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The Role of RhoA and Cytoskeleton in Myofibroblast Transformation in Hyperoxic Lung Fibrosis

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Abstract

Myofibroblast transformation is a key process in the pathogenesis of lung fibrosis. We have previously reported that hyperoxia induces RhoA activation in HFL-1 lung fibroblasts and RhoA mediates collagen synthesis in hyperoxic lung fibrosis. In the present study, we investigated the role of RhoA and actin cytoskeleton in hyperoxia-induced myofibroblast transformation. Exposure of HFL-1 lung fibroblasts to hyperoxia stimulated actin filament formation, shift of G-actin to F-actin, nuclear co-localization of myocardin-related transcription factor-A (MRTF-A), recruitment of MRTF-A to α -smooth muscle actin (α -SMA) gene promoter, myofibroblast transformation, and collagen-I synthesis. Inhibition of RhoA by C3 transferase CT-04 or dominant-negative RhoA mutant T19N, and inhibition of ROCK by Y27632 prevented myofibroblast transformation and collagen-I synthesis. Moreover, inhibition of RhoA by CT-04 prevented hyperoxia-induced actin filament formation, shift of G-actin to F-actin, and nuclear co-localization of MRTF-A. In addition, disrupting actin filaments by cytochalasin D or scavenging reactive oxygen species (ROS) by tiron attenuated actin filament formation, nuclear co-localization of MRTF-A,

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myofibroblast transformation, and collagen-I synthesis. Furthermore, overexpression of constitutively active RhoA mutant Q63L or stabilization of actin filaments recapitulated the effect of hyperoxia on actin cytoskeleton, and nuclear co-localization of MRTF-A, myofibroblast transformation and collagen-I synthesis. Interestingly, knocking-down MRTF-A prevented hyperoxia-induced increase in the recruitment of MRTF-A to serum response factor (SRF) transcriptional complex on α -SMA gene promoter, myofibroblast transformation and collagen-I synthesis. Finally, Y27632 and tiron attenuates hyperoxia-induced increases in α -SMA and collagen-I in mouse lungs. Together, these results indicate that the actin cytoskeletal reorganization due to ROS/RhoA-ROCK pathway mediates myofibroblast transformation and collagen synthesis in lung fibrosis of oxygen toxicity. MRTF-A contributes to the regulatory effect of actin cytoskeleton on myofibroblast transformation during hyperoxia.

Keywords

oxygen toxicity; lung; fibroblasts; collagen; reactive oxygen species; MRTF-A

Introduction

Oxygen toxicity is a major side-effect of oxygen therapy administered to newborns and adults. Prolonged exposure to higher levels of oxygen leads to pulmonary injury resulting in diffuse alveolar damage, intense cellular infiltration, and deposition of interstitial collagen fibers [1,2]. Lung fibrosis is a life threatening consequence of pulmonary oxygen toxicity in human and animal models [3,4]. Excessive production and deposition of extracellular matrix (ECM) proteins is key process in the pulmonary fibrosis occurring in hyperoxia-induced pulmonary injury [5,6]. Collagen is the major extracellular matrix (ECM) component of the lungs and is vital for maintaining the normal lung architecture. The increase in collagen synthesis from lung alveolar interstitial fibroblasts is correlated with changes in the viscoelastic behavior and impairs lung function in hyperoxia-induced lung injuries [7]. Fibroblasts are the major cells to produce collagen ECM in the lungs. Exposure of lung fibroblasts to hyperoxia stimulates fibroblasts proliferation and increases collagen protein [6,8,9]. After hyperoxic injury, lung fibroblasts differentiate into contractile myofibroblasts that secrete excessive ECM proteins such as collagen. More importantly, differentiated myofibroblasts characteristically synthesize α -smooth muscle actin (α -SMA), a most commonly used molecular marker, which contributes stronger contractile activity in lung fibrosis [10]. It has been reported that hyperoxia augments pulmonary lipofibroblast-to-myofibroblast transdifferentiation [11]. In the present study, we found that hyperoxia causes transformation of lung fibroblasts into myofibroblasts. Nevertheless, the detail mechanism for hyperoxia-induced myofibroblast transformation remains unknown.

We recently reported that RhoA activation is implicated in hyperoxic pulmonary fibrosis [6]. We found that exposure of lung fibroblasts to 95% oxygen induces activation of RhoA [6]. Inhibition of RhoA attenuates collagen synthesis in hyperoxic lung fibroblasts and pulmonary fibro-proliferative lesions in mice exposed to hyperoxia [6]. RhoA belongs to a family of small GTPases which are essential in the regulation of various cellular functions including formation of F-actin stress fibers and focal adhesion complexes and transcription

of genes containing serum response element [12]. RhoA can be activated by growth factors and reactive oxygen species (ROS) [6,13]. The downstream events for Rho activation include activation of Rho kinase (ROCK) and increased formation of actin stress fibers. We and others have found that hyperoxia has remarkable effects on actin cytoskeleton, including increased actin polymerization, loss of cortical actin, and formation of stress fibers in cells macrophages and endothelial cells [14,15]. Alterations in actin cytoskeletal organization affect many aspects of cell function such as cell motility, protein synthesis and signal transduction [16,17]. Increased F-actin assembly and/or decreased G-actin have been reported to affect the activities of transcription factors including myocardin-related transcription factor-A (MRTF-A) [18]. Because myofibroblast transformation is an important process in pulmonary fibrosis, we hypothesize that RhoA and cytoskeletal reorganization play a role in myofibroblast transformation in lung fibrosis induced by oxygen toxicity.

In the present study, we demonstrate for the first time that RhoA mediates the transformation of lung fibroblasts to myofibroblast via actin cytoskeletal reorganization. We found that inhibition of RhoA prevents increases in α -SMA and collagen proteins in mouse lungs exposed to hyperoxia, suggesting that RhoA activation contributes to myofibroblast transformation in hyperoxic lung fibrosis. These observations provided not only new information for the mechanism of lung fibroblasts to myofibroblast transformation but also the rationale to manipulate RhoA activation to prevent and treat fibrotic injury in oxygen toxicity.

Materials and Methods

Reagents and methods

Mouse monoclonal anti- α -SMA, goat polyclonal MRTF-A antibody, rabbit polyclonal Nox4 antibody, rabbit polyclonal CD34 antibody, MRTF-A siRNA (h) and Nox4 siRNA (human) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Negative control siRNA silencer was from Invitrogen. Rabbit anti-collagen-I antibody was from Novus Biologicals (Littleton, CO). Antibody against GAPDH was from cell signaling (Beverly, MA). G-actin/F-actin in vivo assay kit (#BK037) and RhoA inhibitor C-3 transferase (CT-04) were from Cytoskeleton (Denver, CO). Mammalian expression plasmids cDNA of wild-type RhoA, constitutively active RhoA with Q63 substituted with L(Q63L), and dominant-negative RhoA with T19 substituted with N containing GFP cDNA (T19N) were obtained from Addgene (Cambridge, MA). Cytochalasin D was purchased from Merck (Darmstadt, Germany). Jasplakinolide was from Enzo life sciences. Other reagents were purchased from Sigma (St. Louis, MO).

Cell culture and hyperoxic exposure

Human HFL-1 lung fibroblasts were purchased from the ATCC. Primary human lung fibroblasts were obtained from Cell Applications, Inc. (San Diego, CA). Third- to eighth-passage cells were maintained in F12K medium containing 10% fetal bovine serum and antibiotics (10 u/ml penicillin, 100 μ g/ml streptomycin, 20 μ g/ml gentamycin, and 2 μ g/ml amphotericin B) and were used 2 or 3 days after confluence. For hyperoxic exposure,

confluent HFL-1 fibroblasts and primary human lung fibroblasts were incubated in 95% or 40% O₂ and 5% CO₂ at 37 °C. Normoxia was air and 5% CO₂.

Western blot analysis

The transformation of fibroblasts to myofibroblasts was monitored by measuring the protein contents of α -SMA, a most commonly used molecular marker of myofibroblast transformation. Cell lysate proteins (25 to 30 μ g) were separated by 4–20% Tris-glycine SDS-PAGE and electrotransferred onto nitrocellulose membranes. The membranes were incubated in blocking solution at room temperature for 1 h and then hybridized with primary antibodies against α -SMA, MRTF-A, collagen-I, Nox4 and GAPDH overnight at 4 °C. The bands were detected by an immunochemiluminescence method. The density was quantitated by Bio-Rad Quantity One software.

RNA isolation and real-time PCR

Total RNA was isolated from lung fibroblast homogenates using an RNAeasy kit from Qiagen (Valencia, CA) according to the manufacture's protocol. To measure α -SMA, COL1A1, COL1A2, MRTF-A mRNA, reverse transcription was done by using a high capacity cDNA reverse transcriptase from Applied Biosystems (Foster City, CA). qRT-PCR was performed by using a TaqMan gene expression assay kit (Applied Biosystems). The primer IDs are Hs00426835_g1 for α -SMA, Hs00164004_m1 for human COL1A1, Hs00164099_m1 for human COL1A2, Hs00252979_m1 for MRTF-A and Hs03003631_g1 for 18s rRNA. The primer sequences were not disclosed by the company. PCR reactions in triplicate were carried out by using an iQ5 real-time PCR system (Bio-Rad). The sequence detector was programmed for the PCR conditions 50 °C for 2 min then 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. For the relative quantification of α -SMA, COL1A1, COL1A2 and MRTF-A mRNA contents, the comparative threshold cycle (CT) method was employed. The CT values of endogenous control (18s rRNA) were first subtracted from the CT values of the detected genes to derive a Δ CT value. The relative α -SMA, COL1A1, COL1A2 and MRTF-A mRNA contents were expressed as $2^{-\Delta CT}$ using 18s rRNA as a reference.

Transfection of fibroblasts with plasmid encoding wild-type RhoA, constitutively active RhoA mutant, or dominant-negative RhoA mutant

Mammalian expression plasmid vectors containing wild-type RhoA cDNA or constitutively active RhoA (Q63L) or dominant-negative RhoA mutant cDNA (T19N) (Addgene) were transfected into HFL-1 cell using Lipofectamine LTX with Plus reagent (Invitrogen) according to the manufacturer's protocol. 48 h after transfection, the cells were exposed to hypoxia or normoxia, and then the levels of RhoA, α -SMA, and collagen-I were analyzed.

Confocal microscopy

HFL-1 cells were fixed with 4% paraformaldehyde, then incubated with 0.1% Triton X-100 for 10 min and with 5% goat serum for 30 min. F-actin and MRTF-A were then stained with Texas-red phalloidin and mouse anti-MRTF-A antibody (Santa Cruz, sc-21558) labeled with anti- goat IgG (Alexa Fluor 488). The slides were counter stained with 4',6-diamidino-2-

phenylindole (DAPI), and the fluorescent images were captured with a Zeiss LSM 510 laser scanning confocal microscope.

Measurement of G- and F-actin

After treatments, the cells were homogenated. G-actin and F-actin fractions in the lysates were separated using a kit from Cytoskeleton, Inc., according to the manufacturer's protocol. Western blot analysis was performed using anti-pan actin antibody supplied in the kit.

Knocking down of MRTF-A and Nox4

MRTF-A and Nox4 protein was knocked down using small interfering RNA (siRNA) against MRTF-A and Nox4 mRNA. The siRNAs were obtained from Santa Cruz Biotechnology (sc- 43944 for MRTF-A and sc-61887 for Nox4). A silencer negative control siRNA (AM4611, Applied Biosystems, Foster City, CA) was used as control. The sequences of these siRNA are not disclosed by the companies. Pre-confluent HFL-1 fibroblasts were transfected with 1 μ g of siRNA against MRTF-A or Nox4 mRNA or a control siRNA using RNAiMax transfection reagent (Qiagen, Valencia, CA) in F12K medium containing 4% fetal bovine serum according to the manufacturer's protocol. The ratio of siRNA to transfection reagent was 1:3. 48 hours after transfection, HFL-1 cells were exposed to hyperoxia or normoxia. Then, the protein levels of MRTF-A, Nox4, α -SMA, and collagen-I were analyzed.

ROS measurement

After hyperoxic exposure, cells plated in 24-well plates were loaded with 10 μ M dihydroethidine (DHE) for 30 min. After washing, fluorescence intensity was assayed using SpectraMax spectrophotometer.

Chromatin immunoprecipitation assay (ChIP)

ChIP analysis was performed using the reagents and methods provided in a Chromatin Immunoprecipitation Assay kit (#17–295) purchased from Millipore (Temecula, CA). Briefly cells were cross-linked with 1% formaldehyde for 10 min. After washing with PBS, the cells were scraped, and re-suspended in 1 ml of 1% SDS lysis buffer containing protease inhibitors. Samples were sonicated and then centrifuged at $13,000 \times g$ for 10 min. The supernatant was removed and diluted in a 10-fold excess of ChIP dilution buffer. The samples were pre-cleared using 30 μ l/ml of salmon sperm DNA/Protein A-agarose slurry and then incubated for 1 h with 8 μ g/ml of anti-MRTF-A or anti-nucleolin C23 antibody. 30 μ l/ml of salmon sperm DNA/Protein A-agarose slurry was added and samples were incubated overnight at 4 °C under gentle agitation. The bead slurry was washed and the DNA-protein complex was detached from the bead slurry with two washes of 250 μ l of 1% SDS, 0.1 M NaHCO₃, then reverse cross-linked by incubation with 200 μ M NaCl for 4 h at 65 °C. 10 μ M EDTA, 40 μ M Tris-HCl, and 20 μ g of proteinase K were then added to the samples, and were incubated for 1 h at 45 °C. DNA was isolated and purified from the samples using phenol/chloroform/isoamyl alcohol. Real-time PCR was then performed on samples using SYBR Green Master Mix purchased from Applied Biosystems and PCR

primers specific for α -SMA promoter: 5'-agcagaacagaggaatgcagtgaagagac-3' and 5'-cctccactcgctcccaacaaggagc-3'. PCR was carried out in a 7500 Real Time PCR System device from Applied Biosystems, using an annealing temperature of 61 °C and an extension temperature of 72 °C, for a total of 55 cycles. PCR products Data are compared to input and normalized to anti-nucleolin C23 and expressed as 2^{-C_T} to determine the relative promoter occupancy.

Animals and hyperoxic exposure

Male C57BL/6 mice with ages between 8 and 10 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME). All experiments were performed in accordance with the guiding principles of the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of the Georgia Regents University. For hyperoxic exposure, mice were kept in a clear plastic polypropylene chamber (30"x20"x20") ad libitum with free access to food and water. The oxygen concentration in the chamber was maintained using Proox Oxygen Controller (BioSpherix, Lacona, NY) at 80% for 5 days and then changed to 50% for another 10 days (15 days in total). The oxygen mixture was humidified, and the concentration of CO₂ in the chamber was lower than 0.3%. Starting at the time when being changed to 50% oxygen, the hyperoxic mice were given daily injection of ROCK inhibitor Y27632 (5 mg/kg, daily by IP injection) or ROS scavenger tiron (1.5 g/kg, daily by IP injection) or same volume of PBS. The normoxic mice were kept in room air and also received daily IP injection of same dose of Y27632 or tiron or PBS at same time.

Mouse lung experiments

Mice were anesthetized (pentobarbital, 90 mg/kg, i.p.) and the trachea was intubated. The mice were then euthanized by using thoracotomy. The blood in pulmonary circulation was rinsed by infusing PBS through pulmonary artery and the heart and lungs were removed. The left lungs were filled with 4% paraformaldehyde (PFA) solution at 25 cm H₂O and fixed in 4% PFA for 24 h. After fixation, the lungs were sliced midsagittally and embedded in paraffin. The lung sections of 7 μ m thickness were then subjected to double-labeling for α -SMA and collagen-I or CD34 and vimentin. The fluorescent images were captured with a Zeiss LSM 510 laser scanning confocal microscope. A minimum of 10 microscopic fields were examined for each slide. The fluorescence intensity was measured in a grey scale mode using ImageJ.

Statistical analysis

In each experiment, experimental and control cells were matched for cell line, age, seeding density, number of passages, and number of days post confluence to avoid variation in tissue culture factors that can influence measurements of α -SMA and collagen-I. Results are shown as the mean $\pm$ SE for n experiments. One-way ANOVA and post t-test analyses were used to determine the significance of differences between the means of different groups. $P < 0.05$ was considered statistically significant.

Results

Exposure of lung fibroblasts to hyperoxia results in myofibroblast transformation and increased collagen synthesis

Differentiated myofibroblasts characteristically synthesize α -SMA, a most commonly used molecular marker [10]. To study the effects of hyperoxia on myofibroblast transformation, HFL-1 lung fibroblasts were exposed to hyperoxia (40% or 95% oxygen) or normoxia conditions. We found that exposure of HFL-1 human lung fibroblasts to 40% or 95% oxygen for 12–24 h resulted in time- and concentration-dependent increases in α -SMA protein and mRNA levels (Figure 1), indicating that hyperoxia stimulates α -SMA synthesis and transformation of lung fibroblasts into myofibroblasts. The increases in α -SMA protein and mRNA in hyperoxic cells were accompanied by increases in the levels of collagen-I protein and COL1A1 and COL1A2 mRNA (Figure 1), suggesting that myofibroblast transformation promotes collagen synthesis during hyperoxia.

Inhibitors of RhoA and ROCK prevent hyperoxia-induced myofibroblast transformation and collagen synthesis

We recently reported that hyperoxia induces RhoA activation and RhoA plays a mediator role in hyperoxic lung fibrosis [6]. To investigate the role of RhoA in hyperoxia-induced myofibroblast transformation, HFL-1 lung fibroblasts were exposed to hyperoxia (95% oxygen) for 24 h in the presence and absence of cell-permeable Rho inhibitor, C-3 transferase (CT-04, 1 μ g/ml) or ROCK inhibitor, Y27632 (10 μ M). The results showed that both RhoA inhibitors CT-04 and ROCK inhibitor Y27632 prevented increases in α -SMA protein and mRNA levels in hyperoxic HFL-1 cells (Figure 2). Moreover, CT-04 and Y27632 blocked the increase in the levels of collagen-I protein and COL1A1 and COL1A2 mRNA in hyperoxic HFL-1 cells (Figure 2). These results suggest that RhoA activation is associated to hyperoxia-induced myofibroblast transformation and collagen-I synthesis.

To investigate whether inhibition of RhoA prevents hyperoxia-induced myofibroblast transformation and collagen synthesis in primary lung fibroblasts, primary lung fibroblasts were exposed to hyperoxia (95% oxygen) for 24 h in the presence and absence of cell-permeable Rho inhibitor, CT-04 (1 μ g/ml). We found that CT-04 prevented increases in α -SMA protein and mRNA levels in hyperoxic primary lung fibroblasts (Supplemental Figure 1). Moreover, CT-04 blocked the increase in the levels of collagen-I protein and COL1A1 and COL1A2 mRNA in hyperoxic primary lung fibroblasts (Supplemental Figure 1). These results suggest that RhoA activation also contributes to hyperoxia-induced myofibroblast transformation and collagen-I synthesis in primary lung fibroblasts.

Overexpression of constitutively active RhoA mutant Q63L promotes myofibroblast transformation

To examine whether active RhoA promotes myofibroblast transformation, HFL-1 lung fibroblasts were transfected with plasmids containing wild type RhoA and constitutively active RhoA mutant Q63L [6,19]. As shown in Figures 3A, 3B and 3C, the protein levels of α -SMA collagen-I and the mRNA levels of α -SMA, COL1A1 and COL1A2 were much higher in HFL-1 cell with constitutively active Rho-A mutant Q63L than those with wild-

type RhoA, suggesting that over-expression of constitutively active Rho-A mutant promotes myofibroblast transformation and increases collagen-I synthesis.

Overexpression of dominant-negative RhoA mutant T19N prevents hyperoxia-induced myofibroblast transformation and collagen synthesis

To further study whether RhoA activation is responsible for hyperoxia-induced myofibroblast transformation and collagen synthesis, we determined the protein levels of α -SMA, collagen-I and the mRNA levels of α -SMA, COL1A1 and COL1A2 in HFL-1 lung fibroblasts transfected with plasmids containing wild-type RhoA and dominant-negative RhoA mutant T19N under normoxic and hyperoxic conditions. As shown in Figures 3D, 3E and 3F, hyperoxia increased the protein levels of α -SMA collagen-I and the mRNA levels of α -SMA, COL1A1 and COL1A2 in HFL-1 cells transfected with plasmids containing wild-type RhoA. However, hyperoxia-induced elevation of these proteins and mRNAs were prevented in HFL-1 cells transfected with plasmids containing the dominant-negative RhoA mutant T19N. Together, these results indicate that RhoA activation mediates hyperoxia-induced myofibroblast transformation and collagen-I synthesis.

RhoA inhibitor CT-04 prevents hyperoxia-induced alterations in the actin cytoskeletal organization

We then studied the alterations in the actin cytoskeletal organization in hyperoxic HFL-1 cells. The cells were exposed to 95% oxygen in the presence and absence of RhoA inhibitor CT-04 (1 μ g/ml) for 24 h. Then actin filaments were stained using Texas-red phalloidin, and G- and F-actin contents were measured. We found that hyperoxia induced remarkable formation of actin filaments (Figure 4A). Concomitantly, F-actin/G-actin ratio increased in hyperoxic cells (Figure 4B). Incubation of HFL-1 fibroblasts with CT-04 prevents hyperoxia-induced increases in actin filaments and F-actin/G-actin ratio (Figure 4), indicating that actin cytoskeletal reorganization is due to RhoA activation during hyperoxia.

Jasplakinolide promotes myofibroblast transformation and collagen synthesis

To study the role of actin cytoskeletal reorganization in myofibroblast transformation and collagen synthesis, HFL-1 fibroblasts were incubated with or without actin filament stabilizing agent jasplakinolide (50 – 200 nM) for 24 h. As shown in Figures 5A, 5B, and 5C, incubation with jasplakinolide resulted in dose-dependent increases in the formation of actin filaments and F-actin/G-actin ratio, indicating that jasplakinolide causes a shift of G-actin to F-actin fraction. Importantly, this shift of G-actin to F-actin fraction was accompanied by increases in the synthesis of α -SMA and collagen-I (Figures 5D and 5E). These results suggest that actin cytoskeletal reorganization is involved in hyperoxia-induced myofibroblast transformation and collagen-I synthesis.

Cytochalasin D prevents hyperoxia-induced myofibroblast transformation and collagen synthesis

It has been shown that disruption of the actin cytoskeleton by cytochalasin D leads to increase in G-actin [16]. To further confirm the role of the actin cytoskeleton in hyperoxia-induced myofibroblast transformation and collagen synthesis, HFL-1 lung fibroblasts were

treated with or without cytochalasin D (2 μ M), a disrupter of the actin cytoskeleton, for 1 h, and then exposed to normoxia or hyperoxia (95% oxygen) for 24 h. As shown in Figure 6A, treatment of HFL-1 fibroblasts with 2 μ M cytochalasin D for 1 h caused disruption of the actin cytoskeleton. Meanwhile, the levels of mRNA and proteins of α -SMA and collagen-I were comparable between normoxic and hyperoxic cells treated with cytochalasin D (Figures 6B, 6C and 6D), indicating that the actin cytoskeleton plays an important role in hyperoxia-induced myofibroblast transformation and collagen-I synthesis.

Cytochalasin D and CT-04 prevent hyperoxia-induced increase in nuclear localization of MRTF-A

To clarify the mechanism for myofibroblast transformation induced by actin cytoskeletal reorganization during hyperoxia, we studied the intracellular localization of MRTF-A, a protein binding to G-actin. As shown in Figure 7A, there was a robust co-localization of MRTF-A in nuclei of HFL-1 fibroblasts exposed to hyperoxia (95% oxygen) for 24 h compared to those exposed to normoxic condition. The increase in nuclear co-localization of MRTF-A in hyperoxic cells was accompanied by an increase in actin filament formation. Incubation of HFL-1 cells with cytochalasin D (2 μ M, 1 h) and CT-04 (1 μ g/ml, 24 h) prevented the increase in nuclear co-localization of MRTF-A induced by hyperoxia (Figure 7A). These data suggest that actin cytoskeletal reorganization due to RhoA activation is associated to the nuclear co-localization of MRTF-A during hyperoxia.

Jasplakinolide and constitutively active RhoA mutant Q63L induce nuclear co-localization of MRTF-A

We then studied the effects of jasplakinolide on the intracellular co-localization of MRTF-A. As shown in Figure 7B, incubation of HFL-1 fibroblasts to jasplakinolide (50–200 nM) resulted in increases in actin filament formation and nuclear co-localization of MRTF-A. Overexpression of constitutively active Rho-A mutant Q63L also induced actin filament formation and nuclear co-localization of MRTF-A (Figure 7B). These results provide further evidence supporting that RhoA and actin cytoskeletal reorganization contribute to nuclear co-localization of MRTF-A.

Knocking-down MRTF-A prevents hyperoxia-induced myofibroblast transformation and collagen synthesis

To further clarify the role of MRTF-A in hyperoxia-induced myofibroblast transformation and collagen synthesis, MRTF-A protein expression was knocked down using its siRNA. As shown in Figure 8, exposure of HFL-1 fibroblasts to 95% oxygen for 24 h caused increases in the levels of MRTF-A protein and mRNA. Knocking-down MRTF-A prevented increases in the protein and mRNA levels of α -SMA and collagen-I in hyperoxic HFL-1 cells (Figure 8), suggesting that MRTF-A mediates transformation of lung fibroblasts into myofibroblasts and collagen-I synthesis induced by hyperoxia.

Knocking-down MRTF-A prevents hyperoxia-induced recruitment of MRTF-A to α -SMA gene promoter

To clarify whether MRTF-A-mediated increase in α -SMA mRNA is due to activation of α -SMA transcription, a ChIP assay was performed in HFL-1 cells exposed to normoxia and hyperoxia. We found that the recruitment of MRTF-A to serum response factor (SRF) transcriptional complex on α -SMA gene promoter was increased in HFL-1 cells exposed to hyperoxia for 24 h (Figure 9). Knocking-down MRTF-A inhibited hyperoxia-induced increase in the recruitment of MRTF-A to α -SMA gene promoter (Figure 9).

ROS scavenger tiron prevents hyperoxia-induced actin filament formation and nuclear co-localization of MRTF-A and myofibroblast transformation

We have recently found that hyperoxia induces ROS production and hyperoxia-induced RhoA activation is dependent on ROS [6]. To study the role of ROS in hyperoxia-induced myofibroblast transformation, HFL-1 lung fibroblasts were exposed to hyperoxia (95% oxygen) for 24 h in the presence and absence of tiron (5 mM), a ROS scavenger. As shown in Figure 9A, incubation of HFL-1 fibroblasts with tiron attenuated increases in actin filament formation and nuclear localization of MRTF-A induced by hyperoxia. Importantly, we found that tiron reduced hyperoxia-induced increases in the protein and mRNA levels of MRTF-A, α -SMA and collagen-I (Figure 10B, 10C and 10D). Together, these results indicate that actin cytoskeletal reorganization, nuclear co-localization of MRTF-A and myofibroblast transformation during hyperoxia are ROS-dependent.

Knocking-down Nox4 prevents hyperoxia-induced increase in ROS formation

To investigate whether Nox4 is a source of ROS in hyperoxic cells, Nox4 protein was knocked-down. We found that knocking-down Nox-4 protein inhibited hyperoxia-induced increase in ROS formation (Figure 11), suggesting that Nox-4 might be a major source of ROS in HFL-1 exposed to hyperoxia.

ROCK inhibitor Y27632 and ROS scavenger tiron attenuates hyperoxia-induced synthesis of α -SMA and collagen in mouse lungs

To confirm our *in vitro* observation of myofibroblast transformation mediated by an activated ROS/RhoA-ROCK pathway in hyperoxic lung fibroblasts, we utilized an animal model of hyperoxia-induced lung fibrosis. Male C57BL/6 mice were exposed to 80% oxygen for 5 days and then exposed to 50% oxygen for another 10 days. Starting at the time of change to 50% oxygen, the hyperoxic mice were given daily injections of the ROCK inhibitor Y27632 (5 mg/kg daily by ip injection) or the ROS scavenger tiron (1.5 g/kg daily by ip injection) or the same volume of PBS. We found that the hyperoxic lungs showed remarkable increases in the fluorescence intensities for α -SMA and collagen-I (Figures 12 and Figure 13). Y27632 and tiron attenuated the fluorescence intensities for α -SMA and collagen-I in hyperoxic lungs (Figures 12 and Figure 13). These results show that RhoA activation and ROS contribute to myofibroblasts transformation in hyperoxia-induced lung fibrosis.

ROCK inhibitor Y27632 and ROS scavenger tiron attenuates hyperoxia-induced increases in fibrocytes in mouse lungs

To investigate circulating fibrocytes contributes to hyperoxic fibrosis, we stained mouse lungs exposed to normoxia or hyperoxia for fibrocytes. We found that hyperoxic lungs showed significant increase in the numbers of cells which are positive for both CD34 and vimentin (Supplemental Figure 2), indicating that hyperoxia increases the recruitment of circulating fibrocytes into the lungs. Moreover, daily injections of the ROCK inhibitor Y27632 (5 mg/kg daily by ip injection) or the ROS scavenger tiron (1.5 g/kg daily by ip injection) reduced the numbers of fibrocytes in hyperoxic mouse lungs (Supplemental Figure 2).

Discussion

Fibroblasts are the major cells to produce collagen ECM in the lungs. Exposure of lung fibroblasts to hyperoxia stimulates fibroblasts proliferation and increases collagen protein [6,8,9]. After hyperoxic injury, lung fibroblasts differentiate into contractile myofibroblasts that secrete excessive ECM proteins such as collagen. More importantly, differentiated myofibroblasts characteristically synthesize α -SMA, a most commonly used molecular marker, which contributes to stronger contractile activity in pulmonary fibrosis [10,20,21]. Contractile myofibroblasts are responsible for the irreversible alterations of the lung parenchyma that results in accumulation of stiff scar tissue ultimately leading to deterioration of lung function. In the present study, we found that the synthesis α -SMA and collagen-I is much higher in hyperoxic HFL-1 lung fibroblasts and primary lung fibroblasts indicating that hyperoxia stimulates the transformation of lung fibroblasts into myofibroblasts. This finding is consistent with previous report that hyperoxia augments pulmonary lipofibroblast-to-myofibroblast transdifferentiation [11].

The mechanism for hyperoxia-induced myofibroblast transformation has not been clarified. It has been well-documented that TGF- β 1 induces myofibroblast transformation and collagen synthesis from lung fibroblasts which is critical in the formation of fibroproliferative lung lesion [22–24]. However, our data shows that the levels of both active and total TGF- β 1 in the medium of normoxic and hyperoxic fibroblasts are comparable [6], suggesting that TGF- β 1 is not responsible for hyperoxia-induced transformation of HFL-1 lung fibroblasts to myofibroblasts. Notably, we have previously reported that hyperoxia induces RhoA activation in HFL-1 lung fibroblasts and inhibition of RhoA attenuates collagen synthesis in hyperoxic lung fibroblasts and pulmonary fibro-proliferative lesions in mice exposed to hyperoxia [6]. In the present study, we found that inhibition of RhoA using CT-04 and ROCK inhibitor Y27632 prevent hyperoxia-induced myofibroblast transformation and collagen synthesis. Moreover, by using a different method (i.e. dominant-negative mutant), we have demonstrated that overexpression of dominant-negative RhoA mutant T19N in HFL-1 cells inhibits the transformation of lung fibroblasts into myofibroblasts and collagen synthesis. More importantly, overexpression of constitutively active RhoA mutant Q63L promotes myofibroblast transformation and collagen synthesis. Taken together, these results indicate that RhoA activation mediates the transformation of lung fibroblasts into myofibroblasts induced by hyperoxia.

The downstream events for activation of RhoA and Rho kinase include the increased F-actin assembly and formation of actin stress fibers. We and others have previously found that hyperoxia has remarkable effects on actin cytoskeleton, including increased actin polymerization, loss of cortical actin, and formation of stress fibers in macrophages and endothelial cells [14,15]. Our present studies show that there is remarkable actin filament formation and F-actin/G-actin ratio is much higher in hyperoxic cells. Inhibition of RhoA using CT-04 prevents hyperoxia-induced increases in actin filaments and F-actin/G-actin ratio, indicating that hyperoxia-induced actin cytoskeletal reorganization is attributed to RhoA activation in myofibroblast transformation.

The actin cytoskeleton is traditionally considered to be a structural system that organizes and maintains the shape of specialized cells. Now, it is clear that the cytoskeleton participates in signal transduction. Alterations in the actin cytoskeletal organization affect many aspects of cell functions such as cell motility, protein synthesis and signal transduction [15–17]. To study the role of actin cytoskeleton in hyperoxia-induced myofibroblast transformation, we manipulated the actin cytoskeleton organization using jasplakinolide and cytochalasin D. We have demonstrated that stabilization of actin filaments using jasplakinolide causes increases in actin stress fibers, F-actin/G-actin ratio, and induces myofibroblast transformation and collagen synthesis. In contrast, disrupting actin cytoskeleton using cytochalasin D inhibits hyperoxia-induced myofibroblast transformation and collagen synthesis. These data provide the first evidence that shift of G-actin to F-actin during hyperoxia promotes myofibroblast transformation.

Increased F-actin assembly and/or decreased G-actin have been reported to affect the activities of transcription factors including MRTF-A and SRF [18]. MRTF-A possess a unique N-terminal RPEL domain that mediates binding to G-actin and leads to cytoplasmic sequestration. MRTF-A can translocate to the nuclei, and subsequently binds and enhances SRF transcriptional activity [25,26]. Indeed, our observations indicate that hyperoxia induces shift of G-actin to F-actin and co-localization of MRTF-A in the nuclei. Moreover, shift of G-actin to F-actin by using jasplakinolide and by overexpression of constitutively active RhoA mutant Q63L increase nuclear co-localization of MRTF-A. In addition, disrupting actin cytoskeleton using cytochalasin D and inhibition of RhoA using CT-04 attenuate nuclear co-localization of MRTF-A in hyperoxic HFL-1 cells. These data suggest that shift of G-actin to F-actin during hyperoxia reduces G-actin availability and enhances MRTF-A translocation into the nuclei. More importantly, we found that knocking-down MRTF-A prevents hyperoxia-induced myofibroblast transformation and collagen synthesis. Taken together, MRTF-A contributes to hyperoxia-induced myofibroblast transformation and collagen synthesis which is mediated by the actin cytoskeletal reorganization due to RhoA activation. De novo expression of α -SMA is a hallmark of myofibroblast differentiation and has been a widely accepted marker for myofibroblasts [10,20]. α -SMA-containing micro-filaments are essential for enhancing the contractile phenotype of myofibroblasts [10,20]. To explore the mechanism for MRTF-A-contributed myofibroblasts transformation, we studied the recruitment of MRTF-A to SRF transcriptional complex on α -SMA gene promoter of hyperoxic lung fibroblasts using a ChIP analysis. The results show that hyperoxia increases the recruitment of MRTF-A to α -SMA gene promoter and knocking-down MRTF-A inhibited hyperoxia-induced increase in the recruitment of MRTF-

A to α -SMA gene promoter. Thus, hyperoxia enhances MRTF-A binding to SRF transcriptional complex on α -SMA gene promoter and results in α -SMA gene transcription and myofibroblasts transformation.

It has been reported that ROS are involved in the pathogenesis of idiopathic pulmonary fibrosis (IPF) and the animal model of bleomycin-induced lung fibrosis [27–31]. Exposure of cultured lung fibroblasts to high concentrations of oxygen leads to increased formation of ROS [32]. In animal models, lungs exposed to high concentrations of oxygen also contain large amount of ROS [33]. Consistently, we have recently reported that there is a robust production of ROS in HFL-1 lung fibroblasts and mouse lungs exposed to hyperoxia which induces RhoA activation in hyperoxic pulmonary fibrosis [6]. In the present study, we found that ROS scavenger tiron attenuates hyperoxia-induced increases in F-actin formation and nuclear co-localization of MRTF-A. Furthermore, tiron inhibits hyperoxia-induced myofibroblast transformation and the increases in the synthesis of MRTF-A and collagen-I. These data indicate that ROS contribute to hyperoxia-induced myofibroblast transformation and collagen synthesis, further supporting our allegation that the actin cytoskeletal reorganization due to RhoA activation mediates hyperoxia-induced myofibroblast transformation and collagen synthesis.

The source of hyperoxia-induced ROS production might be the mitochondrial electron transport [34] or NADPH oxidase (Nox)-catalyzed reaction [35,36]. Notably, our data indicate that knocking-down Nox-4 protein inhibited hyperoxia-induced increase in ROS formation, suggesting that Nox-4 might be a major source of ROS in HFL-1 exposed to hyperoxia.

We have previously reported that ROCK inhibitor Y27632 and ROS scavenger tiron attenuate collagen synthesis and fibrosis in mouse lungs exposed to hyperoxia. To address the pathophysiological relevance of ROS/RhoA-ROCK pathway-mediated myofibroblast transformation in the mechanism of hyperoxic pulmonary fibrosis, we evaluated the changes in α -SMA and collagen-I in hyperoxic lungs of mice treated with or without ROCK inhibitor Y27632 and ROS scavenger tiron. We found that the hyperoxic lungs showed remarkable increases in α -SMA and collagen-I and that Y27632 and tiron attenuated the increases in α -SMA and collagen-I in hyperoxic lungs. These findings recapitulate our *in vitro* observations which support the mediator's role of ROS/RhoA-ROCK pathway in myofibroblasts transformation in hyperoxia-induced lung fibrosis.

Circulating fibrocytes are reportedly involved in the pathogenesis of lung fibrosis [37,38]. In the present study, we found that hyperoxia increases the recruitment of circulating fibrocytes into the lungs indicating that circulating fibroblasts might contribute to hyperoxic lung fibrosis. Moreover, ROCK inhibitor Y27632 or ROS scavenger tiron reduced the numbers of fibrocytes in hyperoxic mouse lungs, further supporting the role of RhoA and ROS in hyperoxia-induced lung fibrosis.

In summary, our novel findings provide evidence supporting that the actin cytoskeletal reorganization due to ROS/RhoA-ROCK pathway mediates myofibroblast transformation and collagen synthesis in pulmonary fibrosis of oxygen toxicity. MRTF-A contributes to the

regulatory effect of the actin cytoskeleton on myofibroblast transformation during hyperoxia. Therefore, ROS scavengers and RhoA/ROCK inhibitors have therapeutic potential for the prevention and treatment of lung fibrosis caused by oxygen toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlights

- Hyperoxia promotes myofibroblasts transformation.
- Hyperoxia enhances gene transcription of α -smooth muscle actin (α -SMA).
- Myocardin-related transcription factor-A binds to α -SMA gene promoter in hyperoxia.
- Cytoskeletal reorganization causes myofibroblast transformation in hyperoxia.
- Reactive oxygen species/RhoA pathway causes cytoskeletal reorganization.

Figure 1.

Exposure of lung fibroblasts to hyperoxia results in myofibroblast transformation and increased collagen synthesis. Human HFL-1 lung fibroblasts were exposed to normoxia or hyperoxia (40% and 95% oxygen) for 12–24 h after which the protein levels of α-SMA and collagen-I (A and B) and the mRNA levels of α-SMA, COL1A1 and COL1A2 (C) were determined as described in Materials and Methods. (A) is representative blot of four separate experiments. (B) is bar graph depicting the changes in α-SMA and collagen-I protein. (C) is bar graph depicting the changes in mRNA levels of α-SMA, COL1A1 and COL1A2.

Results are expressed as mean $\pm$ SE; n=4. * $P<0.05$ vs normoxia 12 h; # $P<0.05$ vs normoxia 24 h.

Figure 2.

RhoA inhibitor CT-04 and ROCK inhibitor Y27632 prevent hyperoxia-induced myofibroblast transformation and collagen synthesis. HFL-1 fibroblasts were exposed to normoxia or 95% oxygen in the presence and absence of the cell-permeative Rho inhibitor CT-04 (0.5 μ M) or ROCK inhibitor Y27632 (10 μ M) for 24 h, after which the protein levels of α -SMA and collagen-I (A and B) and the mRNA levels of α -SMA, COL1A1 and COL1A2 (C) were determined as described in Materials and Methods. (A) is representative blot of four separate experiments. (B) is bar graph depicting the changes in α -SMA and

collagen-I protein. (C) is bar graph depicting the changes in the mRNA levels of α -SMA, COL1A1 and COL1A2. Results are expressed as mean $\pm$ SE; n=4. * $P<0.05$ vs normoxia vehicle group in CT-04 experiment; # $P<0.05$ vs normoxia vehicle group in Y27632 experiment.

Figure 3.

RhoA activation is responsible for hyperoxia-induced myofibroblast transformation and collagen synthesis. (A, B, and C): HFL-1 fibroblasts were transfected with or without plasmids containing the wild-type RhoA gene and constitutively active RhoA mutant Q63L. 48 h after transfection, the protein levels of α-SMA and collagen-I (A and B) and the mRNA levels of α-SMA, COL1A1 and COL1A2 (C) were determined as described in Materials and Methods. (D, E, and F): HFL-1 fibroblasts were transfected with or without plasmids containing the wild-type RhoA gene and constitutively dominant-negative RhoA mutant

T19N. 48 h after transfection, the cells were exposed to normoxia or 95% oxygen for 24 h after which the protein levels of α -SMA and collagen-I (D and E) and the mRNA levels of α -SMA, COL1A1 and COL1A2 (F) were determined as described in Materials and Methods. Results are expressed as means $\pm$ SE; n=4. * $P < 0.05$ vs the group of wild-type plasmids (WT plasmid); # $P < 0.05$ vs normoxia WT plasmid group.

Figure 4.

RhoA inhibitor CT-04 prevents hyperoxia-induced alterations in the actin cytoskeletal organization. HFL-1 fibroblasts were exposed to normoxia or 95% oxygen in the presence and absence of the cell-permeable Rho inhibitor CT-04 (0.5 $\mu\text{g}/\text{ml}$) for 24 h, after which the actin cytoskeleton was stained (A), and F-actin and G-actin were separated and measured using Western blot analysis (B and C). (A) is representative image of the actin cytoskeleton of four separate experiments. (B) is representative blot of four separate experiments. (C) is

bar graph depicting the changes in F-actin/G-actin ratio. Results are expressed as mean $\pm$ SE; n=4. * $P<0.05$ vs normoxia vehicle group.

Figure 5.

Jasplakinolide promotes myofibroblast transformation and collagen synthesis. HFL-1 fibroblasts were incubated with jasplakinolide (0–200 nM) for 24 h, after which the actin cytoskeleton was stained (A), F-actin and G-actin were separated and measured using Western blot (B and C), and the protein levels of α -SMA and collagen-I (D and E) and the mRNA levels of α -SMA, COL1A1 and COL1A2 (F) were determined as described in Materials and Methods. (A) is representative image of the actin cytoskeleton of four separate experiments. (B) and (D) are representative images of Western blot. (C) is bar graph

depicting the changes in F-actin/G- actin ratio. (E) is bar graph depicting the changes in α -SMA and collagen-I protein. (F) is bar graph depicting the changes in the mRNA levels of α -SMA, COL1A1 and COL1A2. Results are expressed as mean $\pm$ SE; n=4. * $P<0.05$ vs control (0).

Figure 6.

Cytochalasin D prevents hyperoxia-induced myofibroblast transformation and collagen synthesis. HFL-1 lung fibroblasts were treated with or without cytochalasin D (2 μ M) for 1 h, a disrupter of the actin cytoskeleton, and then exposed to normoxia or hyperoxia (95% oxygen) for 24 h, after which the actin cytoskeleton was stained (A), the protein levels of α -SMA and collagen-I (B and C) and the mRNA levels of α -SMA, COL1A1 and COL1A2 (D) were determined as described in Materials and Methods. (A) is representative image of the actin cytoskeleton of four separate experiments. (B) is representative image of Western

blot. (C) is bar graph depicting the changes in α -SMA and collagen-I protein. (D) is bar graph depicting the changes in mRNA levels of α -SMA, COL1A1 and COL1A2. Results are expressed as mean $\pm$ SE; n=4. * $P<0.05$ vs normoxia vehicle group.

Figure 7.

Manipulating the actin cytoskeleton affects nuclear co-localization of MRTF-A in normoxic and hyperoxic HFL-1 fibroblasts. (A): HFL-1 lung fibroblasts were treated with or without cytochalasin D (2 μ M) for 1 h and then exposed to normoxia or hyperoxia (95% oxygen) for 24 h. Some HFL-1 cells were exposed to normoxia or hyperoxia (95% oxygen) in the absence or presence of CT-04 (1 μ g/ml) for 24 h. (B): HFL-1 fibroblasts were incubated with jasplakinolide (0–200 nM) for 24 h. Some HFL-1 fibroblasts were transfected with or without plasmids containing the wild-type RhoA gene and constitutively active RhoA mutant Q63L for 48 h. After these treatments, the cells were stained for the actin filaments and MRTF-A and counter-stained with DAPI. The images shown are representatives from four separate experiments.

Figure 8.

Knocking-down MRTF-A prevents hyperoxia-induced myofibroblast transformation and collagen synthesis. HFL-1 cells were transfected with siRNA against MRTF-A or control siRNA. 48 h after the transfection, the cells were exposed to normoxia or hyperoxia (95% oxygen) for 24 h after which the protein levels of MRTF-A, α-SMA and collagen-I (A and B) and the mRNA levels of MRTF-A, α-SMA, COL1A1 and COL1A2 (C) were determined as described in Materials and Methods. Results are expressed as means ± SE; n=4. * $P < 0.05$ vs normoxia group without siRNA; # $P < 0.05$ vs normoxia group with control siRNA.

Figure 9.

Knocking-down MRTF-A prevents hyperoxia-induced recruitment of MRTF-A to α -SMA gene promoter. HFL-1 cells were transfected with siRNA against MRTF-A or control siRNA. 48 h after the transfection, the cells were exposed to normoxia or hyperoxia (95% oxygen) for 24 h after which a ChIP assay was performed as described in Materials and Methods. Results are expressed as means $\pm$ SE; n=4., * P < 0.05 vs normoxia with control siRNA.

Figure 10.

ROS scavenger tiron attenuates hyperoxia-induced myofibroblast transformation and collagen synthesis. HFL-1 cells were exposed to normoxia or hyperoxia (95% oxygen) in the absence or presence of tiron (5mM) for 24 h after which the actin cytoskeleton was stained (A), the protein levels of MRTF-A, α-SMA and collagen-I (B and C) and the mRNA levels of MRTF-A, α-SMA, COL1A1 and COL1A2 (D) were determined as described in Materials and Methods. (A) is representative image of the actin cytoskeleton of four separate experiments. (B) is representative image of Western blot. (C) is bar graph depicting the changes in MRTF-A, α-SMA and collagen-I protein. (D) is bar graph depicting the changes in mRNA levels of MRTF-A, α-SMA, COL1A1 and COL1A2. Results are expressed as mean ± SE; n=4. * $P < 0.05$ vs normoxia vehicle group.

Figure 11.

Knocking-down Nox4 inhibits hyperoxia-induced ROS formation. HFL-1 cells were transfected with siRNA against Nox4 or control siRNA. 48 h after the transfection, the cells were exposed to normoxia or hyperoxia (95% oxygen) for 24 h after which ROS were assayed as described in Materials and Methods. Results are expressed as means $\pm$ SE; n=4. * $P < 0.05$ vs normoxia group.

Figure 12.

ROCK inhibitor Y27632 attenuates hyperoxia-induced synthesis of α -SMA and collagen-I in mouse lungs. Male C57BL/6 mice were exposed to 80% oxygen for 5 days, and then exposed to 50% oxygen for another 10 days. Starting at the time when being changed to 50% oxygen, the hyperoxic mice were given daily injection of ROCK inhibitor Y27632 (5 mg/kg, daily by IP injection) or same volume of PBS. The normoxic mice were kept in room air and also received daily IP injection of same dose of Y27632 or PBS at same time. The lung sections were stained for α -SMA and collagen-I protein and subjected to fluorescence

confocal microscopy. (A): representative images from eight separate experiments (magnification x400). (B): fluorescence intensity of α -SMA and collagen-I protein. Results are expressed as means $\pm$ SE; n=8. * $P < 0.05$ vs normoxia group.

Figure 13.

ROS scavenger tiron attenuates hyperoxia-induced synthesis of α -SMA and collagen-I in mouse lungs. Male C57BL/6 mice were exposed to 80% oxygen for 5 days, and then exposed to 50% oxygen for another 10 days. Starting at the time when being changed to 50% oxygen, the hyperoxic mice were given daily injection of ROS scavenger tiron (1.5 g/kg, daily by IP injection) or same volume of PBS. The normoxic mice were kept in room air and also received daily IP injection of same dose of tiron or PBS at same time. The lung sections were stained for α -SMA and collagen-I protein and subjected to fluorescence

confocal microscopy. (A): representative images from eight separate experiments (magnification x400). (B): fluorescence intensity of α -SMA and collagen-I protein. Results are expressed as means $\pm$ SE; n=8. * $P < 0.05$ vs normoxia group.