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Original Contribution

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 this 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 colocalization of myocardin-related transcription factor-A (MRTF-A), recruitment of MRTF-A to the 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 colocalization of MRTF-A. In addition, disrupting actin filaments with cytochalasin D or scavenging reactive oxygen species (ROS) with tiron attenuated actin filament formation, nuclear colocalization of MRTF-A, myofibroblast transformation, and collagen-I synthesis. Furthermore, overexpression of constitutively active RhoA mutant Q63L or stabilization of actin filaments recapitulated the effects of hyperoxia on the actin cytoskeleton and nuclear colocalization 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 the serum response factor transcriptional complex on the α-SMA gene promoter, myofibroblast transformation, and collagen-I synthesis. Finally, Y27632 and tiron attenuated hyperoxia-induced increases in α-SMA and collagen-I in mouse lungs. Together, these results indicate that the actin cytoskeletal reorganization due to the ROS/RhoA–ROCK pathway mediates myofibroblast transformation and collagen synthesis in lung fibrosis of oxygen toxicity. MRTF-A contributes to the regulatory effect of the actin cytoskeleton on myofibroblast transformation during hyperoxia.

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 a key process in the pulmonary fibrosis occurring in hyperoxia-induced pulmonary injury [5,6]. Collagen is the major 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 fibroblast 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 commonly used molecular marker, which contributes to stronger contractile activity in lung fibrosis [10]. It has been reported that hyperoxia augments pulmonary lipofibroblast-to-myofibroblast

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transdifferentiation [11]. In this study, we found that hyperoxia causes transformation of lung fibroblasts into myofibroblasts. Nevertheless, the details of the mechanism for hyperoxia-induced myofibroblast transformation remain 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 fibroproliferative 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 the 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 the actin cytoskeleton, including increased actin polymerization, loss of cortical actin, and formation of stress fibers in 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 roles in myofibroblast transformation in lung fibrosis induced by oxygen toxicity.

In this study, we demonstrate for the first time that RhoA mediates the transformation of lung fibroblasts to myofibroblasts 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 NADPH oxidase 4 (Nox4) antibody, rabbit polyclonal CD34 antibody, MRTF-A siRNA (human), and Nox4 siRNA (human) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Negative control siRNA silencer was from Invitrogen. Rabbit anti-collagen-I antibody was from Novus Biologicals (Littleton, CO, USA). Antibody against GAPDH was from Cell Signaling (Beverly, MA, USA). G-actin/F-actin in vivo assay kit (No. BK037) and RhoA inhibitor C-3 transferase (CT-04) were from Cytoskeleton (Denver, CO, USA). Mammalian expression plasmids with cDNA of wild-type RhoA, constitutively active RhoA with Q63 replaced with L (Q63L), and dominant-negative RhoA with T19 replaced with N containing GFP cDNA (T19N) were obtained from Addgene (Cambridge, MA, USA). Cytochalasin D was purchased from Merck (Darmstadt, Germany). Jasplakinolide was from Enzo Life Sciences. Other reagents were purchased from Sigma (St. Louis, MO, USA).

Cell culture and hyperoxic exposure

Human HFL-1 lung fibroblasts were purchased from the ATCC. Primary human lung fibroblasts were obtained from Cell Applications (San Diego, CA, USA). Third- to eighth-passage cells were maintained in F12K medium containing 10% fetal bovine serum and antibiotics (10 U/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml gentamycin, and 2 μg/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% O_2 and 5% CO_2 at 37 °C. Normoxia was air and 5% CO_2.

Western blot analysis

The transformation of fibroblasts to myofibroblasts was monitored by measuring the protein contents of α-SMA, a commonly used molecular marker of myofibroblast transformation. Cell lysate proteins (25 to 30 μg) 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, USA) according to the manufacturer's protocol. To measure α-SMA, COL1A1, COL1A2, and MRTF-A mRNA, reverse transcription was done using a high-capacity cDNA reverse transcriptase from Applied Biosystems (Foster City, CA, USA). qRT-PCR was performed using a TaqMan gene expression assay kit (Applied Biosystems). The assay IDs were Hs00426835_g1 for α-SMA, Hs00164004_m1 for human COL1A1, Hs00164099_m1 for human COL1A2, Hs00252979_m1 for MRTF-A, and Hs03003631_g1 for 18 s rRNA. The primer sequences were not disclosed by the company. PCR in triplicate was carried out using an iQ5 real-time PCR system (Bio-Rad). The sequence detector was programmed for the PCR conditions 50 °C for 2 min and 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 (C_T) method was employed. The C_T values of endogenous control (18 s rRNA) were first subtracted from the C_T values of the detected genes to derive a ΔC_T value. The relative α-SMA, COL1A1, COL1A2, and MRTF-A mRNA contents were expressed as $2^{-\Delta\Delta CT}$ using 18 s rRNA as a reference.

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

Mammalian plasmid expression vectors containing wild-type RhoA cDNA or constitutively active RhoA (Q63L) or dominant-negative RhoA mutant cDNA (T19N) (Addgene) were transfected into HFL-1 cells using Lipofectamine LTX with Plus reagent (Invitrogen) according to the manufacturer's protocol. Forty-eight hours after transfection, the cells were exposed to hyperoxia 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 and 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 Biotechnology, sc-21558) labeled with anti-goat IgG (Alexa Fluor 488). The slides were counterstained with 4′,6-diamidino-2-