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Original Article

RhoA/ROCK1 signaling pathway is involved in proliferation and differentiation in human lung fibroblast cells

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Abstract: Pulmonary fibrosis, a secondary effect of other diseases such as sepsis-associated ARDS, interferes with a patient's ability to breathe. The control of proliferation and differentiation become a strategy for treating pulmonary fibrosis. In this study, we want to explore the effects of Lipopolysaccharide (LPS) on the activity of RhoA/ROCK1 signaling pathways, and tried to explore the mechanism of the signaling pathways in MRC-5 cells proliferation and differentiation. We divided cells into four groups, control group, LPS group, LPS + low-dose Fasudil (15 μmol/ml) group and LPS + high-dose Fasudil (30 μmol/ml) group. RhoA activity was determined by Rho pull-down analysis and the protein levels of GTP-RhoA, ROCK1, MYPT-1 (Myosin phosphatase target subunit), p-MYPT-1 (a downstream substrate of ROCK1) and alpha-smooth muscle (α-SMA, a marker of myofibroblast cells differentiation) were determined by Western blot. Real-time PCR was used to determine the level of α-SMA mRNA. Cell proliferation rate was examined using CCK-8 and EdU Imaging Kit. LPS up-regulated RhoA activity, protein expressions of ROCK1, p-MYPT-1 and α-SMA as well as proliferation rate ($P<0.05$). Furthermore, the effects mentioned above were inhibited by Fasudil (a highly selective inhibitor of ROCK) in dose-dependent manner. The significant higher inhibitory effects in high-dose Fasudil group were observed compared with low-dose Fasudil. Our data suggest that LPS induced MRC-5 cells proliferation and differentiation via RhoA/ROCK1 signaling pathways. Fasudil attenuated LPS-induced cells proliferation and differentiation by inhibiting this signaling pathway. Regulating RhoA/ROCK1 signaling pathway could be a potential new target to treat pulmonary fibrosis.

Keywords: Pulmonary fibrosis, MRC-5 cells, RhoA/ROCK1 signaling pathway, proliferation, differentiation

Introduction

Acute respiratory distress syndrome (ARDS) partly caused by sepsis is one of the most challenging problems in intensive care unit (ICU) [1, 2]. ARDS triggers overlapping inflammation and subsequent pulmonary fibrosis which significantly contribute to morbidity and mortality [3-5]. However, the mechanism of ARDS-associated lung fibrosis is complex and remains unclear. Increasing evidences from experimental and clinical studies suggest the proliferation and differentiation in human lung fibroblast cells have important pathological significance in the development of ARDS [6-8]. It is vital to understand pathogenesis of pulmonary fibrosis, and provide guidance in choosing suitable treatment to mitigate lung fibrosis.

Ras homolog gene family member A (RhoA) is a small GTPase protein of Rho family and its action is dependent upon several other effectors. Serine/threonine kinase also called Rho-associated coiled-coil containing protein kinase 1 (ROCK1) is one of them to be involved in many cellular processes. The studies have shown that RhoA/ROCK1 signaling pathway has been implicated in numerous cell functions, such as proliferation, differentiation, apoptosis and the regulation of cytoskeletal protein synthesis, degradation, movement and contraction [9-11]. The development of pulmonary *fibrosis* is asso-

ciated with fibroblast proliferation and differentiation in lung fibroblasts [6-8]. But the role of RhoA/ROCK1 signaling pathway in fibrotic disease (i.e. fibrosis) via regulating fibroblast proliferation and differentiation has not been well-reported.

LPS, a component of Gram-negative bacterial cell walls, which can induce severe sepsis and ARDS *in vivo* so that LPS is widely use to make septic and ARDS animal model [12, 13]. The activation of RhoA/ROCK1 signaling pathway by LPS has been demonstrated in previous studies [13, 14].

Fasudil is a potent Rho-kinase inhibitor and vasodilator. It has been used for the treatment of cerebral vasospasm, pulmonary hypertension and age related or neurodegenerative memory loss. During recent years, Fasudil also has been demonstrated to improve renal interstitial fibrosis, cardiac fibrosis and pulmonary fibrosis due to its function of inhibiting Rho-kinase [15-18]. The regulation of RhoA/ROCK1 signaling pathway by Fasudil has been validated in animal model of pulmonary and renal interstitial fibrosis as well as in fibroblast cell line.

Based on these previous findings, in order to better understand how the cell signaling mechanisms control fibroblast proliferation and differentiation, we hypothesized that the regulation of MRC-5 cells proliferation and differentiation depends on the activation of RhoA/ROCK1 signaling pathway. In the current study we examined cell proliferation and differentiation as well as RhoA/ROCK1 activation and MYPT1 phosphorylation in MRC-5 cells followed by LPS stimulation. Fasudil was used to investigate effect of the deactivation of RhoA/ROCK1 and de-phosphorylation of MYPT1 on LPS-induced cell proliferation and differentiation. Our results provided evidence that MRC-5 cells proliferation and differentiation are associated with the regulation of RhoA/ROCK1 signaling pathway in fibroblasts.

Materials and methods

MRC-5 cells and reagents

MRC-5 cells and DMEM were purchased from Boster (USA). LPS was purchased from Sigma-Aldrich (USA). Fasudil was purchased from Tianjin Sun Pharmaceutical Co. Ltd (Tianjin, China). Cell-Light™ EdU Apollo®643 In Vitro Imaging Kit (100T) was purchased from Ribobio (Guangdong, China). Cell Counting Kit-8 was purchased from Dojindo Laboratories (Kumamoto, Japan). Agarose-conjugated Rhotekin Rho binding domain was purchased from Upstate (Lake Placid, NY, USA). Trizol reagent was purchased from Beyotime (USA). RIPA buffer was purchased from Santa Cruz (USA). BCA assay kit was purchased from Solarbio (China). All antibodies were provided by Abcam (England).

Cell culture and treatments

MRC-5 cells were grown in DMEM medium supplemented with low serum growth, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin, in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. Subcultures were performed with 0.25% trypsin and 0.01% EDTA when the cells reaching up to 80% confluence. All experiments were performed on the cells between passage two to five. Media were refreshed every other day.

MRC-5 cells were divided into four groups: control group, LPS group (LPS: 10 μg/mL), low-dose group (Fasudil: 15 μmol/mL + LPS: 10 μg/mL) and high-dose group (Fasudil: 30 μmol/mL + LPS: 10 μg/mL). First, the cells were treated with LPS (10 μg/ml) for 24 h. Then the cells were incubated with Fasudil for 2 h prior to LPS treatment for 24 h.

CCK-8 assay

The proliferation rate of MRC-5 cells was assessed using a Cell Counting Kit-8 (CCK-8) assay. The cells were cultured in 96-well plate (n=5) at a density of 5×10^3 cells per well and incubated overnight. The cells were incubated with LPS at indicated concentration (10 μg/ml), and assayed at different time points (0 h, 12 h, 24 h and 48 h) according to the manufacturer's instructions. Absorbance for CCK8 assay was read at 450 nm to measure MRC-5 cell proliferation.

5-ethynyl-20-deoxyuridine (EdU) assay

We measured the proliferation rate of MRC-5 cells by Cell-Light™ EdU Apollo®643 In Vitro Imaging Kit (100T). Cells were cultured in

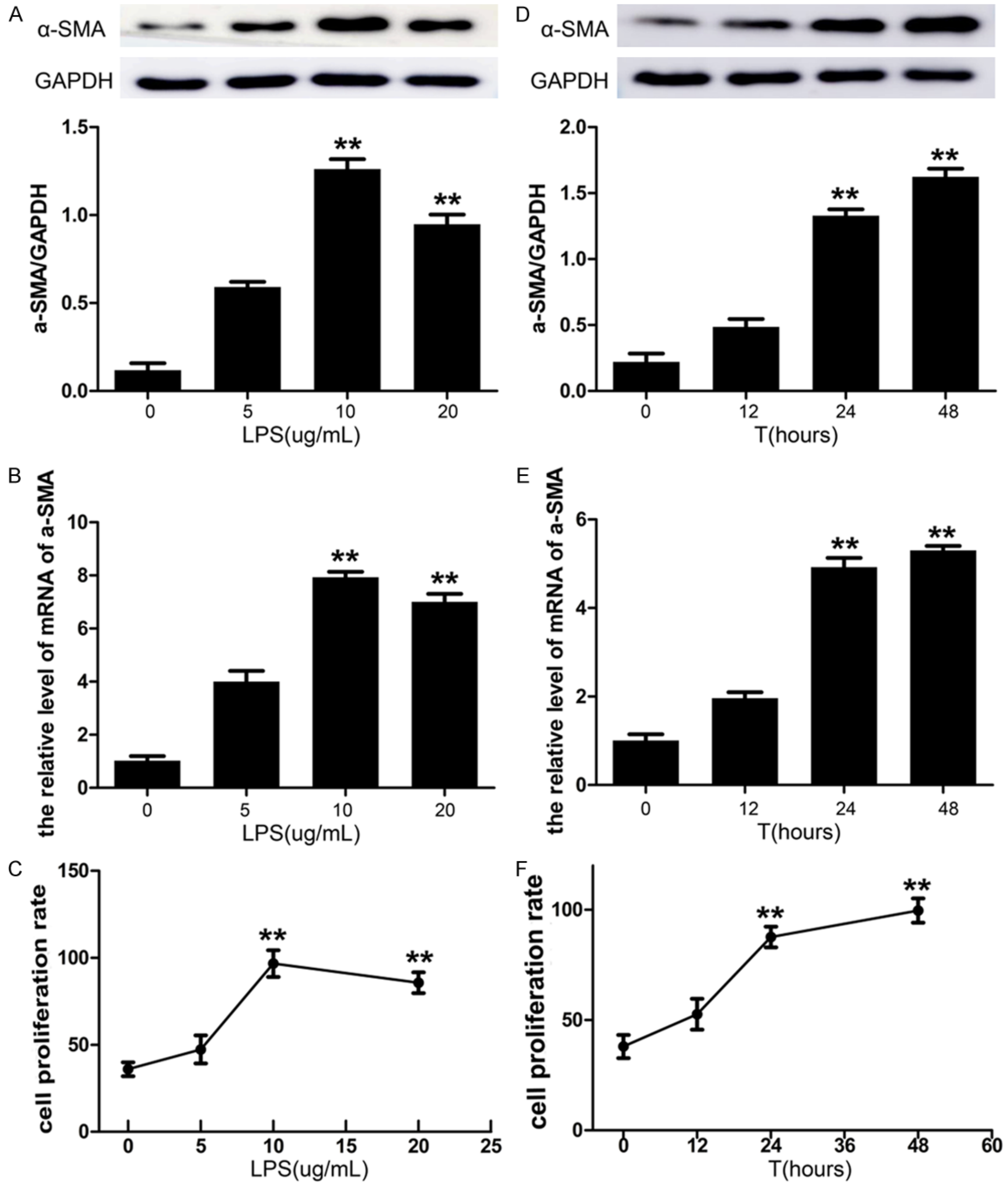

Figure 1. (A) The protein α-SMA of MRC-5 cells in different concentration of LPS. (B) Real-time quantitative RT-PCR of α-SMA mRNA level in different concentration of LPS. (C) MRC-5 cells proliferation in different concentration of LPS. (D) The protein α-SMA of MRC-5 cells at different processing time. (E) α-SMA mRNA level at different processing time. F. MRC-5 cells proliferation at different processing time. First, the cells were treated with increasing doses of LPS (0, 5, 10, 20 μg/ml) for 24 h. Then the cells were incubated with LPS (10 μg/ml) at different time points (0, 12, 24, 48 h). As shown in (A), (C) and (E), the levels of protein and mRNA of α-SMA, cell proliferation were significantly increased by LPS in the dose of 10 and 20 μg/ml (vs 0 μg/ml). LPS markedly increased α-SMA mRNA and protein levels, cell viability at time point of 24 and 48 h (vs 0 h) seen in (B), (D) and (F). Data were expressed as the mean ± SD. *P<0.05, and **P<0.01.

96-well plates at 5×10^3 cells per well and we divided cells into four groups: control group, LPS group, low-dose group, high-dose group. And each groups has 5 wells, then exposed to 50 μmol/L of EdU for additional 4 h at 37°C. The cells were fixed with 4% formaldehyde for

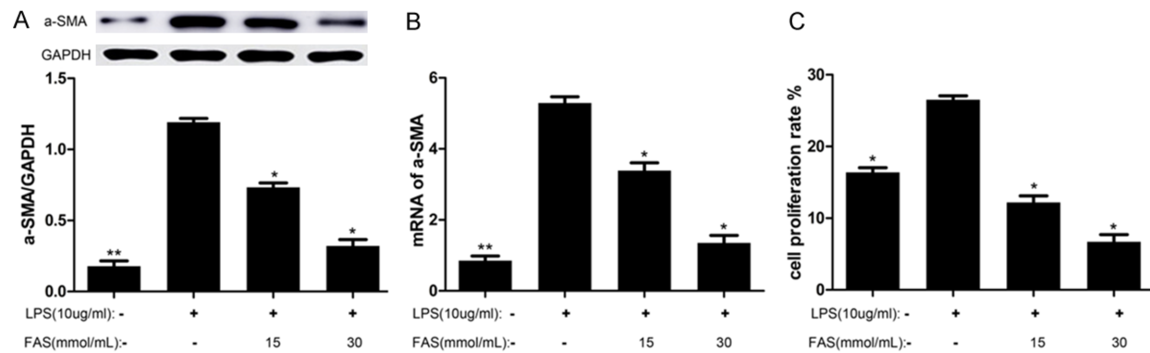

Figure 2. A. α-SMA was measured by Western blot. B. qrt-PCR was used to detect α-SMA mRNA level. C. CCK-8 was used to assess cells proliferation. MRC-5 cells were divided into four groups, control group, LPS group (LPS: 10 μg/mL), low-dose group (Fasudil: 15 μmol/mL + LPS: 10 μg/mL) and high-dose group (Fasudil: 30 μmol/mL + LPS: 10 μg/mL). Cells were treated in serum-free medium for 24 h. The levels of protein and mRNA of α-SMA, cell proliferation were significantly increased by LPS (vs control). A significant decreases in the protein and mRNA of α-SMA, cell proliferation were noted in LPS plus Fasudil group (vs LPS alone group). Data were expressed as the mean ± SD. *P<0.05, **P<0.01.

15 min and treated with 0.5% Triton X-100 for 20 min at room temperature. After washing with PBS (phosphate buffer saline, PBS) for three times, the cells of each well were reacted with 100 μL of 1 × Apollo® reaction cocktail for 30 min. Subsequently, the DNA contents of cells in each well were stained with 100 μL of Hoechst 33342 (5 μg/mL) for 30 min and visualized under a fluorescent microscope. Fluorescence images showed the nucleus in cells. The blue-fluorescent showed the total cell nucleus, and the green-fluorescent showed the hyperplastic nucleus.

Rho pull-down assay

Cells cultured in vitro for 24 h and 2 × 10^6 cells were lysed in MLB buffer (25 mmol/L HEPES, 150 mmol/L NaCl, 10 mmol/L MgCl2, 1 mmol/L EDTA, 2% glycerol, 1% Igepal CA-630, 2 μg/mL Aprotinin, 2 μg/mL Leupeptin and 1 mmol/L PMSF). Total proteins from clarified lysates were incubated with 20 μg Rho-binding domain of rhotekin-agarose slurry for 2 h at 4°C to precipitate GTP-bound RhoA (GTP-RhoA). Retained GTP-RhoA was subject to Western blotting using monoclonal anti-RhoA antibody.

Protein analysis by Western blotting

MRC-5 cells were harvested and lysed on ice in RIPA buffer. Protein concentrations were determined by BCA assay. 40 μg of protein were resolved on 8-12% Tris-HCl DS-polyacrylamide gels and blotted onto Immobilon PVDF for immunoblotting. The blots were incubated with 5% dry milk in TBST (TBS and 0.05% Tween 20) for 1 h at room temperature and then stained with α-SMA (1:3000); Anti-RhoA (1:2000); Anti-Active RhoA (1:2000); Anti-ROCK1 (1:2000); MYPT1 antibody (1:1000); Phospho-MYPT1 antibody (1:1000) and Anti-GAPDH (1:5000) overnight at 4°C. After incubation with primary antibodies, membranes were washed, incubated with secondary HRP-conjugated antibody (1:5000) for 1 h. Bands were stained using an enhanced chemiluminescence detection system (Syngene, USA) according to the manufacturer's instructions and recorded by exposure of the membrane to x-ray film. The membranes were stripped and probed with GAPDH antibody to verify equal protein loading. Band intensity was quantified by use of Image J software. Western blot results were reported as relative densitometry units (RDU) normalized to GAPDH.

mRNA analysis by real-time polymerase chain reaction (Real-time PCR)

The total cellular RNA was isolated from MRC-5 cells using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. After extraction, total RNA was converted into cDNA by reverse transcription reaction, and qrt-PCR was performed using the ABI 7500 Fast Real-Time PCR system (Applied Biosystems) with GAPDH as a reference control. The primers used for RT-PCR were following: α-SMA forward 5'-TGAGCGTGGCTATTCCTTCGT-3', and reverse, 5'-GCAGTGGCCATCTCATTTTCAA-3'; GAPDH for-

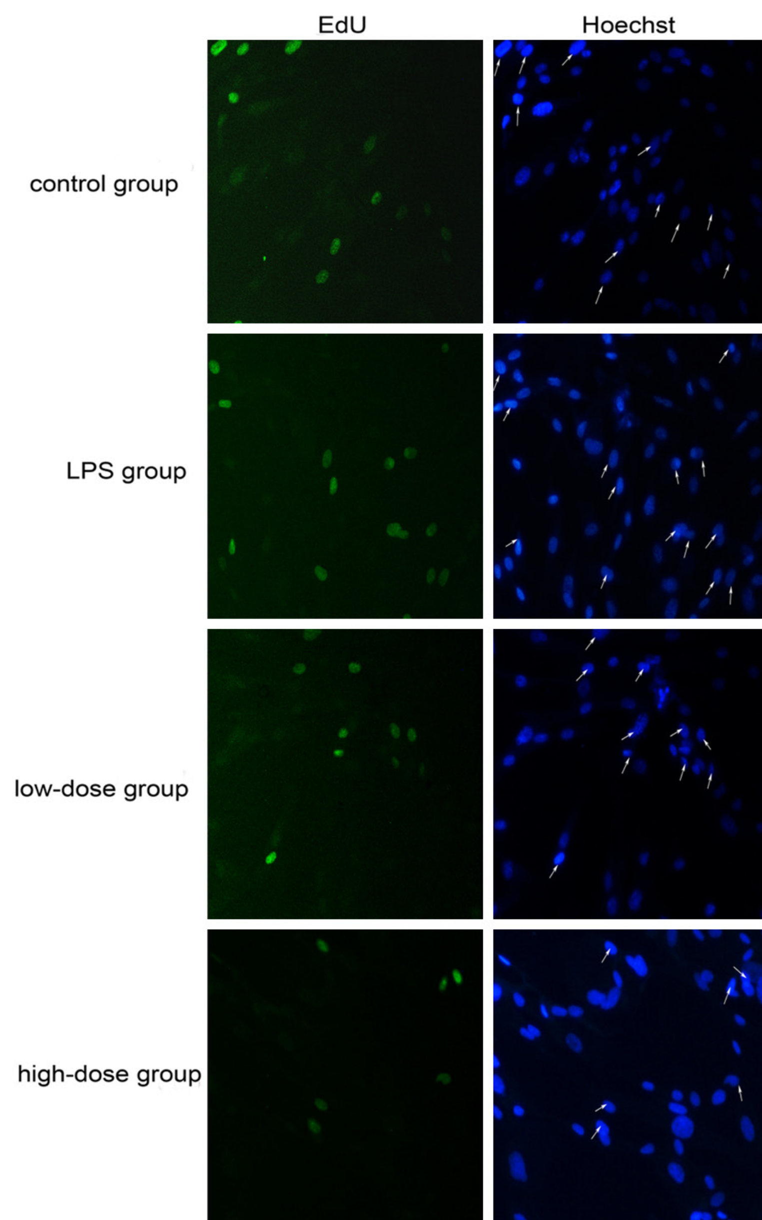

Figure 3. MRC-5 cells *nucleus* are imaged with EdU kit. Total cell *nuclei* are imaged with *blue fluorescence (Hoechst)* and hyperplastic nucleus are imaged with green *fluorescence (EdU) in the cells (*100 ×*).* We divided fibroblast MRC-5 cells into four groups, control group, LPS group (LPS: 10 μg/mL), low-dose group (Fasudil: 15 μmol/mL + LPS: 10 μg/mL) and high-dose group (Fasudil: 30 μmol/mL + LPS: 10 μg/mL). Cells were treated in serum-free medium for 24 h. The numbers of hyperplastic nucleus in LPS group were significantly higher in control group. Fasudil decreased the numbers of hyperplastic nucleus obviously (LPS alone group vs. LPS plus Fasudil group).

ward 5'-GGACCTGACCTGCCGTCTAG-3', and reverse, 5'-GTAGCCCAGGATGCCCTTGA-3'.

Statistical analyses

Data were expressed as mean ± SD (n≥3). Comparison of the means was performed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post testing using SPSS (version 17.0) and Prism5 (GraphPad) Software. P<0.05 was considered statistically significant.

Results

LPS induces MRC-5 cells differentiation and proliferation in dose-and time-dependent manner

Cell differentiation is the process where a cell changes from one cell type to another. α-SMA is commonly used as a marker of myofibroblast formation and differentiation. Cell proliferation is the process that results in an increase of the number of cells. CCK-8 was used to examine cell proliferation. Cell proliferation and the expression of α-SMA induced by LPS were investigated in this experiment. LPS resulted in profound elevations of the levels of α-SMA protein (**Figure 1A**) and mRNA (**Figure 1B**) as well as cell proliferation (**Figure 1C**) at different doses, with a maximal peak at 10 μg/mL dose (P<0.01). Similarly, LPS increased the levels of α-SMA protein (**Figure 1D**) and mRNA (**Figure 1E**), and cell proliferation (**Figure 1F**) over time. The rapid raise in the levels of α-SMA protein and mRNA were observed at 24 h. Gradually increased cell proliferation by LPS reached a plateau after 24 h of LPS challenge.

Fasudil attenuated LPS-induced MRC-5 cells differentiation and proliferation

We measured α-SMA expression to investigate cell differentiation and proliferation was assayed by CCK-8 and Cell-Light™ EdU Apollo®643 In Vitro Imaging Kit (EdU). Increased

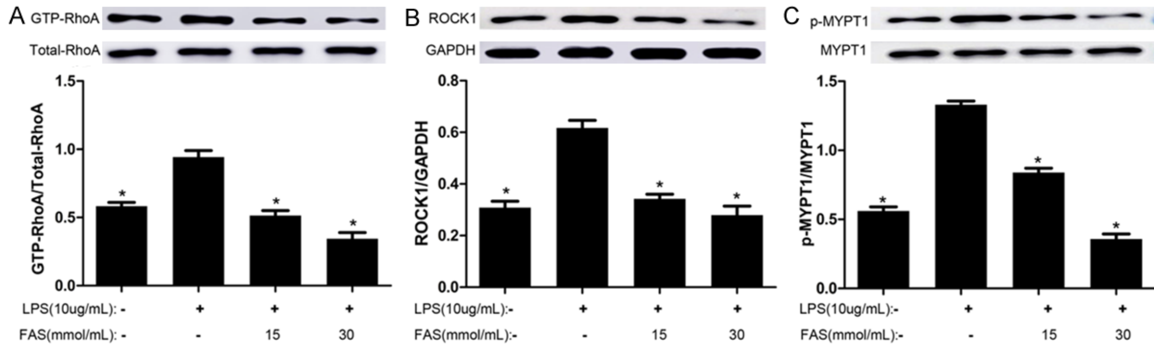

Figure 4. (A) The protein levels of GTP-RhoA from diverse groups. (B) The protein levels of ROCK1 from diverse groups. (C) The protein levels of p-MYPT1 from diverse groups. MRC-5 cells were divided into four groups, control group, LPS group (LPS: 10 μg/mL), low-dose group (Fasudil: 15 μmol/mL + LPS: 10 μg/mL) and high-dose group (Fasudil: 30 μmol/mL + LPS: 10 μg/mL). Cells were treated in serum-free medium for 24 h. LPS significantly increased GTP-RhoA (A), ROCK1 (B) and p-MYPT1 (C) (control group vs LPS group); Fasudil markedly decreased protein expressions of GTP-RhoA, ROCK1 and p-MYPT1 (low-dose group vs LPS group; high-dose group vs LPS group). Data were expressed as mean ± SD. *P<0.05, **P<0.01.

α-SMA protein (**Figure 2A**) and mRNA (**Figure 2B**) by LPS in MRC-5 cells were diminished with pretreatments of Fasudil 15 μmol/mL (vs LPS alone group, P<0.05) or 30 μmol/mL (vs LPS alone group P<0.01). LPS-induced proliferation in MRC-5 cells (**Figure 2C**) were reduced by pre-incubation with Fasudil (15 μmol/mL group vs LPS alone group, P<0.05; 30 μmol/mL group vs LPS alone group, P<0.01). The inhibitory role of Fasudil in LPS-induced proliferation were confirmed by EdU as well (**Figure 3**).

The role of activation/deactivation of RhoA/ROCK1 signaling pathway in cell differentiation and proliferation

We further determined whether the RhoA/ROCK1 signaling pathway is involved in the LPS-induced cell differentiation and proliferation. RhoA/ROCK1 expressions and phosphorylation of MYPT1 were determined in the regulation of differentiation and proliferation in MRC-5 cells. LPS resulted in a significant increase of the levels of GTP-RhoA (**Figure 4A**), ROCK1 (**Figure 4B**) and p-MYPT1 (**Figure 4C**). Up-regulations of GTP-RhoA, ROCK1 and p-MYPT1 by LPS were revealed in this experiment.

Fasudil markedly decreased the protein expressions of GTP-RhoA, ROCK1 and p-MYPT1 stimulated by LPS. The higher inhibitory effects of Fasudil were observed in high-dose group (30 μmol/mL). The data from this experiment indicated LPS activated RhoA/ROCK1 signaling pathway and phosphorylated MYPT1. Fasudil, a highly selective inhibitor of ROCK, significantly suppressed RhoA/ROCK1 activation and phosphorylation of MYPT1.

Discussion

Evidences from clinical and experimental studies show that ARDS begins as an acute inflammatory lung injury, which quickly progresses to a fibrosis phase [19, 20]. Fibrosis, which is characterized by the overgrowth in various tissues especially in lung, is attributed to excess deposition of extracellular matrix components including collagen. Previous studies have demonstrated that the myofibroblast, a main cellular mediator of fibrosis, serves as a primary collagen-producing cell. Fibroblasts can be activated by cytokines in inflammation. Further activated fibroblasts transform into α-SMA expressing myofibroblasts [10, 21, 22]. Fibroblast proliferation and transdifferentiation present in normal lungs into a myofibroblast-like cell type are regarded as an early step in the fibrotic process [23]. In current study, we use MRC-5 cells, a fibroblast-like cell line in morphology, to document for the first time that cell proliferation and differentiation which precedes development of fibrosis are regulated by RhoA/ROCK1 signaling pathway with RhoA/ROCK1 activation and MYPT1 phosphorylation *in vitro*.

LPS induces cell proliferation and differentiation in fibroblast *in vivo* and *in vitro* in many studies [13, 24]. Our data showed that LPS

induced cell proliferation confirmed by CCK-8 and EdU assay. Expression of α-SMA protein, a key indicator of myofibroblast differentiation in fibroblasts, was elevated by LPS. The results from our study are consistent with other studies [24, 25].

RhoA has two forms, which are inactive when bound to GDP, are activated following GTP binding, GTP-RhoA translocated to the plasma membrane, where it mediates downstream ROCK. Activated ROCK forms a complex with MYPT1 leading to ROCK-mediated phosphorylation and inhibition of myosin light-chain phosphatase (MLCP). Besides, formed complex prevents de-phosphorylation level of myosin light chain (MLC) and induces the reorganization of actin [9, 11, 26, 27]. And then fibroblast cells evolved toward myofibroblast cells. Some evidences have shown that cytokines and other factors can activate RhoA [28-30]. The role of epithelial to mesenchymal transition (EMT) in fibrotic response has been reported [31, 32]. Inhibiting Rho/Rock pathway in a rat model of chronic allograft nephropathy decreases renal interstitial fibrosis with reduced the level of α-SMA (It is also a marker of EMT) [32, 33]. In our study, LPS activated RhoA/ROCK1 signaling pathway by up-regulating GTP-RhoA and ROCK1 as well as increasing MYPT1 phosphorylation. Combining the results from LPS-induced cell proliferation and differentiation, we could conclude LPS-induced cell proliferation and differentiation is at least in part with the involvement of RhoA/ROCK1 signaling pathway in MRC-5 cells.

Further investigation is performed to confirm the role of RhoA/ROCK1 signaling pathway in mediating cell proliferation and differentiation. Fasudil, a highly selective inhibitor of ROCK, could suppress RhoA/ROCK1 signaling pathway. Some studies have shown that Fasudil has functions in anti-fibrosis and modulating inflammation [17, 18, 34]. But cellular mechanisms contributing to fibrosis are unexplored. Our findings revealed that Fasudil decreased LPS-induced activation of RhoA/ROCK1 and MYPT1 phosphorylation, consequently attenuated fibroblast cell proliferation and differentiation stimulated by LPS.

The alternations of proliferation and differentiation in fibroblasts are complexed and result from different factors. More importantly, a variety of pathways could be responsible for the changes of proliferation and differentiation. In this project, we used cell model to demonstrate proliferation and differentiation in MRC-5 cells with stimulation of LPS. The other stimuli and cell signaling pathways are unmentioned. In the future study, the possible pathogenic factors and signaling pathways will be investigated *in vitro*. Also animal model *in vivo* will be utilized to mimic fibrotic diseases.

In summary, we have demonstrated that the RhoA/ROCK1 signaling pathway is related to the proliferation and differentiation in MRC-5 cells. Our study provides evidence that regulations of RhoA/ROCK1 activation and de-phosphorylation of MYPT1 could be strategy to prevent/treat pulmonary fibrosis in the patients with ARDS.

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Disclosure of conflict of interest

None.

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