Original Article

Roles of Rho/Rock Signaling Pathway in Silica-induced Epithelial-mesenchymal Transition in Human Bronchial Epithelial Cells*

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Abstract

Objective To investigate the roles of Rho/Rock signaling pathway in silica-induced Epithelial-mesenchymal transition (EMT) in human bronchial epithelial cells (BEC) *in vitro*.

Methods Human BEC were incubated with silica with various concentrations for indicated times. Cell viability was assayed by MTT test. Morphologic Changes were observed by microscope. Mesenchymal marker α-smooth muscle actin (α-SMA), vimentin (Vim), and epithelial marker E-cadherin (E-cad) were analyzed by Western Blot. The pull-down assay was used to measure Rho activity. In the prevention experiments, the specific inhibitor for Rho effector ROCK (Y27632) was used to inhibit the activity of Rho.

Results Human BEC stimulated with silica were converted from a ''cobblestone'' epithelial structure into an elongated fibroblast-like shape structure. Incubation of human BEC with silica induced de novo expression of α-SMA and Vim, and loss of E-cad. Also, silica treatment resulted in Rho activation in human BEC. Y27632 up-regulated the E-cad expression but attenuated α-SMA and Vim expression in silica-stimulated cells.

Conclusion The activation of Rho/ROCK signaling pathways is most likely involved in Silica-induced EMT in human bronchial epithelial cells.

Key words: Silica; Bronchial epithelial cell; Epithelial-to-mesenchymal transition; Rho

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INTRODUCTION

The inhalation of silica particles can result in the development of an inflammatory response and fibrosis in lungs and, the primary response of pulmonary epithelium is believed to play the key role in the onset and the development of crystalline silica-induced lung diseases, along with those of the alveolar macrophages. Airway epithelium serves not only as barriers preventing the egress of particles into the interstitial space, but also as effectors of mineral silica[1-3]. It is well known that bronchial epithelium enhances pulmonary host defense mechanisms by producing inflammatory factors. Fibrosis was used to be believed to result primarily from chronic inflammation; however, it is now clear that inflammation and chronic fibrosis are often dissociated. Inflammation is neither a necessary condition nor a sufficient condition to induce fibrosis[4].

Recently, there are growing evidences that a

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process called "epithelial– mesenchymal transition" (EMT) plays a prominent role in fibrogenesis in lungs, kidneys and liver[5-7]. In EMT, fully differentiated epithelial cells undergo transition to a mesenchymal phenotype and give rise to fibroblasts and myofibroblasts. This is increasingly recognized as the source of fibrotic extracellular matrix (ECM)[8]. EMT is characterized by loss of epithelial markers E-cad, cytoskeletal reorganization, and transition to a spindle-shaped morphology concomitant with acquisition of mesenchymal markers α-SMA and Vim. Although bronchial epithelial cells have been showed to undergo EMT in bleomycin-induced pulmonary fibrosis and asthma[9-10], much is still remained unknown regarding silica-induced EMT in silicosis.

The signaling pathways involved in EMT are very important fields for research in fibrotic diseases. The small GTPase (RhoA) controls cell adhesion and its motility via actin-cytoskeleton reorganization and actin-myosin filament bundles regulation. ROCK (Rho-associated coiled-coil-forming protein kinase) is downstream target protein of Rho. Activation of Rho is a key step in EMT-associated renal, lens and liver fibrosis[11-13]. Based on these findings, we hypothesize that Rho/Rock signaling pathway regulate, most likely, the EMT in silica-treated human bronchial epithelial cells.

MATERIALS AND METHODS

Preparation of Crystalline Silica

Crystalline silica (Sigma, USA, 0.1-10 μm in diameter) was washed with HCl in order to remove the contamination of Fe_2O_3 from it. Briefly, silica was boiled in 1 mol/L HCl, washed, and dried in an oven at 110 °C for 90 min and the particles were then sterilized by being heated at 180 °C for 6 h[14]. Before use, the particles were suspended with 1 mL of sterile saline and sonicated for 10 min.

Cell Culture and Reagents

An human bronchial epithelial cells (BEC) line was kindly provided by Professor Gruenernt (San Francisco Branch Campus, the University of California). This cell line was cultured in RPMI medium 1640 (Gibco Biocult, Paisley, UK) containing 10% fetal bovine serum and insulin at 37 °C in a humidified 5% CO_2 atmosphere. At 80% confluence, cells were switched to free- serum medium for 24 h prior to addition of silica for indicated times. The specific inhibitor for Rho effector ROCK (Y27632) was purchased from Calbiochem (LaJolla, CA).

MTT Assay

Cell viability was assayed by MTT test in this study. The cultured cells were exposed to silica (100 μg/mL, 200 μg/mL, 300 μg/mL, 400 μg/mL) for 72 h. At the end of the experimental period, MTT (5 mg/mL) was added to the wells (20 μL/well). After a 4 h incubation at 37 °C, media were removed and DMSO was added (150 μL/well). The plates were agitated at room temperature for 10 min. The value of absorbance of every well at 570-nm wavelength was then read on an enzyme-linked immunosorbent assay reader. The viable cells produced a dark blue formazan product, whereas no such staining was formed in the dead cells.

Western Blot Analysis

After being treated for the indicated times, the cultured cells were harvested and total protein was extracted. Equal amounts of cell proteins, quantified by BCA protein assay kit (Pierce Biotechnology, Rockford, IL), were resolved on 10% SDS-PAGE and were transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk for 2 h and then incubated with various antibodies for 18 h at room temperature. After incubation with the secondary antibody at room temperature for 2 h, immunoreactive bands were visualized by enhanced chemiluminescence reaction. The anti-E-cad and Vim antibodies were from Santa Cruz Biotechnology, Inc., California, USA; and anti-α-SMA antibody was from Novocastra, Newcastle, UK.

Rho Activation Assay

The pull-down assay for measuring Rho activity was performed by using a Rho activation assay kit, according to the manufacturer's protocol (Upstate, Massachusetts, USA). A protein assay was performed prior to the pull-down assay in order to equalize total protein concentration in each treatment group. β-actin protein was used as the internal control. Whole cell lysates were incubated with agarose-conjugated Rhotekin- RBD for 60 min at 4 °C and then washed once with wash buffer. Agarose beads were boiled in SDS-PAGE sample buffer to release active Rho prior to undergoing precipitation with the Rhotekin GTP-Rho binding domain. After precipitation, samples were processed for Western blotting with a specific anti-Rho antibody.

Statistical Method

All the experiments were performed three times and all the data were shown as means with the standard error. Statistical comparisons were conducted through analysis of variance (ANOVA), and values of $P<0.05$ were considered significant.

RESULTS

Effects of Silica on the Viability and Morphology of Human BEC

MTT assay was conducted in order to ensure that the doses used in these experiments had no effect on cell viability. The human BEC was treated with 0 to 400 μg/mL silica for 72 h. The values of absorbance derived from control or silica-exposed cells were compared. The data in Figure 1A showed that exposure to 100 to 300 μg/mL of silica did not cause a reduction in cell viability. Human BEC cultured in the absence of silica maintained a classic cobblestone epithelial morphology and growth pattern (Figure 1B), but after the exposure to 100-300 μg/mL for 72 h, the cells assumed an elongated shape and many cells lost contact with their neighbor and displayed a spindle-shape, fibroblast-like morphology (Figure 1C-1E).

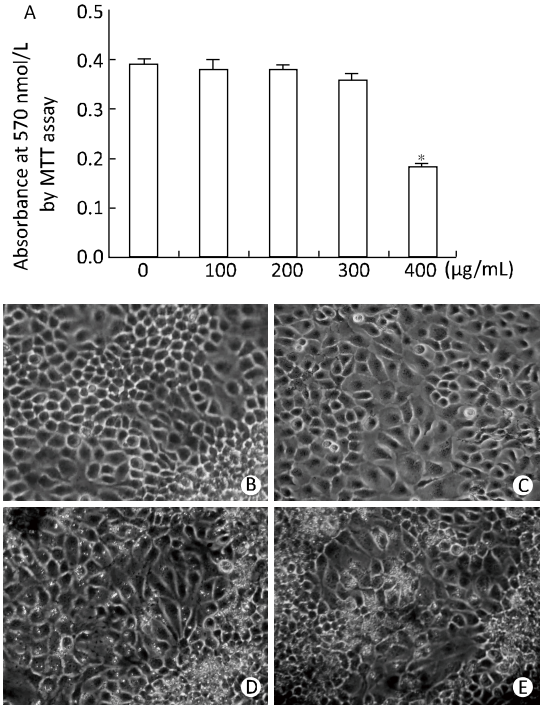


Figure 1. Effects of silica on viability and morphology in human BEC. (A) Cells were treated with silica (0-400 μg/mL) for 72 h. Cell viability was assayed by MTT test**.** Data are represented as mean±SD of three separate experiments with triplicate values for each silica concentration. $^{*}P<0.05$, compared with unexposed group. (B-E) Morphologic changes caused by silica in human BEC. Cells were incubated respectively with 0 (B), 100 (C), 200 (D), 300 (E) μg/mL silica for 72 h (magnification 200×).

Effects of Silica on the Expression of E-cad, α-SMA, and Vim of Human BEC

In order to investigate the EMT in silica-treated Human BEC, the expression of epithelial marker E-cad, mesenchymal marker α-SMA and Vim were examined in this study. Cells were incubated with silica with various concentrations (50-300 μg/mL) for 72 h. As shown in Figure 2, silica caused a significant concentration-dependent reduction of E-cad from human BEC. Meanwhile, silica induced α-SMA and Vim expression and the maximal induction was observed at the dose of 200 μg/mL.

Effects of Silica on Rho Activity of Human BEC

In order to explore the Rho activity in silica-stimulated Human BEC, we used a pull-down assay with the fusion protein GST-RBD, which specifically recognizes the Rho-GTP, the active form of Rho. And a 200 μg/mL silica exposure caused a noticeable increase in the amount of GTP-Rho. The effect was visible after 1 h, peaked around the 6th h, and persisted to the 24th h (Figure 3).

Effects of Rho/Rock Inhibitor on Silica-induced EMT of Human BEC

As it is necessary to determine whether there is a causal relationship between the observed Rho activation and the subsequent change in EMT, the effect of inhibition of Rho activation by using Y27632, a selective inhibitor of the Rho effector ROCK, on cell morphology and the protein expression (E-cad, α-SMA and Vim) was investigated. And it was found that Y27632 blocked silica-induced EMT. As shown by Figure 4 (A-D), Y27632 (20, 30 μmol/L) inhibited silica-induced morphologic changes. And when pretreated with Y27632 (20, 30 μmol/L), many silica-stimulated cells lost fibroblast-like morphology. Moreover, Y27632 (20, 30 μmol/L) up-regulated the E-cad expression, and attenuated α-SMA and Vim expression (Figure 4E-4J).

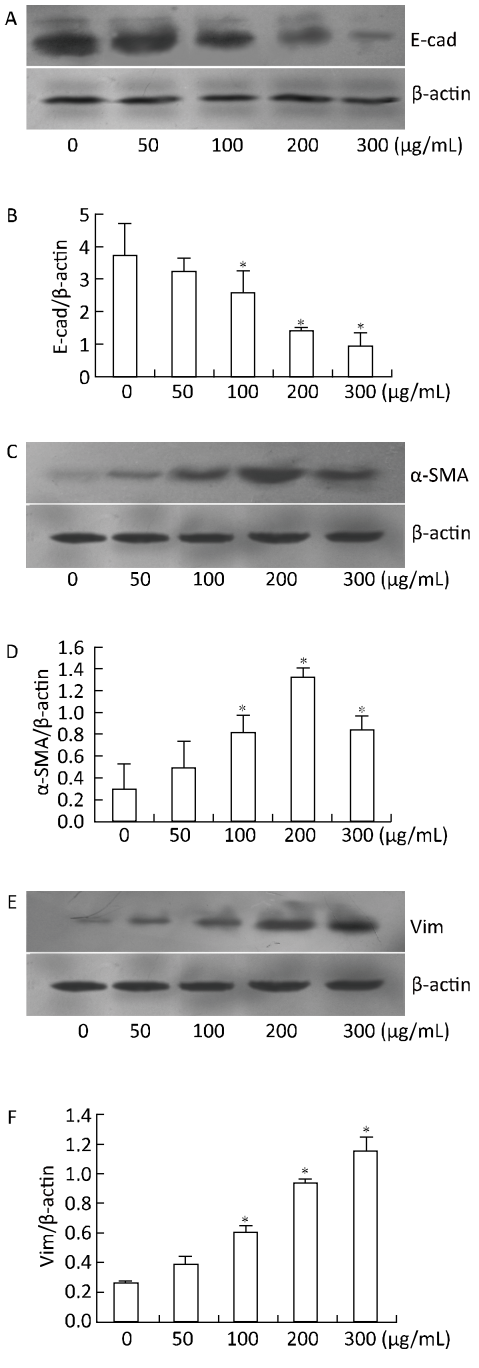


Figure 2. Effects of silica on the expression of E-cad, α-SMA and Vim in human BEC. (A, C, E) Cells were treated with 0, 50, 100, 200, 300 μg/mL silica for 72 h. E-cad, α-SMA and Vim protein expression was then determined by western blotting. (B, D, F) The mean±SD of values were quantified by densitometric analysis of three individual experiments. *: $P<0.05$, compared with unexposed group.

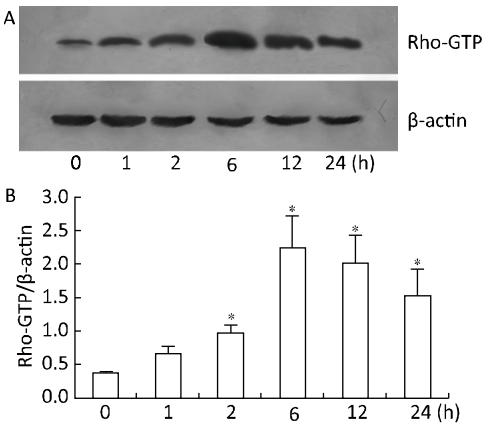
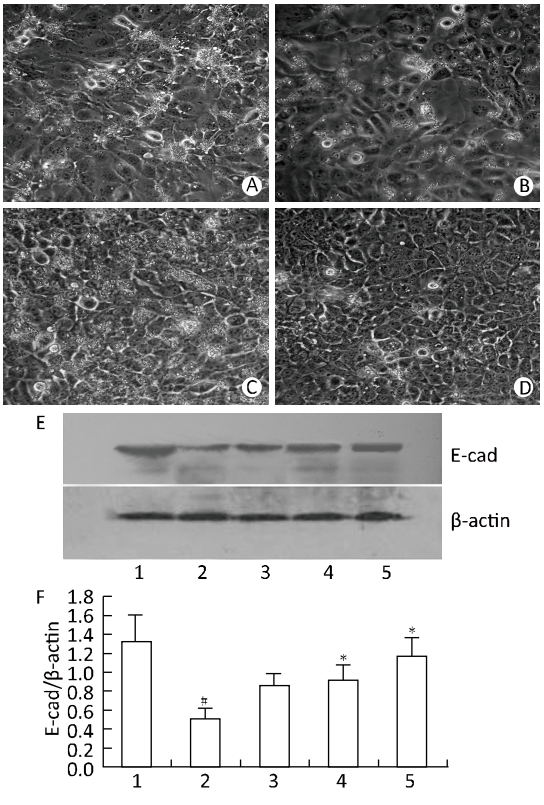


Figure 3. Effects of silica on Rho activity in human BEC. (A) Cells were stimulated by 200 μg/mL silica for 0, 1, 2, 6, 12, and 24 h respectively. The cellular proteins were then extracted and the pull-down assay was performed as described above in Materials and methods section**.** Bound Rho proteins (the active form of Rho) and the β-actin were detected by Western blotting. (B) The mean±SD of values were quantified by densitometric analysis of three individual experiments. *$P<0.05$, compared with unexposed group.



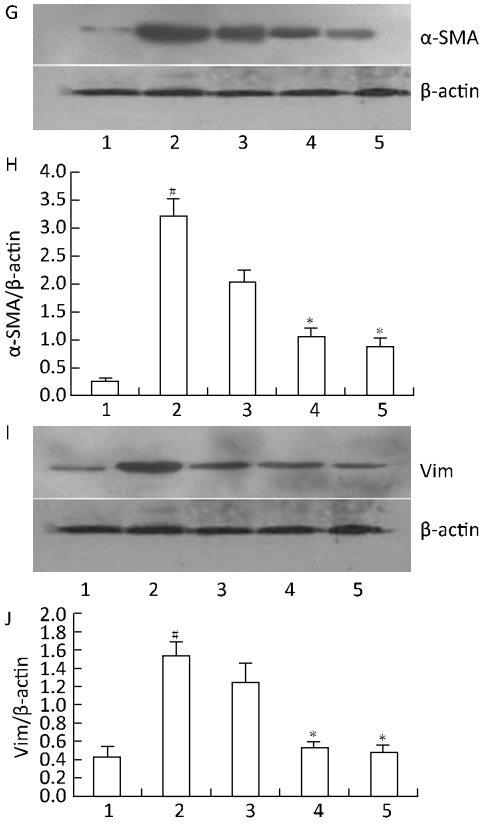


Figure 4. Effects of Rho/Rock inhibitor on silica-induced morphologic changes and EMT in human BEC. Cells were pre-incubated with Y27632 (0, 10, 20, 30 μmol/L) for 4 h and then stimulated with or without silica (200 μg/mL) for 72 h. (A-D): A silica, B silica +10 μmol/L Y27632, C silica +20 μmol/L Y27632, D silica +30 μmol/L Y27632. (E-J): Lane 1 without Y27632 and silica, Lane 2 silica, Lane3 silica +10 μmol/L Y27632, Lane4 silica +20 μmol/L Y27632, Lane5 silica +30 μmol/L Y27632. E-cad (E), α-SMA (G) and Vim (I) protein expression was determined by western blotting. (F, H, J) The mean±SD of values were quantified by densitometric analysis of three individual experiments. $^{*}P<0.05$, compared with Lane 2. $^{\#}P<0.05$, compared with Lane 1.

DISCUSSION

Myofibroblast cell was suggested to play a critical role in the development of pulmonary fibrosis[4]. And the EMT may be a major source of pathogenic mesenchymal cell types, such as myofibroblasts, during renal and lung fibrosis[4,15]. Under the TGF-β_1 treatment, it was shown that lung epithelial cells undergo EMT to produce myofibroblasts *in vitro*[16]. And for silica as a profibrotic particle, whether it induces EMT in lungs remains unknown.

E-cadherin is an epithelial cell transmembrane protein, and its loss is strongly associated with EMT[17]. The expression of α-SMA and Vim can be the marker of myofibroblasts, a cell type that represents an advanced phase of EMT[18]. Human BEC are key effector cells activated by silica. In the present study, we found that exposure of human BEC to silica resulted in the decreased expression of E-cad, with the concurrent transition to the myofibroblastic morphology and the increased expression of α-SMA and Vim in a dose-dependent manner. And these data suggest that silica could induce EMT in lung epithelial cells, which may contribute to the pathogenesis of silicosis. As it was shown in our present study, 100 to 300 μg/mL of silica did not cause a reduction in cell viability and 200 μg/mL silica-stimulated human BEC was therefore used as an EMT model.

When the EMT model was established, we started to identify key signaling events underlying silica-induced EMT. We considered the small GTPase Rho as a candidate to mediate the above mentioned effects of silica since Rho GTPases act as molecular switches directing upstream signals to a plethora of downstream effector pathways that regulate many fundamental cellular states such as the organization of the actin cytoskeleton, cell motility and vascular smooth muscle tone[19]. In addition, findings from some other studies showed that Rho activation was detectable in lung epithelial cells exposed to various stimuli (thrombin, mechanical stretch and integrin)[20-22]. In consistent with those reported and mentioned above, we found in our study that Rho activity increased significantly in silica-stimulated human BEC. And the effect preceded EMT changes. Preliminary experiments demonstrated that changes in the E-cad, α-SMA and Vim expression were not observed in silica-stimulated cells until 72 h (data not shown). It therefore provided a clue that Rho may regulate silica-induced EMT in human BEC.

We examined accordingly the silica-induced EMT changes by preventing the Rho activity. It is believed that Y-27632 (cyclohexane carboxamide dihydrochloride, monohydrate) is a highly selective inhibitor of Rho activation both in vitro and *in vivo*[23]. Y-27632 significantly blocked the expression of α-SMA in TGF-β1 treated smooth muscle cells[24]. In addition, Y-27632 increased the expression of E-cad in cancerous esophageal squamous cells[25]. It was

identified in ous study that Y-27632 inhibited silica-induced morphologic changes in human BEC while it attenuated silica-induced loss of E-cad also in human BEC. Furthermore, Y-27632 decreased silica-induced expression of α-SMA and Vim. These data serve as direct evidence that Rho may regulate silica-induced EMT in human BEC. It was reported that Y-27632 could alleviate pulmonary fibrosis induced by bleomycin in rat[24]. Moreover, the RhoA pathway may be a potential therapeutic target for the treatment of peritoneal fibrosis[26]. The antifibrotic effect of Y-27632 may be associated with the prevention of EMT through inhibition of Rho activity.

To our knowledge, this is the first study demonstrating that Rho signal pathway plays an important role in silica-induced EMT in human lung epithelial cells and this finding provides new and important clues in the understanding of molecular mechanisms involved in silica-induced fibrosis and also the possible prevention of it.

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