1065-72
32. Kulemina LV, Ostrov DA. Prediction of off-target effects on angiotensin-converting enzyme. *J Biomol Screen* 2011;**16**:878-85
33. Prata LO, Oliveira FMS, Ribeiro TMS, Almeida PWM, Cardoso JA, Rodrigues-Machado MG, Caliari MV. Exercise attenuates pulmonary injury in mice with bleomycin-induced pulmonary fibrosis. *Exp Biol Med* 2012;**237**:873-83
34. Foureaux G, Nogueira JC, Nogueira BS, Fulgêncio GO, Menezes GB, Fernandes SOA, Cardoso VN, Fernandes RS, Oliveira GP, França JR, Faraco AAG, Raizada MK, Ferreira AJ. Antiglaucomatous effects of the activation of intrinsic angiotensin-converting enzyme 2. *Invest Ophthalmol Visual Sci* 2013;**54**:1-11
35. Marshall RP, Gohlke P, Chambers RC, Howell DC, Bottoms SE, Unger T, McAnulty RJ, Laurent GJ. Angiotensin II and the fibroproliferative response to acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2004;**286**:156-64
36. Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, Godbout K, Parsons T, Baronas E, Hsieh F, Acton S, Patane M, Nichols A, Tummino P. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem* 2001;**277**:14838-43
37. Ingelfinger JR. Angiotensin-converting enzyme 2: implications for blood pressure and kidney disease. *Curr Opin Nephrol Hypertens* 2009;**18**:79-84
38. Bradford CN, Ely DR, Raizada MK. Targeting the vasoprotective axis of the renin-angiotensin system: a novel strategic approach to pulmonary hypertensive therapy. *Curr Hypertens Rep* 2010;**12**:212-9
39. Silva ACS, Silveira KD, Ferreira AJ, Teixeira MM. ACE2, angiotensin-(1-7) and Mas receptor axis in inflammation and fibrosis. *Br J Pharmacol* 2013;**169**:477-92

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