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ORIGINAL RESEARCH

Exercise Attenuate Diaphragm Atrophy in COPD Mice via Inhibiting the RhoA/ROCK Signaling

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Background: Exercise is an indispensable component of pulmonary rehabilitation with strong anti-inflammatory effects. However, the mechanisms by which exercise prevents diaphragmatic atrophy in COPD (chronic obstructive pulmonary disease) remain unclear.
Methods: Forty male C57BL/6 mice were assigned to the control (n=16) and smoke (n=24) groups. Mice in the smoke group were exposed to the cigarette smoke (CS) for six months. They were then divided into model and exercise training groups for 2 months. Histological changes were observed in lung and diaphragms. Subsequently, agonist U46639 and antagonist Y27632 of RhoA/ROCK were subjected to mechanical stretching in LPS-treated C2C12 myoblasts. The expression levels of Atrogin-1, MuRF-1, MyoD, Myf5, IL-1β, TNF-α, and RhoA/ROCK were determined by Western blotting.
Results: Diaphragmatic atrophy and increased RhoA/ROCK expression were observed in COPD mice. Exercise training attenuated diaphragmatic atrophy, decreased the expression of MuRF-1, and increased MyoD expression in COPD diaphragms. Exercise also affects the upregulation of RhoA/ROCK and inflammation-related proteins. In in vitro experiments with C2C12 myoblasts, LPS remarkably increased the level of inflammation and protein degradation, whereas Y27632 or combined with mechanical stretching prevented this phenomenon considerably.
Conclusion: RhoA/ROCK plays an important role in the prevention of diaphragmatic atrophy in COPD.
Keywords: chronic obstructive pulmonary disease, diaphragmatic atrophy, exercise training, inflammation, RhoA/ROCK signaling

Introduction

Chronic obstructive pulmonary disease (COPD) is the third most life-threatening disease worldwide, causing damage to the respiratory system and common diaphragmatic dysfunction.[1] Diaphragmatic dysfunction can occur at all stages of COPD,[2] leading to dyspnea or even respiratory failure, which is an independent risk factor for increased hospitalization and mortality in patients with COPD.[3] Many factors including dynamic hyperinflation, chronic hypoxia, malnutrition, systemic inflammation contribute to diaphragmatic dysfunction.[4] Persistent chronic inflammation is negatively correlated with diaphragm mass and strength,[5] and this condition may be a key pathological factor that causes diaphragmatic dysfunction in patients with COPD.[6] Moreover, diaphragm function depends on the physiological structure.[4]

Exercise, as the core component of pulmonary rehabilitation, has been found to improve dyspnea, respiratory function, and respiratory muscle strength in patients with COPD.[7,8] Many studies have shown that exercise can regulate the levels of inflammatory factors in the circulation and diaphragm tissue of COPD,[9–11] but the specific mechanism by which exercise regulates inflammation and alleviates diaphragmatic atrophy in patients with COPD remains unknown. Ras homolog family member A (RhoA) is a widely expressed cytoplasmic protein belonging to the Ras-related small GTP-binding protein family.[12] RhoA is activated by pathogens and endogenous danger signals in innate immune cells, which migrate to infection and inflammation sites and play an inflammatory regulatory role.[13] Rho-associated coiled-coil kinase (ROCK) is a RhoA effector,

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and activated ROCK not only leads to nuclear factor-kappaB activation[14,15] but also plays a critical role in regulating the contraction of the actin-myosin cytoskeleton.[16] Upregulation of the RhoA/ROCK causes a series events including increased inflammation, immune cell migration, apoptosis, which contribute to the hallmark of acute lung injury.[17] In addition, in mice with muscular dystrophy, the continuously activated RhoA pathway can block the differentiation of muscle cells and inhibit myogenic fusion, while Y27632 (antagonist of RhoA/ROCK) can improve myogenic potential.[18] These results suggest that the role of RhoA/ROCK in regulating inflammation, muscle production, and differentiation may be a key pathological mechanism of diaphragmatic atrophy in COPD.

Hence, this study aimed to establish a COPD diaphragmatic atrophy model through long-term cigarette smoke (CS) exposure and then conduct exercise training on mice with COPD to observe its effects on RhoA/ROCK. Finally, mechanical stretching and RhoA/ROCK agonists and antagonists were applied to lipopolysaccharide (LPS)-treated C2C12 myoblasts to verify the mechanism of action of RhoA/ROCK in exercise for diaphragmatic atrophy.

Methods

Animals and Mouse Myoblasts

Forty male, 8 weeks old C57BL/6J mice were obtained from Zhejiang Vitalriver Laboratory Animal Company (Zhejiang, China) and housed at Shanghai University of Traditional Chinese Medicine Laboratory Animal Center. The room was kept at 60%±10% humidity, 21°C±2°C, and 12 h light/dark cycle. The experimental protocol was approved by the Shanghai University of Traditional Chinese Medicine Animal Care Committee (no. PZSHUTCM210312012) and conformed to ARRIVE guidelines.

Mouse C2C12 myoblasts were obtained from the National Collection of Authenticated Cell Cultures (Shanghai, China). Cells were cultured in growth medium (DMEM, 10% FBS, and 1% antibiotic-antimycotic (Gibco, USA)) at 37°C and 5% CO_2. When the cells reached 70–80% confluence, they were passaged at a split ratio of 1:2 and seeded onto new plates. Low passage P3 to P8 cells were used for the cell experiments.

Experimental Design

To explore the effects of exercise on RhoA/ROCK signaling in COPD diaphragms. Forty mice were randomly assigned to either the control group (exposed to air) or smoke group (exposed to CS). The mice in the smoke group were divided into model and exercise groups.

To investigate the role of RhoA/ROCK signaling in exercise alleviating COPD diaphragmatic atrophy, the following experiment was conducted. C2C12 myoblasts were divided into six groups: control, LPS-treated, LPS and U46639 treated, LPS and U46639 treated then submitted to mechanical stretching, LPS and Y27632 treatment, and LPS and Y27632 treatment, followed by mechanical stretching. LPS (Sigma, USA) was used to induce inflammation and mechanical stretching was used to simulate exercise in vivo. U46639 (MCE, USA) is an agonist of RhoA/ROCK, and Y27632 (MCE, USA) is an antagonist of RhoA/ROCK.

COPD Model Protocol

Mice in the smoke group were exposed to cigarette smoking produced by commercially filtered cigarettes (tar: 10 mg, nicotine: 0.9 mg, CO: 12 mg) for 6 months. The progressive smoke exposure protocol was applied:[19] in the 1st week, 10 cigarettes once a day with 1 h a session; in 2–7 week, 10 cigarettes twice a day with 1 h a session; in 8–13 week, 15 cigarettes twice a day with 1 h a session; in 14–25 week, 20 cigarettes twice a day with 1 h a session, 6 days a week. Briefly, mice were placed in an exposure chamber (80 cm long, 60 cm wide, and 58 cm high) with cigarette smoke conveyed from two plastic 100 mL syringe.

C2C12 myoblasts were exposed to 2 mg/mL LPS for 24 h to induce the culture environment of inflammation, according to preliminary experimental results.

Exercise Protocol

Before exercise training, the mice in the exercise group were subjected to an adaptation period (10 min on the first day, increased by 10 min every day until the exercise time reached 60 min on the sixth day, and 5 m/min). Subsequently, their maximal exercise capacity (100%) was tested, and the formal training protocol was set at 55% of maximal exercise capacity for 60 minutes per session, one session per day, 6 days a week, and 2 months. The specified velocity of formal exercise protocol was 12.5 m/min.

After treatment with LPS, C2C12 myoblasts were seeded into six-well plates (BioFlex, FlexCell International Corporation, USA) for 24 h. Subsequently, the cells were stretched at 15% elongation and 0.5 Hz frequency for 2 h for three consecutive days by using a computer-controlled stretching instrument (Flexcell FX-6000TM Tension System, FlexCell International Corporation, USA).

Indicator Measurement

For mice, 24 h after the end of CS or treadmill training protocol, all mice were anesthetized and euthanized to achieve the sample for further analysis. For cells, 24 h after stretching, the supernatant of culture medium and the cells was collected for further analysis.

Histology

For histological analysis, samples of the left lung and diaphragm were fixed with 4% paraformaldehyde at room temperature for 24 h. Then, the samples were embedded in paraffin, cut into 4–6 μm sections, and stained with hematoxylin and eosin (HE). Five randomly selected representative non-overlapping fields were selected from each section at 200 × magnification. Morphological analysis was conducted on the lung and diaphragm tissues, and the cross-sectional areas (CSA) of the alveoli and muscle fibers were calculated.

Pulmonary Function Test

1.25% avodine tribromoethanol (0.2mL/10 g body weight) was intraperitoneally injected to anesthetize mice. The mice were then tracheostomized and mechanically ventilated using a computer-controlled forced pulmonary maneuver system (DSI Buxco, USA). Forced vital capacity (FVC), forced expiratory volume in 20 ms (FEV20), FEV50, FEV20/FVC, and FEV50/FVC were analyzed.

Cytokine Analysis

After washing the right lung with sterile saline (0.5 mL) three times, bronchoalveolar lavage fluid (BALF) samples were collected. The culture medium was immediately replaced after stretching with fresh medium and incubated for 24 h. The culture medium was then collected. The levels of Il −1β, IL-6, IL-8, and TNF-α in BALF and supernatant were quantified using an enzyme-linked immunosorbent assay (ELISA). The kits were purchased from Wellbio (Shanghai, China).

Western Blotting

The diaphragms and cells were subjected to Western blot analysis. RIPA buffer was used to prepare protein lysates. BCA kit was used to determine protein concentration. The protein lysates were subjected to SDS-PAGE and the separated bands were transferred onto polyvinylidene fluoride membranes. The membranes were incubated with the following primary antibodies: anti-RhoA antibody (1:1000, Immunoway), anti-ROCK1 antibody (1:700, Immunoway), anti-ROCK2 antibody (1:700, Immunoway), anti-Atrogin-1 antibody (1:1500, Immunoway), anti-MuRF-1 antibody (1:1000, Immunoway), anti-MyoD1 antibody (1:1500, Proteintech), anti-Myf5 antibody (1:1000, Immunoway), anti-IL-1β antibody (1:1500, Affinity), anti-TNF -α antibody (1:1500, Proteintech), or anti-GAPDH antibody (1:2000, Wellbio) at 4 °C overnight and the corresponding secondary antibodies for 2 h at room temperature. After washing the membranes, an imager (Tanon Biotechnology, China) was used for detection. GAPDH was used to normalize the band densities of the target proteins.

Statistical Analysis

The values are presented as mean ± SEM and analyzed by GraphPad Prism 7.0 (CA, USA). Normality was determined using the Shapiro–Wilk test. Student's *t*-test was used for comparisons between the two groups. For multiple

comparisons, one-way analysis of variance (ANOVA) was conducted using Tukey's post-hoc analysis. A nonparametric Mann–Whitney *U*-test or Kruskal–Wallis *H*-test was conducted to detect differences in the skewed data. *P*-value <0.05 was considered statistical significance.

Results

Long-Term CS Exposure Induces Diaphragmatic Atrophy in Mice with COPD

CS exposure induced a significant increase in the CSA of the alveolus (Figure 1A) and decreased the respiratory function (Figure 1B) of FVC, FEV20 ($p<0.05$), FEV50, FEV20/FVC, and FEV50/FVC ($p<0.05$). After exposure to CS, mice showed significantly higher levels of IL-6 ($p<0.01$) and IL-8 ($p<0.05$) in BALF (Figure 1C). These results indicate that long-term CS induced COPD in a mouse model.

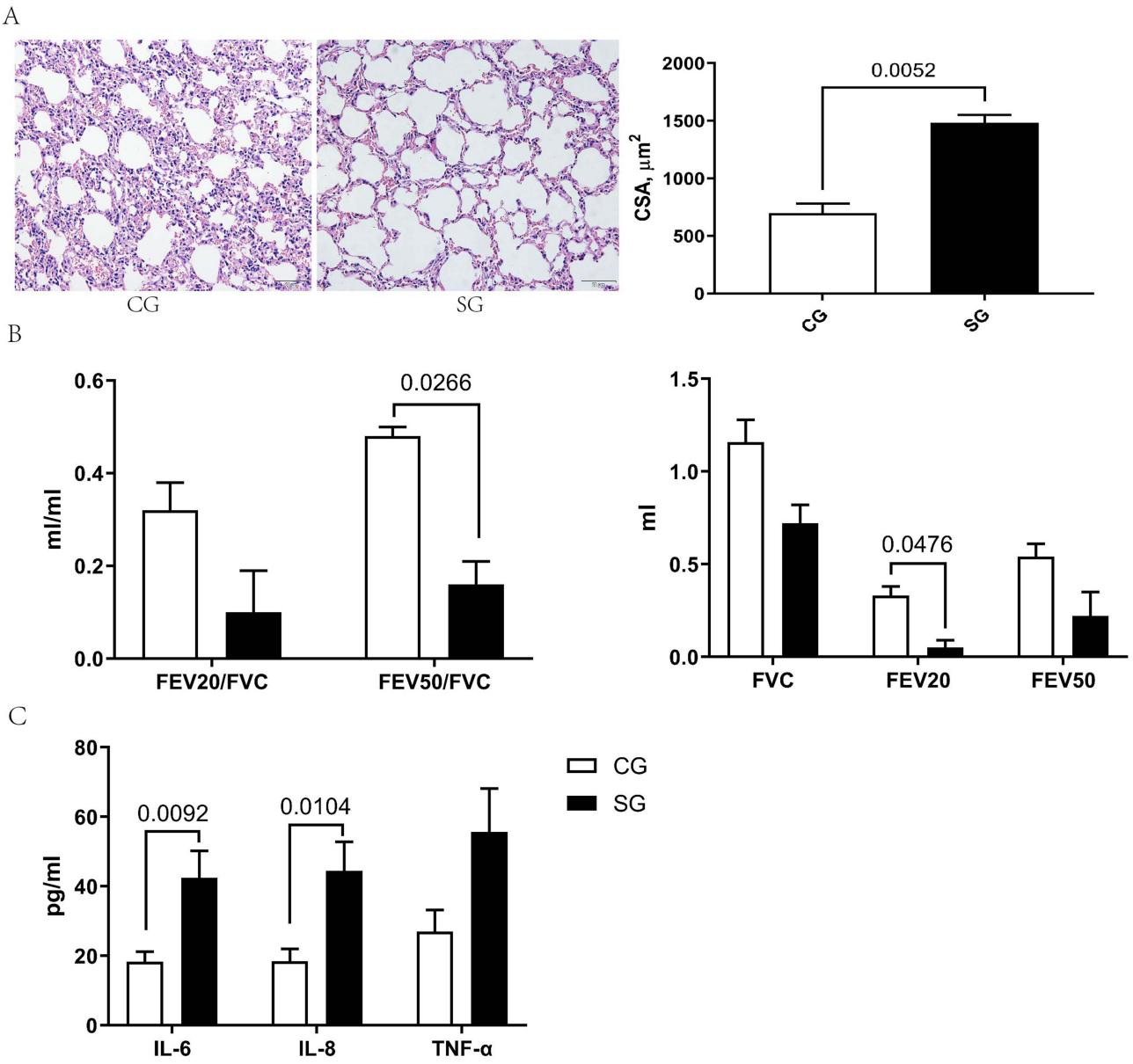

Figure 1 Long-term CS establish a mouse model of COPD. (**A**). hematoxylin and eosin staining of lung sections from mice exposed to air or CS. scale bars, 50 μm; and CSA of alveolus; (**B**). lung function; (**C**). inflammation levels of BALF. Significance was calculated by one-way ANOVA with Tukey's post-hoc analysis for the parameters including CSA of alveolus, FVC, FEV50, FEV50/FVC, IL-6, IL-8, and TNF-α.

Abbreviations: CS, cigarette smoke; CSA, cross-sectional area; FEV, forced expiratory volume; FVC, forced vital capacity; CG, control group; SG, smoke group.

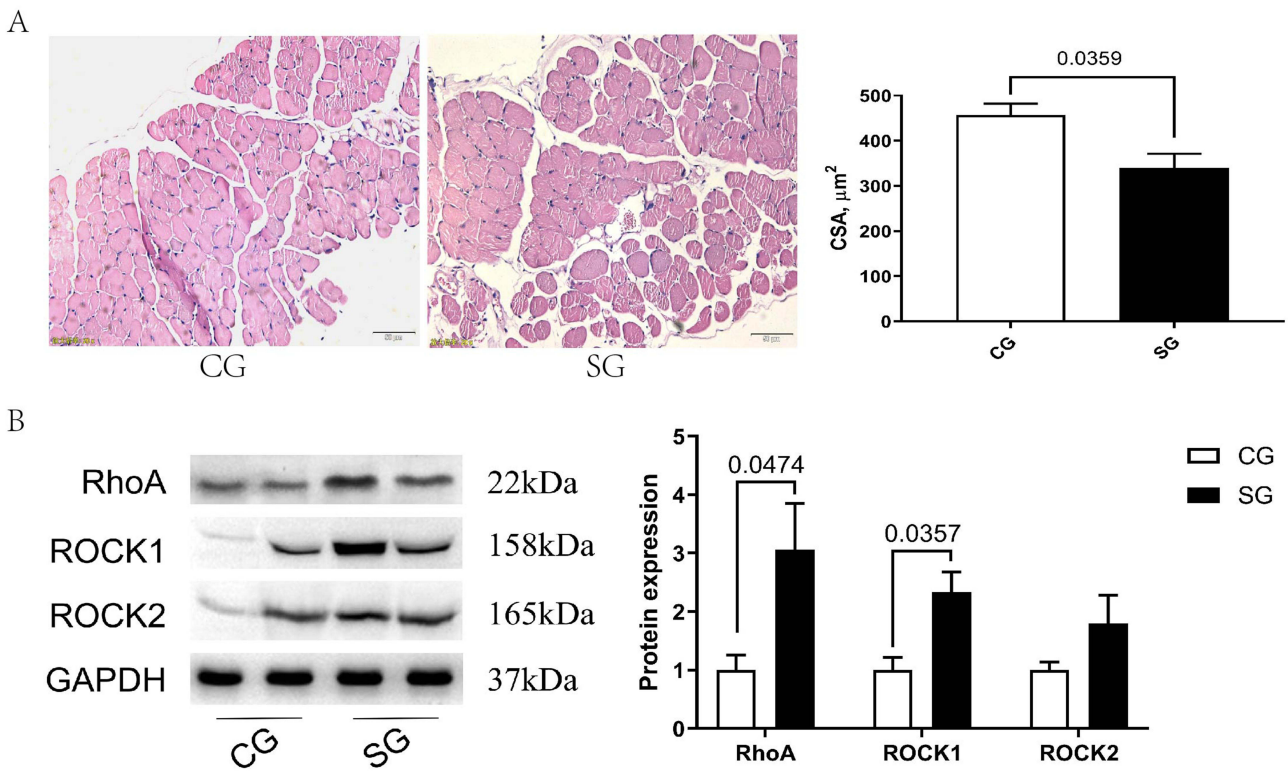

Figure 2 Long-term CS induces diaphragmatic atrophy and upregulation of RhoA/ROCK. (**A**).hematoxylin and eosin staining of diaphragm sections from mice exposed to air or CS. scale bars, 50 μm; and CSA of diaphragm fibers; (**B**). Western blot analysis of protein expression of RhoA, ROCK1, ROCK2 in diaphragm from mice exposed to air or CS. Significance was calculated by one-way ANOVA with Tukey's post-hoc analysis for the parameters including CSA of diaphragm fibers, RhoA, and ROCK2.
Abbreviations: CS, cigarette smoke; CSA, cross-sectional area; CG, control group; SG, smoke group.

In addition, CS-exposed mice showed a significant decrease in the CSA of diaphragm fibers (Figure 2A). To explore the potential role of RhoA/ROCK, protein concentrations of RhoA, ROCK1, and ROCK2 in the diaphragm were evaluated. After exposure to CS, mice showed higher levels of RhoA ($p<0.05$), ROCK1 ($p<0.01$), and ROCK2 expression in the diaphragm (Figure 2B).

Exercise Training Prevents Diaphragmatic Atrophy and Inhibits Inflammation in Mice with COPD

Next, mice with COPD were subjected to treadmill training for 2 months, and HE staining of the diaphragm showed that the decreased CSA of diaphragm fibers in mice with COPD was partially reversed by treadmill training (Figure 3A). Molecules involved in muscle protein synthesis and breakdown were evaluated because muscle atrophy largely results from an imbalance between these two processes. The protein concentrations of Atrogin-1 and MuRF1 in the diaphragm increased in mice with COPD, and the protein concentration of MuRF1 was significantly reduced by treadmill training ($p<0.05$, Figure 3B–D). In contrast, the protein expression of MyoD and Myf5, which are positive regulators of muscle mass, was decreased in mice with COPD, but was significantly increased after exercise training (Figure 3E and F).

Furthermore, the expression of RhoA and ROCK1 proteins was distinctly increased in the diaphragms of mice with COPD, whereas exercise training partially upregulated both proteins ($p<0.05$, Figure 4A–C). Similar changes were also observed in inflammation-related proteins such as IL-1β and TNF-α (Figure 4D and E). These results indicate that exercise training prevents diaphragmatic atrophy in COPD mice by downregulating RhoA/ROCK expression.

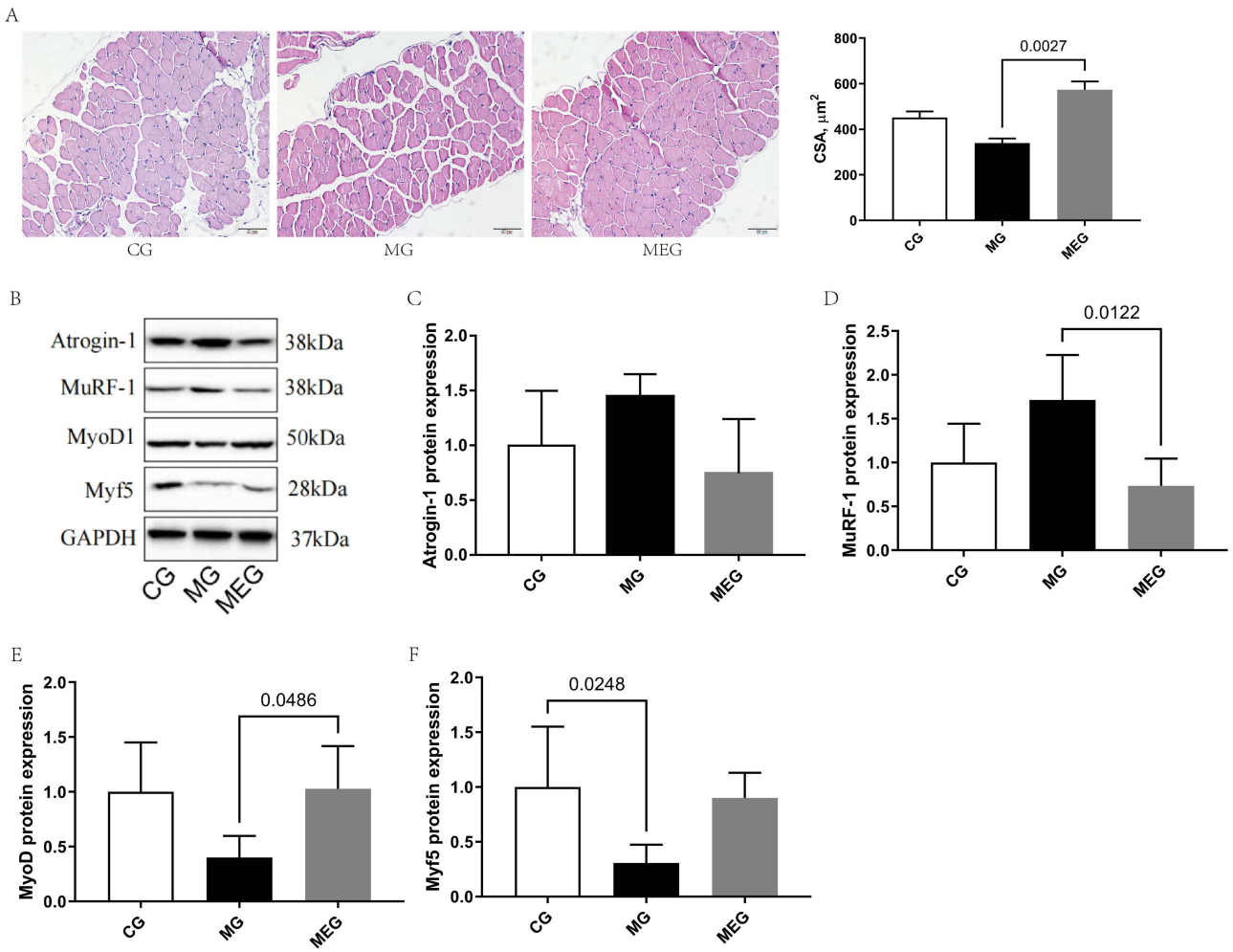

Figure 3 Treadmill training improves diaphragmatic atrophy in mice with COPD. (**A**). hematoxylin and eosin staining of diaphragm sections from mice exposed to air or CS. scale bars, 50 μm; CSA of diaphragm fibers; (**B-F**). Western blot analysis of protein expression of Atrogin-1, MuRF-1, MyoD1 and Myf5. Significance was calculated by one-way ANOVA with Tukey's post-hoc analysis for the parameters including CSA of diaphragm fibers, Atrogin-1, MyoD, and Myf5.
Abbreviations: CS, cigarette smoke; CSA, cross-sectional area; CG, control group; MG, COPD model group; MEG, COPD model submitted to exercise group.

RhoA/ROCK is Involved in the Effects of Exercise Decreasing the Inflammation of LPS-Treated C2C12 Myoblasts

To verify the role of RhoA/ROCK in preventing diaphragmatic atrophy in COPD, machine stretching was used to simulate the exercise. ELISA showed that 2 mg/mL LPS significantly increased the level of inflammation, particularly IL-1β ($p<0.01$, Figure 5A and B). The levels of IL-1β and TNF-α also increased after treatment with agonist U46639 ($p<0.01$), and IL-1β decreased significantly after mechanical stretching ($p<0.01$). The levels of IL-1β and TNF-α were lower after treatment with antagonist Y27632 and mechanical stretching. These results revealed the regulatory role of RhoA/ROCK and mechanical stretching in inflammation in LPS-treated C2C12 myoblasts.

Next, we evaluated the expression of the molecules involved in muscle protein synthesis and breakdown. The results of Western blot analysis showed that U46639 combined with mechanical stretching, as well as Y27632 and Y27632 combined with mechanical stretching, significantly suppressed the LPS-induced upregulation of Atrogin-1 ($p<0.01$, Figure 5C–E). The same, but not significant, phenomenon was observed for MuRF-1. The protein expression of MyoD and Myf5 showed opposite, but not significant, results after treatment (Figure 5F and G). In addition, the protein concentrations of RhoA and ROCK1 significantly decreased after mechanical stretching in C2C12 myoblasts compared with those in LPS-treated cells ($p<0.05$, Figure 5H and I). These results indicated that mechanical stretching decreased

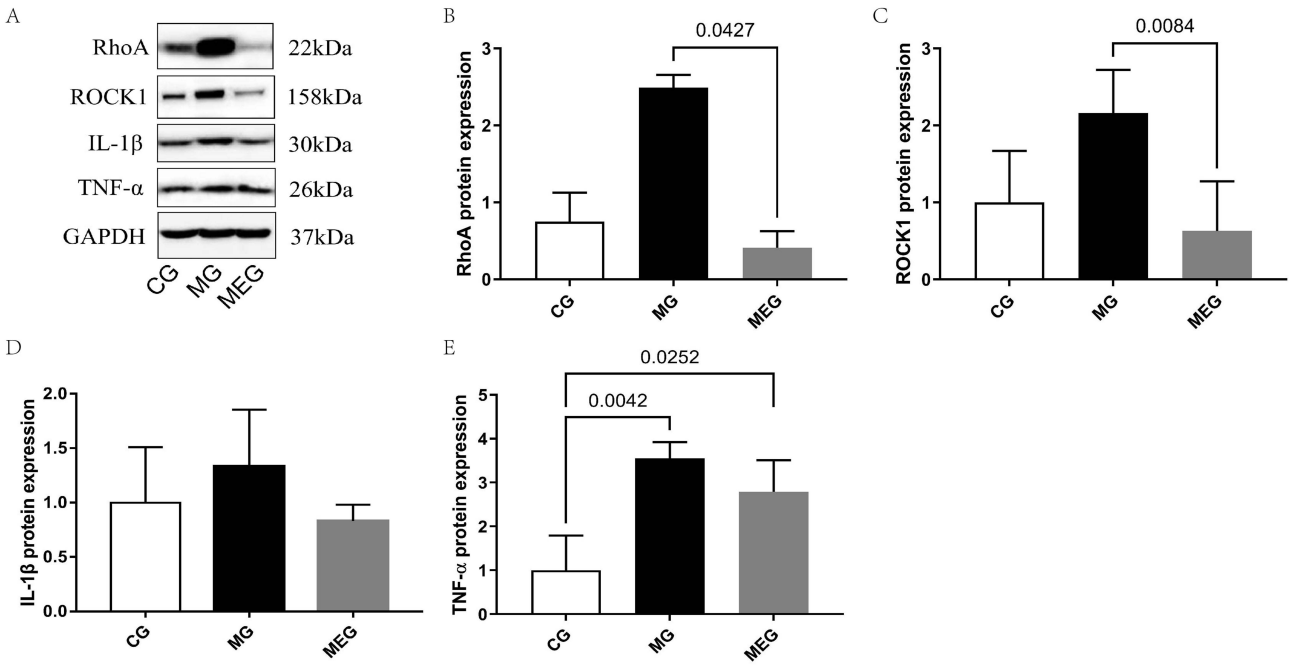

Figure 4 Treadmill training decreases the expression of RhoA/ROCK and inflammation in COPD diaphragm. (**A-E**). Western blot analysis of protein expression of RhoA, ROCK, IL-1β and TNF-α. Significance was calculated by one-way ANOVA with Tukey's post-hoc analysis for the TNF-α.
Abbreviations: CG, control group; MG, COPD model group; MEG, COPD model submitted to exercise group.

inflammation, balanced muscle protein synthesis and breakdown of LPS-treated C2C12 myoblasts involving RhoA/ROCK.

Discussion

In the present study, long-term CS exposure induced COPD diaphragmatic atrophy and increased RhoA/ROCK levels, whereas exercise alleviated COPD diaphragmatic atrophy, inhibited protein degradation, promoted muscle regeneration, and reduced RhoA/ROCK levels. In C2C12 myoblasts, the application of exogenous agonists further aggravates inflammation, whereas exogenous antagonists reduce inflammation and protein degradation, particularly when combined with mechanical stretching. Collectively, these data reveal the key role of RhoA/ROCK in alleviating diaphragmatic atrophy in COPD after exercise.

Stimulants including CS, LPS, and elastase are commonly used to establish COPD models.[20] One commonly used method of CS exposure is whole-body CS exposure, which leads to key pathological changes in COPD mice such as chronic airway inflammation, airway remodeling, lung parenchyma destruction.[21] In this study, six months of CS exposure also induced pathological changes in emphysema, remarkably decreased lung ventilation, and increased the levels of inflammatory cytokines. As important muscles for respiration, the mass and fiber diameter of the diaphragm gradually decrease as the CS time prolongs.[22] After 24 weeks of CS, the gene expression of proliferation and energy metabolism activity decreased and the gene expression of the protein degradation regulator increased in diaphragm cells.[23] In the present study, CS exposure induced a sparse arrangement of diaphragmatic fibers and remarkably decreased CSA, contributing to diaphragmatic atrophy. In addition, CS exposure increases the expression of RhoA and ROCK1 proteins, which are related to increased levels of inflammation in lung tissues.[24,25] These data indicate that long-term CS exposure induces diaphragmatic atrophy in mice with COPD, and that RhoA/ROCK may play a key role.

In recent years, an increasing number of studies have focused on the effects of exercise on the diaphragm in COPD. Exercise training alleviated CS-induced decreases in diaphragm contraction function, protein degradation,[26] and diaphragm mass reduction.[10] Rehabilitation improves diaphragm thickness at the end of expiration in COPD patients.[27] In this study, exercise training improved CSA of the diaphragm and reduced the elevated expression of MuRF-1 protein in mice with COPD. In addition, this study detected important regulatory factors involved in myogenesis and

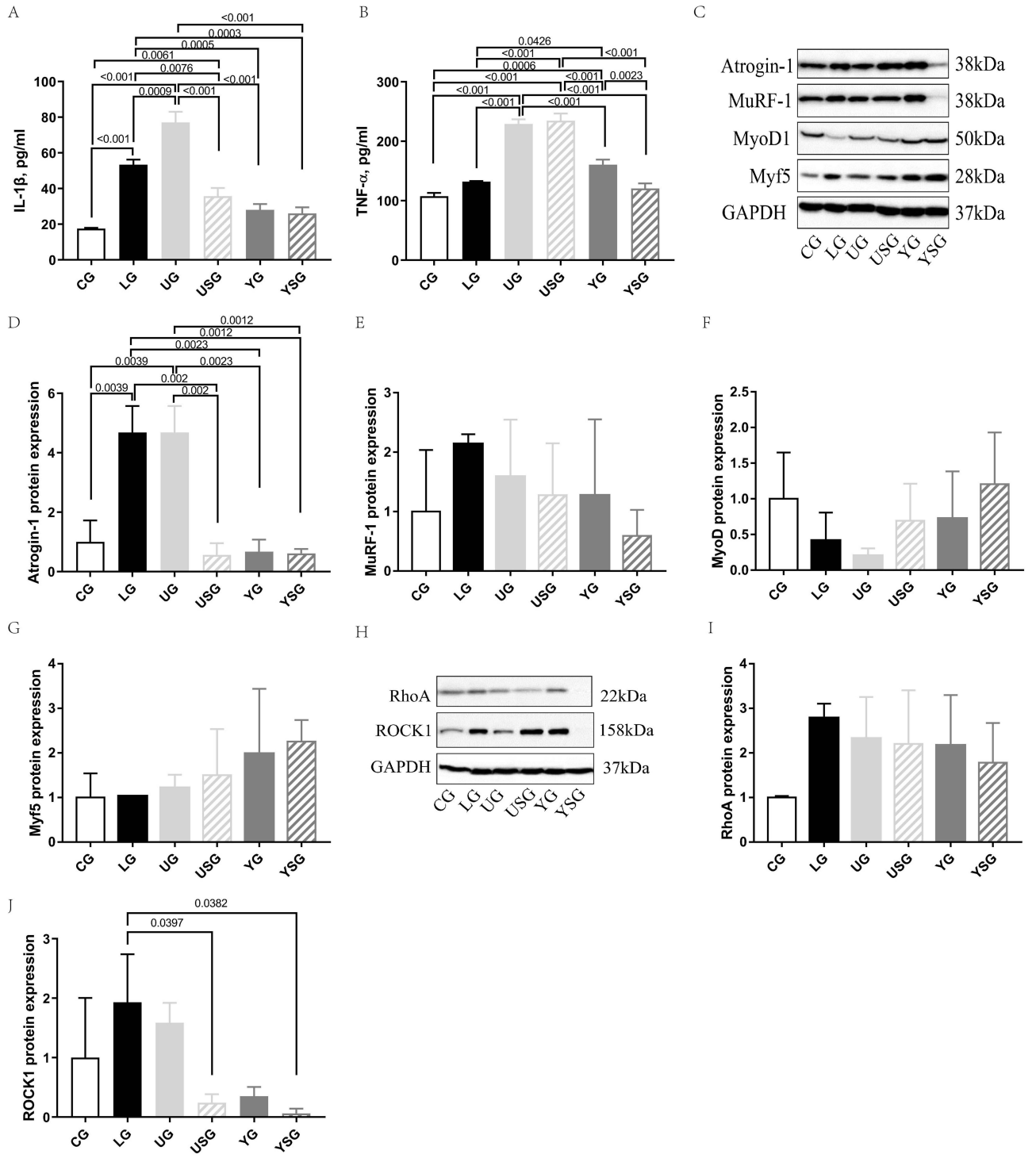

Figure 5 RhoA/ROCK involves in mechanical stretching downregulating inflammation in LPS-treated C2C12 myoblasts. **(A and B)**. levels of IL-1β and TNF-α; **(C-G)**. Western blot analysis of protein expression of Atrogin-1, MuRF-1, MyoD and Myf5; **(H-J)**. Western blot analysis of protein expression of RhoA and ROCK. Significance was calculated by one-way ANOVA with Tukey's post-hoc analysis for the parameters including IL −1β, TNF-α, Atrogin-1, MuRF-1, MyoD, Myf5, RhoA, and ROCK1.

Abbreviations: CG, control group; LG, LPS-treated group; UG, U46639-treated group; USG, U46639 with machinal stretching group; YG, Y27632-treated group; YSG, Y27632 with machinal stretching group.

differentiation, and showed that exercise could reverse the decrease in myogenic regulatory factors in COPD diaphragms. To investigate whether the positive effects of exercise are related to the role of RhoA/ROCK in regulating inflammation, we evaluated the expression of diaphragmatic inflammatory factors and showed that the levels of such inflammatory factors decreased after exercise training. Previous studies have also found that high-intensity exercise training reduces the expression levels of proinflammatory factors in COPD muscles,[28] whereas low-intensity exercise training seems to have no effect on inflammation levels.[29]

Mechanical stretching is used to simulate in vivo movement at the cellular level, and myoblasts are sensitive to this method.[30] Hence, this study further investigated the role of RhoA/ROCK in exercise improving diaphragmatic atrophy in COPD at the cellular level. Previous study have found that TNF-α can activate RhoA, and Y27632 can partially inhibit such activation, indicating a positive feedback loop between RhoA/ROCK and inflammation.[31] Consistent with this, we found that the agonist U46639 further enhanced the level of LPS-induced inflammation, whereas the level of inflammation considerably decreased after agonist or antagonist combined with mechanical stretching. The inactivation of RhoA not only can directly mediate inflammation, but also can improve muscle regeneration.[18] The expression of MyoD is mediated by RhoA, which controls myoblast survival by the phosphatidylinositol 3-kinase-Akt signaling.[32] MyoD, a myogenic regulator, is degraded by ubiquitin ligase to inhibiting myogenic differentiation.[33] Low-amplitude vertical vibration upregulates MyoD expression in C2C12 myoblasts in a time-dependent manner.[34] A mechanical stretching protocol with 15% elongation for 2h/day for 3 days in C2C12 myoblasts remarkably increased the expression of MyoD, whereas stretching for 6 h/day reduced the proliferation of MyoD.[35] Myf5 expression was observed after 12 h of stretching with 15% elongation, and MyoD expression gradually decreased over time.[36] These results indicated a negative feedback relationship between protein degradation and muscle regeneration. In addition, inflammatory cytokines TNF-α regulate myogenesis by inhibiting differentiation and promoting proliferation in satellite cells.[37] Taken together, RhoA/ROCK signaling is interactive with inflammation, the highly activated RhoA/ROCK and inflammation both can inhibit myogenic differentiation.

Conclusions

These findings highlight RhoA/ROCK as an upstream signaling event involved in exercise improving diaphragmatic atrophy in CS-induced COPD.

Data Sharing Statement

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Ethics Statement

All animal experimental procedures were performed in accordance with the Committee's Animal Care and Use Guidelines and approved by the Shanghai University of Traditional Chinese Medicine Animal Care Committee (no. PZSHUTCM210312012). All institutional and national guidelines for the care and use of laboratory animals were followed.

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Author Contributions

All authors made a significant contribution to the work reported. XL and WW conceived of and designed the study. PL wrote the draft of the manuscript. All authors performed the research, took part in revising and critically reviewing the article, gave final approval of the version to be published, have agreed on the journal to which the article has been submitted, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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