Exercise Reduces Lung Fibrosis Involving Serotonin/Akt Signaling

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ABSTRACT

PEREIRA, P. R., M. C. OLIVEIRA-JUNIOR, B. MACKENZIE, J. E. D. CHIOVATTO, Y. MATOS, F. R. GREIFFO, N. C. RIGONATO-OLIVEIRA, T. R. BRUGEMMAN, H. DELLE, M. IDZKO, R. ALBERTINI, A. P. LIGEIRO OLIVEIRA, N. R. DAMACENO-RODRIGUES, E. G. CALDINI, I. E. FERNANDEZ, H. C. CASTRO-FARIA-NETO, M. DOLHNIKOFF, O. EICKELBERG, and R. P. VIEIRA. Exercise Reduces Lung Fibrosis Involving Serotonin/Akt Signaling. *Med. Sci. Sports Exerc.*, Vol. 48, No. 7, pp. 1276–1284, 2016. **Purpose**: Idiopathic pulmonary fibrosis (IPF) is a chronic fibrosing interstitial pneumonia, which involves aberrant serotonin (5-hydroxytryptamine [5-HT]) and Akt signaling. As protective effects of chronic aerobic training (AT) have been demonstrated in the context of lung injury, this study investigated whether AT attenuates bleomycin-induced lung fibrosis partly via a reduction of 5-HT and AKT signaling. **Methods**: Seventy-two C57BL/6 male mice were distributed in Control (Co), Exercise (Ex), Fibrosis (Fi), and Fibrosis + Exercise (Fi + Ex) groups. Bleomycin (1.5 UI·kg^{-1}) was administered on day 1 and treadmill AT began on day 15 and continued for 60 min·d^{-1}, 5 d·wk^{-1} for 4 wk. We evaluated total and differential cell counts in bronchoalveolar lavage (BAL), interleukin (IL)-1β, IL-6, CXCL1/KC, IL-10, tumor necrosis factor α, and transforming growth factor β levels in BAL, collagen content in lung parenchyma, 5-HT levels in BAL fluid and in serum, the expression of 5-HT2B receptor, and Akt phosphorylation in lung tissue. **Results**: AT reduced bleomycin-increased number of total cells ($P < 0.001$), neutrophils ($P < 0.01$), macrophages ($P < 0.01$), and lymphocytes ($P < 0.05$) in BAL. It also reduced the levels of IL-1β ($P < 0.01$), IL-6 ($P < 0.05$), CXCL1/KC ($P < 0.001$), tumor necrosis factor α ($P < 0.001$), and transforming growth factor β ($P < 0.001$). It increased expression of ant-inflammatory cytokine IL-10 ($P < 0.001$). It reduced bleomycin-increased 5-HT levels in BAL ($P < 0.001$) and in serum ($P < 0.05$). Reductions in collagen fiber deposition ($P < 0.01$), 5-HT2B receptor expression ($P < 0.01$), and Akt phosphorylation in lung tissue were observed. **Conclusions**: AT accelerates the resolution of lung inflammation and fibrosis in a model of bleomycin-induced lung fibrosis partly via attenuation of 5-HT/ Akt signaling. **Key Words**: AEROBIC TRAINING, FIBROSIS, EXERCISE, INFLAMMATION, 5-HT

Idiopathic pulmonary fibrosis (IPF) is a chronic fibrosing interstitial pneumonia of unknown etiology with a poor prognosis, leading to death in most cases just 3 to 5 yr after diagnosis (6–8). With no effective treatment available, IPF patients present exercise intolerance, poor quality of life, and a progressive, irreversible decline in lung function (6–8). Morphologically, IPF lungs are characterized by the fibrotic distortion of pulmonary architecture, alveolar epithelial cell injury, and multiple fibroblastic foci in the lung interstitium in which increased proliferation and activation of fibroblasts and myofibroblasts are observed (6–8).

Serotonin (5-hydroxytryptamine [5-HT]) is a neurotransmitter and a vasoactive peptide present in very low concentrations in the circulation, which is transported mainly via platelets (5). 5-HT is involved in the control of several physiological and pathological conditions (15,26,27,34,43), including various respiratory diseases. Beyond playing a role in IPF (6–8), elevated 5-HT signaling was also shown to play a role in asthma phenotype (5) and in chronic obstructive pulmonary disease (COPD) (34). Furthermore, 5-HT is linked to protein kinase B (Akt) signaling (25,33). In IPF, Akt signaling plays a central role in controlling fibroblast growth, proliferation, and survival (9,19,21,45). Regular chronic aerobic training (AT) modulates 5-HT response, mainly by improving 5-HT synthesis and release to normal levels, resulting in antidepressant effects (12,20,36). These effects are also observed in experimental models of posttraumatic stress disorders (24), and chronic unpredictable stress (44), suggesting a modulator effect of exercise on 5-HT signaling. AT inhibits lung inflammation and fibrosis in mouse models of asthma (23,38–41), emphysema and COPD (35), and lipopolysaccharide-induced acute lung injury (29,30), as well as the bleomycin model of lung fibrosis (28).

Therefore, the present study hypothesized that chronic AT inhibits lung inflammation and fibrosis in a model of bleomycin-induced pulmonary fibrosis mediated through inhibition of exacerbated 5-HT/Akt signaling.

METHODS

All experimental procedures were approved by the ethical committee from the School of Medicine of University of Sao Paulo and by the ethical committee from Nove de Julho University (375/13) and were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

Animals and experimental groups. Three sets of six mice per group were used for this study. C57BL/6 mice (20–25 g) were obtained from the Central Animal Facility of School of Medicine of the University of Sao Paulo and distributed equally in control (Co), exercise (Ex), fibrosis (Fi), and fibrosis + exercise (Fi + Ex) groups ($n = 18$ per group).

Protocol for pulmonary fibrosis induced by bleomycin. Bleomycin sulfate (1.5 UI·kg^{-1}; Meizler Biopharma, SP, Brazil) was administered orotracheally under anesthesia (ketamine, 100 mg·kg^{-1}; xylazine, 10 mg·kg^{-1}) on day 1 of the experimental procedures. Bleomycin-induced lung fibrosis, when administered intratracheally at doses ranging between 1.25 and 4 UI·kg^{-1}, remains the best experimental model available until this moment (17).

Treadmill exercise test and training. Treadmill exercise adaptation, test, and training was performed as previously described (30,35,37,39). Briefly, after 3 d of adaptation (15 min·d^{-1}, 25° incline, 0.2 km·h^{-1}) on the treadmill, animals were submitted to physical test (beginning at 0.2 km·h^{-1}, increasing 0.1 km·h^{-1} every 2.5 until animals'

exhaustion, i.e., until they were not able to run even after 10 mechanical stimulus), followed by physical training during 4 wk, five times per week, 60 min per session, 60% of maximal velocity was reached in the physical test. Twenty-four hours before the euthanasia, the final physical test was performed (30,35,37,39). AT began 15 d after bleomycin administration.

Blood and bronchoalveolar lavage collection and analysis. Under anesthesia (ketamine, 100 mg·kg^{-1}; xylazine, 10 mg·kg^{-1}), 1 mL of blood was collected via vena cava, followed by centrifugation at 1000g, at 4°C, during 10 min. Serum was collected and stored at −70°C for cytokine measurements.

After blood collection, mice were tracheostomized and cannulated for bronchoalveolar lavage (BAL) collection. The lungs were washed with 1.5 mL PBS, and 1 mL was recovered and then centrifuged at 900g for 10 min at 4°C. The supernatant was stored at −80°C, and the cell pellet was resuspended in 1 mL of PBS. Total cell count was performed using a hemocytometer (Neubauer chamber) and the differential cell count by microscopic examination of cytospin preparations stained with Diff Quick, and 300 cells were counted per slide (23,27–29).

Collagen fibers quantification in the lung parenchyma. Lungs were excised in block and submitted to histological routine. Five-micrometer lung slices were stained with picrosirius red (24,27,29). The content of collagen fibers (areas of the lung that stained red) in the parenchyma was quantified by image analysis using Image Pro Plus 4.5 software as previously described (24,27,29). The results were expressed as percentage of collagen fibers related to the total amount of lung tissue.

Cytokine measurements in BAL fluid. The levels of IL-1β, IL-6, CXCL1/KC, IL-10, and tumor necrosis factor α (TNF-α) were measured using ELISA kit according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN).

Serotonin (5-HT) measurements. The levels of 5-HT were measured in the BAL fluid and in serum using the Fastrack ELISA kit according to the manufacturer's recommendations (DIAsource ImmunoAssays, Belgium).

Western blotting. Lungs were homogenized in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) and centrifuged at 13300 rpm at 4°C for 15 min to remove the cell debris. The amount of proteins was quantified by BCA method (BCA Protein Assay kit; Thermo Scientific, Rockford, IL). For all samples, 50 μg of proteins (composed by a pool of the same amount of proteins of eight animals from each group) was loaded in NuPAGE 4%–12% Bis–Tris gel (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membrane. Primary antibody anti-5-HT2B receptor (sc-25647, Santa Cruz Biotechnology), total anti-Akt (1:1000 rabbit antihuman Akt, MAB 4685; Cell Signaling Technology Inc., Danvers, MA), and phosphorylated anti-Akt (Thr308) (1:1000 rabbit antihuman phospho-Akt, MAB 2965; Cell Signaling Technology Inc., Danvers, MA) were

visualized using horseradish-conjugated secondary antibody and enhanced by chemiluminescence (Pierce; Thermo Scientific). An additional probe with beta-actin for control of the amount of proteins was performed using monoclonal anti-actin antibody clone C4 (sc-130656, Santa Cruz Biotechnology). The densitometric analysis of the expression of the bands was performed using Image J from the National Institutes of Health (42).

Statistical analysis. Sigma Stat 3.5 software was used to perform the statistical analysis. Graph Pad Prism 5.0 was used to construct the graphs. Normality analysis revealed parametric data that were expressed as the mean ± SD. Comparisons between all groups were conducted by two-way ANOVA, followed by Student–Newman–Keuls *post hoc* test. Values were considered significant at $P < 0.05$.

RESULTS

Aerobic exercise training improves physical capacity in both healthy and bleomycin-treated mice. In untrained animals, significant changes in the initial versus the final physical capacity test (maximal velocity reached in the physical test) between control (Co) and fibrosis (Fi) groups were not observed (Co group, $1.75 \pm 0.174 \times 1.81 \pm 0.181$ km·h^{-1}, $P > 0.05$; Fi group, $1.81 \pm 0.156 \times 1.83 \pm 0.112$ km·h^{-1}, $P > 0.05$). On the other hand, trained groups (Exercise – Ex and Fibrosis + Exercise – Fi + Ex) presented improvement in physical capacity when compared with the initial versus the final physical test (Ex group, $1.70 \pm 0.135 \times 2.45 \pm 0.144$ km·h^{-1}, $P < 0.05$; Fi + Ex group, $1.76 \pm 0.119 \times 2.53 \pm 0.194$ km·h^{-1}, $P < 0.05$).

In summary, AT improved physical capacity for 4 wk in both Ex and Fi + Ex groups to a similar extent.

Aerobic exercise reduces bleomycin-induced lung inflammation. Next, the extent by which AT reduces lung inflammation in this model was tested. Figure 1 shows the number of total and differential cells in BAL fluid. The untrained Fi group presented significantly higher number of total cells (Fig. 1A, $P < 0.001$), neutrophils (Fig. 1B, $P < 0.01$), macrophages (Fig. 1C, $P < 0.01$), and lymphocytes (Fig. 1D, $P < 0.05$) when compared with all other groups. AT significantly reduced bleo-induced lung inflammation.

Aerobic exercise diminishes bleomycin-induced lung fibrosis. Picrosirius red staining in Figure 2 shows that collagen fiber content were reduced in trained animals. Fi group presented higher content of collagen fibers (Fig. 2A) compared with all other groups ($P < 0.001$). Figures 2B to 2E are representative photomicrographs of lung tissue stained for collagen fibers in control (Co), exercise (Ex), fibrosis (Fi), and fibrosis + exercise (Fi + Ex) groups, respectively.

Aerobic exercise inhibits bleomycin-induced cytokines release. Cytokines levels (IL-1β, IL-6, CXCL1/KC, IL-10, TNF-α, and transforming growth factor β [TGF-β]) in BAL fluid were measured and compared (Fig. 3). Figures 3A to 3D and 3F show, respectively, that the Fi group presented higher levels of IL-1β ($P < 0.01$), IL-6 ($P < 0.05$), CXCL1/KC ($P < 0.001$), TNF-α ($P < 0.001$), and TGF-β ($P < 0.001$) compared with all other groups. Figure 3E shows that Ex and Fi + Ex groups presented higher levels of IL-10 compared with Co and Fi groups ($P < 0.001$).
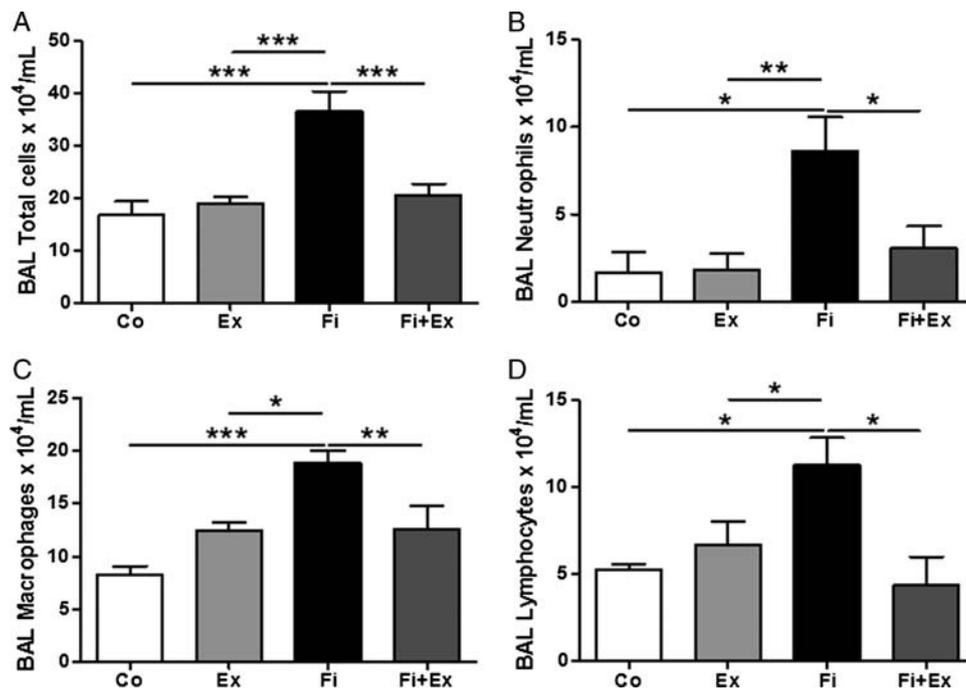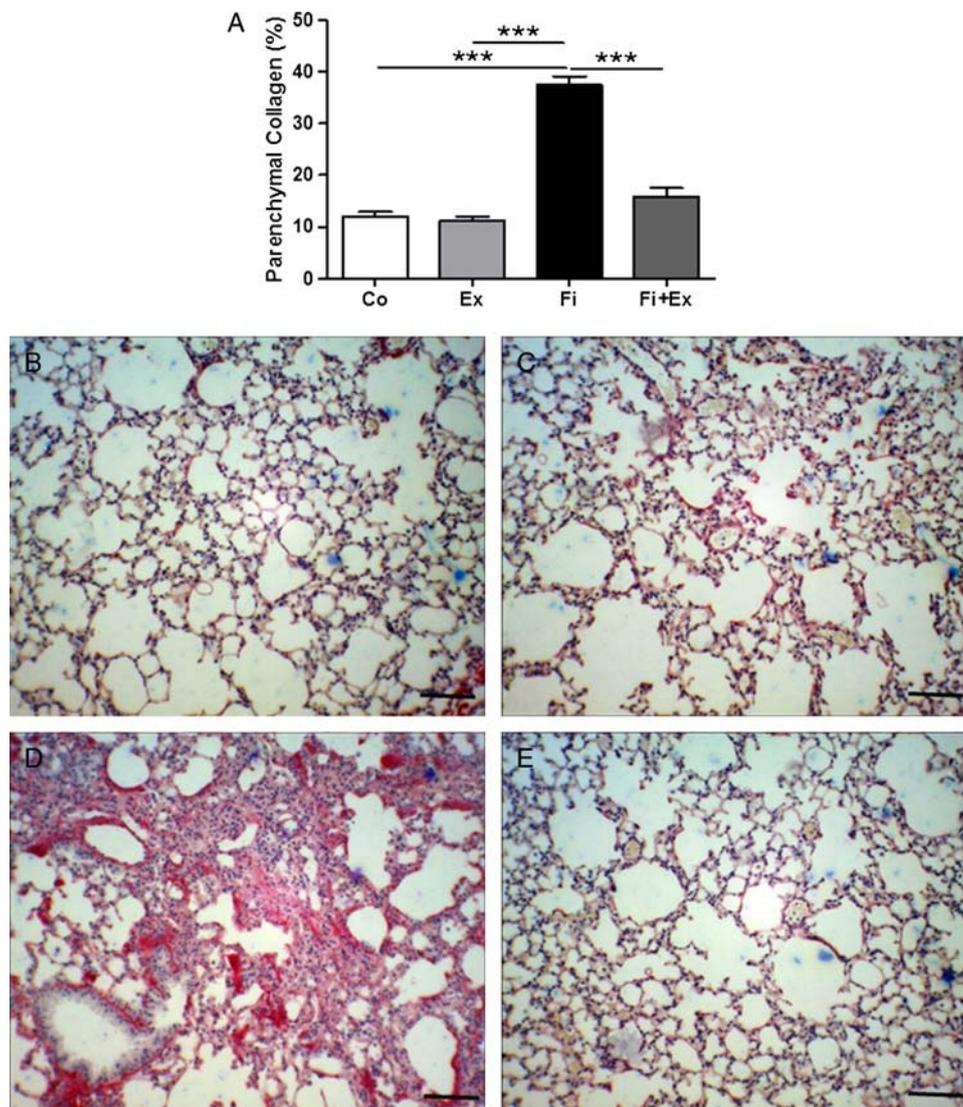

FIGURE 1—Inflammatory response. Number of total cells (A), neutrophils (B), macrophages (C), and lymphocytes (D) in BAL fluid from control (Co), exercise (Ex), fibrosis (Fi), and fibrosis + exercise (Fi + Ex) groups. ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

FIGURE 2—Lung fibrosis. A. The percentage of collagen fibers in the lung parenchyma. B–E. Representative photomicrographs of collagen-stained (picrosirius red) lung parenchyma from control (Co), exercise (Ex), fibrosis (Fi), and fibrosis + exercise (Fi + Ex) groups. Scale bar = 100 μm. ***P < 0.001.

Taken together, trained groups exhibited increased anti-inflammatory cytokine expression and decreased proinflammatory cytokines.

Aerobic exercise inhibits bleomycin-induced 5-HT signaling. Levels of 5-HT in BAL and in serum and also the expression of 5-HTR-2B in lung tissue were assessed (Fig. 4). Figures 4A and 4B show that the Fi group presented increased levels of 5-HT in serum (P < 0.05) and BAL (P < 0.001) compared with all other groups, respectively. Figure 4C shows that the expression of 5-HTR-2B in lung tissue was increased in Fi group compared with all other groups (P < 0.001). This figure represents an average of three independent experiments. Figures 4D and 4E show the representative blotting of 5-HTR-2B and beta-actin expression in all experimental groups (Co, Ex, Fi, and Fi + Ex, from left to right). Importantly, 5-HT in BAL and 5-HTR-2B expression in lung tissue was reduced in the Fi + Ex group.

Aerobic exercise inhibits bleomycin-induced Akt phosphorylation. Figure 5 shows the expression of total Akt and phospho-Akt in lung tissue, measured by densitometric analysis. No differences were found among groups in total Akt expression in lung tissue (Fig. 5A). Figure 5B shows that the Fi group presented a higher expression of phosphorylated Akt compared with all other groups (P < 0.01). These figures represent an average of three independent experiments. Figures 5C to 5E show the representative blotting of Akt, phosphorylated Akt, and beta-actin expression in all experimental groups (Co, Ex, Fi, and Fi + Ex, from left to right). In summary, AT significantly reduced levels of phosphorylated AKT in Fi + Ex animals.

DISCUSSION

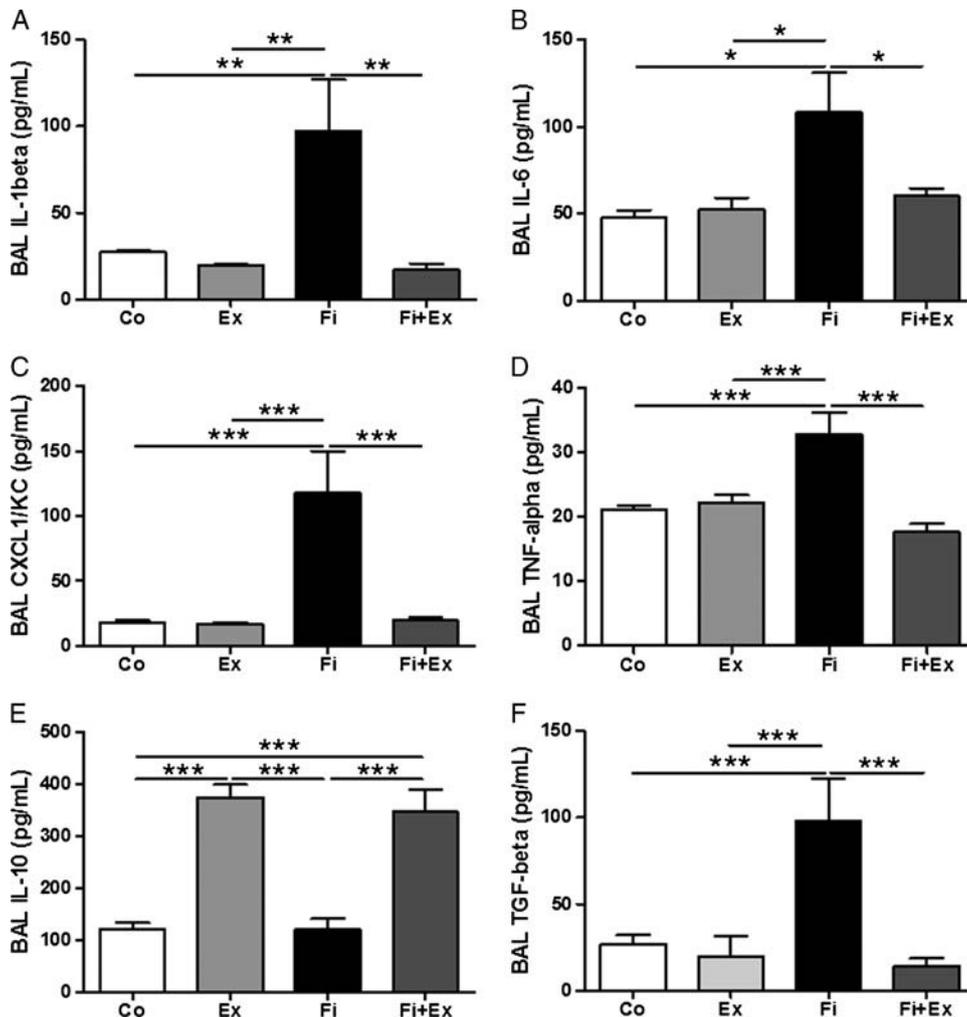This study shows for the first time that AT reduces lung inflammation and fibrosis in a model of bleomycin-induced

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FIGURE 3—Cytokines and growth factor measurements. Levels of cytokines IL-1β (A), IL-6 (B), CXCL1/KC (C), TNF-α (D), IL-10 (E), and TGF-β (F) from control (Co), exercise (Ex), fibrosis (Fi), and fibrosis + exercise (Fi + Ex) groups. ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

lung fibrosis, in part by increasing IL-10 expression and inhibiting both 5-HT and Akt signaling, two important pathways implicated in the IPF pathology.

IPF is a devastating disease with no cure or effective treatment available (6–8). It is characterized by multiple heterogeneous foci, which are composed of hyperplastic and apoptotic alveolar epithelial cells, highly proliferative resident fibroblasts and basal cells, activated myofibroblasts, epithelial cells undergoing mesenchymal transition (EMT) (22), and accumulated extracellular matrix proteins (i.e., collagen fibers) in the lung interstitium leading to the disruption of lung alveolar architecture (6–8). These characteristics are directly related to decline of lung function, leading to a poor quality of life (11,32). Many of the histopathological features of IPF are recapitulated in models of bleomycin-induced pulmonary fibrosis (6,8,13,28) and were also observed in the present study. However, AT significantly attenuated collagen deposition and fibrotic foci (Fi + Ex group).

The pathogenesis of IPF is not fully understood. Evidence indicates that the fibrotic response is partly driven by repetitive microinjuries to the alveolar epithelium. Various signaling pathways are recruited, which produce mediators that induce abnormal tissue repair. Although the role of inflammatory cells is controversial, inflammation likely plays a role in IPF genesis and progression (4). During the initial injury phase, activated alveolar epithelial cells and recruited inflammatory cells (e.g., macrophages, neutrophils) release fibrogenic growth factors that perpetuate the cycle of injury, abnormal repair, and fibrosis (7). In this study, AT significantly reduced both inflammation and collagen fiber accumulation in the lung parenchyma. The antifibrotic effects of AT have also been demonstrated in other models of pulmonary diseases, such as asthma (23,38–41), emphysema and COPD (35), acute lung injury (29,30), and pulmonary fibrosis (28), and even in the lungs of mice exposed to air pollutants (37).

Leukocyte recruitment and hyperactivation are also related to IPF severity and progression as they release inflammatory cytokines and growth factors (4,7,16). Increased numbers of inflammatory cells in the lung interstitium have been reported in the bleomycin model (6–8,13,16). In agreement with these reports, increased numbers of neutrophils,

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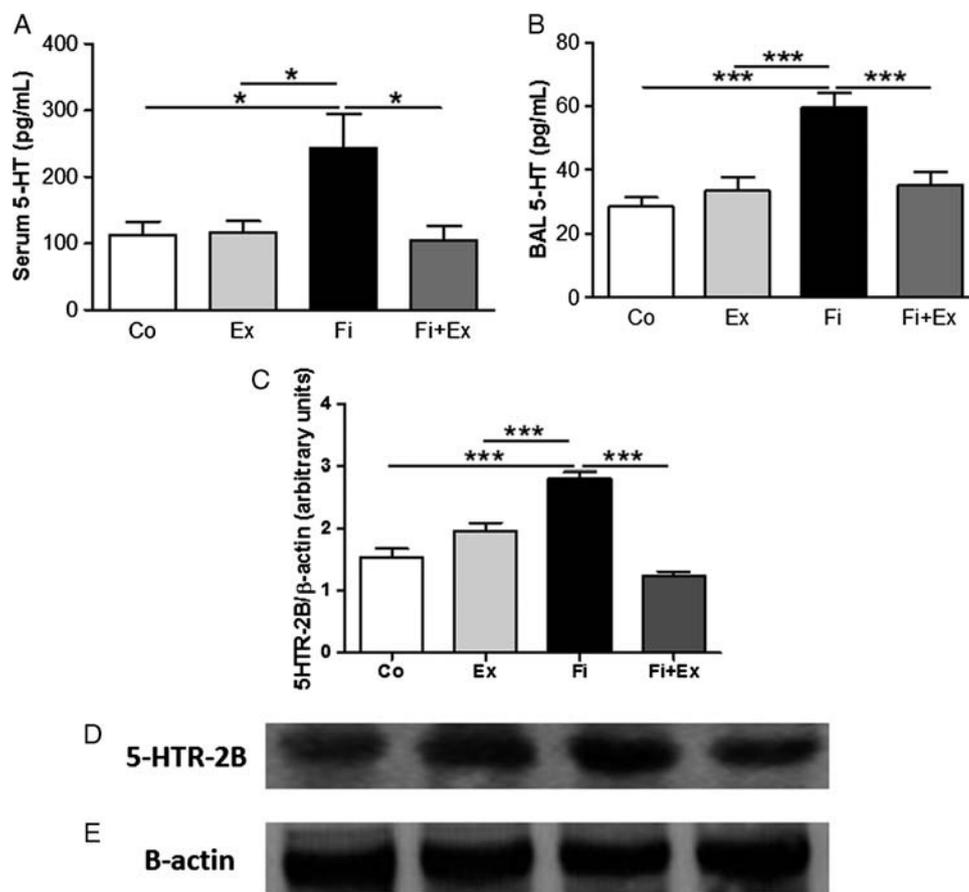
FIGURE 4—5-HT signaling. Levels of 5-HT in serum (A), 5-HT in BAL (B) and the expression of receptor 5-HTR-2B in lung tissue (C), as well as representative images from western blotting of 5-HTR-2B (D) and B-actin (E) expression in lung tissue from control (Co), exercise (Ex), fibrosis (Fi), and fibrosis + exercise (Fi + Ex) groups. ***$P < 0.001$ and *$P < 0.05$.

macrophages, and lymphocytes in untrained bleomycin-treated mice were observed. AT significantly reduced the number of these cells. Prata et al. (2012) reported similar findings and showed that swimming training attenuated bleomycin-induced pulmonary fibrosis lesions (28).

Cytokines and growth factors also play a key role in the pathophysiology of IPF (1–3,7,8,42). IL-1β is a proinflammatory and profibrotic cytokine, primarily released by activated macrophages and epithelial cells (39). IL-1β is centrally involved in the pathophysiology of IPF and induces the release of other cytokines, such as IL-6 and TNF-α (3). IL-6 has also been implicated as central mediator of IPF pathophysiology, contributing both to inflammation and remodeling as it has been demonstrated to induce the conversion of fibroblasts to collagen-secreting myofibroblasts (6–8,10). In addition, IL-8 is a potent neutrophil-attracting chemokine that is increased in IPF patient BAL (1). TNF-α is also considered a profibrotic cytokine, especially given its ability to stimulate TGF-β expression in fibroblasts (42). TGF-β is a key growth factor involved in IPF pathophysiology and is responsible for fibroblast activation and differentiation to myofibroblast, resulting in increased extracellular matrix proteins synthesis and accumulation (3,6–8,10,17,18,42). In the present study, IL-1β, IL-8, IL-6, TNF-α, and TGF-β were increased

in the BAL of bleomycin-treated mice. Conversely, their production was inhibited in bleomycin-treated exercised mice, demonstrating the anti-inflammatory and antifibrotic effects of AT.

IL-10, an immunoregulatory and anti-inflammatory cytokine, is found at very low levels in IPF patients (18). Like 5-HTB receptor antagonists, IL-10 is capable of inhibiting TGF-β synthesis and activation and has been considered as a potential IPF therapy (6,14,18). In the present study, AT in both bleomycin-treated and nontreated mice significantly increased the BAL levels of IL-10. This finding points to the beneficial effects of exercise-induced IL-10 release in controlling inflammation and fibrosis in a model of lung fibrosis, similar to previously demonstrated results in models of asthma (23,38–41), emphysema, and COPD (35) and in lipopolysaccharide-induced acute lung injury (29,30).

Beyond regulation of mood and sleep, serotonin (5-HT) is thought to have an important role in the pathophysiology of respiratory diseases, such as asthma (8), COPD (34), and IPF (6,8). 5-HT controls several aspects involved in lung fibrosis, such as cell migration, activation, and proliferation (5,6,8,25,34). Furthermore, 5-HT2B receptor is increased in the fibroblastic foci of IPF patients and has been implicated
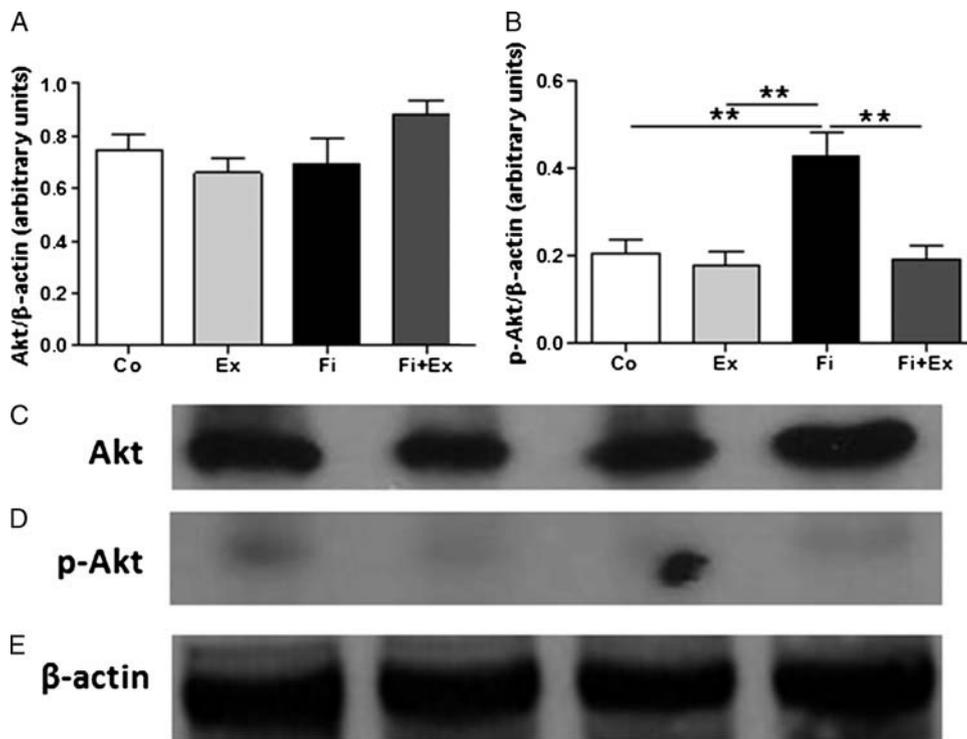
FIGURE 5—Akt signaling. Expression of total Akt, phosphorylated Akt, and beta-actin in lung proteins. **$P < 0.01$.

in the activation of myofibroblasts (5,6,8). In concordance with previous studies (6), increased levels of 5-HT in untrained bleomycin-treated mouse BAL and serum as well as increased expression of 5-HTB receptor in the parenchyma were observed. Importantly, chronic AT not only reduced inflammation but also inhibited reduced the aberrant expression of 5-HT2B receptor during bleomycin injury. Moreover, systemic 5-HT levels of Fi + Ex mice did not deviate from the levels of 5-HT detected in untreated mice Fi + Ex mice, suggesting that chronic AT represses aberrant 5-HT signaling in the fibrosis model.

Finally, increased protein kinase B (Akt) phosphorylation in the lung tissue of bleomycin-treated animals was observed. Akt is a protein kinase involved in cell apoptosis, proliferation, migration, and transcription (9,18,25,31,33). Akt signaling regulates fibroblast resistance to apoptosis and promotes excessive collagen fibers synthesis (25,31,33). This study demonstrates for the first time that AT inhibits Akt phosphorylation in lung tissue, an effect that contributed to the reduced collagen fiber accumulation in the lung parenchyma.

Unlike IPF, the bleomycin model is inherent reversible and the severity of bleomycin-induced lesions in rodents generally corresponds to lung inflammatory levels. As AT was started just 14 d after bleomycin administration, Fi + Ex mice were exposed to high levels of IL-10 and the anti-inflammatory effects of exercise during a time point of peak inflammation in this model. Given that treatments that reduce inflammation in the bleomycin model correlate strongly with a reduction in the development of fibrotic lesions, this likely explains the lack of difference in fibrotic outcome measurements in Fi + Ex versus Fi groups. As IPF patients are generally given 3–5 yr to live after diagnosis, experiments that begin chronic AT after the resolution of the inflammatory phase (after day 21) may be closer to the clinical scenario and likely show increased fibrosis in the Fi + Ex group compared with the model used in this study (exercise began at d14, during peak inflammation). Although 5-HTP receptor antagonists in the bleomycin model have not been shown to reduce inflammation, but nonetheless decrease fibrotic outcome, these experiments suggest that chronic AT not only reduces inflammation but also represses aberrant 5-HTP signaling which may be beneficial to IPF patients.

In conclusion, this study demonstrated that chronic AT accelerates the resolution of lung inflammation and fibrosis in bleomycin-treated animals and that these effects may be mediated by increased IL-10 production and inhibition of aberrant 5-HT and Akt signaling.

revised the manuscript. A. P. L. O., H. C. C. F. N., and R. P. V. performed the statistical analysis. R.P.V. designed the study. All authors have reviewed and approved the final version of the manuscript before submission.

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