RhoA regulates lipopolysaccharide-induced lung cell injury via the Wnt/β-catenin pathway

GUANHUA CHEN[1], XUEDONG SUN[1] and CHUNXIAO DONG[2]

Departments of [1]Emergency and [2]Pediatrics, Central Hospital of Shengli Oil Field of Shandong, Dongying, Shandong 257000, P.R. China

Received September 28, 2016; Accepted July 17, 2017

DOI: 10.3892/mmr.2017.7662

Abstract. Ras homolog family member A (RhoA) has been reported to be involved in numerous biological processes; however, the effects of RhoA on acute lung injury (ALI) have yet to be reported. The present study aimed to explore how RhoA affects cell viability, reactive oxygen species (ROS) activity and cell apoptosis in a cell model of lipopolysaccharide (LPS)-induced ALI. An MTT assay, flow cytometry, reverse transcription-quantitative polymerase chain reaction and western blotting were used to determine the effects of RhoA on cell viability, apoptosis and ROS activity. The results demonstrated that RhoA inactivation was able to promote cell viability, and decrease apoptosis and ROS activity of LPS-treated cells. The results of western blotting indicated that RhoA activated the downstream Wnt/β-catenin signaling pathway and inhibited the expression of apoptotic factors. These findings suggested that RhoA may be involved in ALI progression and could be a novel therapeutic target for this disease.

Introduction

Acute lung injury (ALI) is a common clinical complication of acute respiratory distress, non-cardiac pulmonary edema, excessive inflammation and surfactant dysfunction (1-4). ALI is associated with severe trauma and infection, as well as a high mortality rate (5,6). Until now, the majority of studies have focused on anti-inflammatory ALI treatment. Ji *et al* (7) indicated that the Chinese herbal preparation Xuebijing may protect against left ventricular ischemia/reperfusion-induced ALI by regulating the expression of the inflammatory mediators tumor necrosis factor-α and intercellular adhesion molecule-1. Although progress has been made in ALI research, the mechanisms underlying ALI remain to be elucidated and the development of more effective therapies is required.

Lipopolysaccharide (LPS) is a type of endotoxin, which leads to multiple organ damage through excessive stimulation of the host's immune cells, resulting in the release of cytokines (8,9). LPS is often used in lung cell studies to generate a model of lung cell damage, in order to examine the mechanisms of lung damage at a cellular level (10).

Ras homolog family member A (RhoA), which is an important member of the Ras family, is a small GTPase protein that regulates the structure of the actin cytoskeleton and is also involved in the regulation of cell division (11,12). RhoA has also been reported to participate in proliferation, differentiation, migration and polarity of epithelial cells, and is essential to the invasion and aggressiveness of numerous types of cancer (13-15). As a small GTPase, RhoA participates in numerous biological processes, where it serves essential roles. It has been reported that partially hydrolyzed guar gum promotes colonic epithelial cell wound healing via activation of RhoA in inflammatory bowel disease (15). Notably, the anti-hypertrophic effects of adiponectin on the vasculature have been revealed to be associated with inhibition of the RhoA/Rho-associated protein kinase pathway and reactive oxygen species (ROS) formation (16). However, the role of RhoA in the progression of ALI is poorly characterized and comprehensive studies regarding the underlying mechanism are currently lacking.

The present study demonstrated that in an LPS-induced cell model of ALI, the expression levels of RhoA were significantly increased. It was hypothesized that RhoA may be involved in ALI progression; therefore, the present study preliminarily explored its role in ALI. A cell model of LPS-induced ALI demonstrated that reduced RhoA activity significantly increased cell viability, and inhibited apoptosis and ROS activity. In addition, RhoA was revealed to activate Wnt3a/β-catenin activity, and enhance the activity of apoptotic factors. These results indicated that the loss of RhoA may significantly inhibit the progression of ALI through the activation of canonical Wnt3a/β-catenin signaling.

Materials and methods

Cell culture and LPS treatment. The A549 cell line was purchased from the American Type Culture Collection

Correspondence to: Dr Xuedong Sun, Department of Emergency, Central Hospital of Shengli Oil Field of Shandong, 31 Jinan Road, Dongying, Shandong 257000, P.R. China
E-mail: sunxuedong391@126.com

Key words: acute lung injury, lipopolysaccharide, Ras homolog family member A, Wnt/β-catenin

(Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences; Logan, UT, USA), 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were cultured until they reached 80% confluence, following which the medium was replaced with serum-free DMEM and the cells were incubated for a further 15 h at 37˚C in a humidified atmosphere containing 5% CO_2. The cells were then stimulated with 10 μg/ml of *Escherichia coli*-derived LPS (O26:B6; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 6 h in a humidified atmosphere containing 5% CO2 at 37˚C (10).

Small interfering (si)RNA transfection. The RhoA-specific siRNA (si-RhoA) (sense: CCUUAUAGUUACUGUGUAATT; antisense: UUACACAGUAACUAUAAGGTA) and the negative control (si-NC) (sense: UUAUCGCCAAAUUCUUUU AUCGGACAGAG; antisense: UUGAUAAAAGAAUUU GGCGAUGGACAGAG), which had no silencing effect, were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells were seeded into 100 mm dishes at a concentration of 10^6 cells/dish and incubated at 37˚C in a humidified atmosphere containing 5% CO_2 for 24 h. Then, 25 mg/well of a RhoA specific or a negative control siRNA was diluted into Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and added to cells. After approximately 2 weeks, stable RhoA transfections were generated under G418 (Gibco; Thermo Fisher Scientific, Inc.) selection as described (17).

MTT assay. Cell proliferation was measured using the MTT colorimetric assay. In brief, non-infected cells and cells transfected with si-RhoA or si-NC were seeded in 96-well plates at a concentration of 10^4 cells/well in a humidified atmosphere containing 5% CO_2. Cells were incubated in normal condition for 1, 2, 3 and 4 days prior to being treated with LPS for 6 h. Treated cells were washed twice with PBS and added with MTT (5 mg/ml) (Sigma-Aldrich; Merck KGaA) in the dark and incubated at 37˚C for another 3 h. Then, 100 μl DMSO was added to dissolve the formazan crystals. The optical density of each well was measured at a wavelength of 570 nm on a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

ROS assay. ROS activity was measured by flow cytometry using 2',7'-dichlorofluorescein diacetate (DCFH-DA; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The cells were seeded into a 6-well plate, at a concentration of 10^5 cells/ml for 24 h and were then exposed to LPS for 6 h. The treated cells were washed twice with PBS and were incubated in serum-free culture medium containing 10 μM DCFH-DA for 20 min at 37˚C in the dark. The cells were then washed with PBS, and collected using the trypsin digestion method. All samples were centrifuged at 800 x g for 5 min at 4˚C and the supernatants were removed. The cells were resuspended in 500 μl PBS and fluorescent intensities were measured using a flow cytometer.

Flow cytometry. An apoptosis analysis was performed by flow cytometry using the Annexin V-fluorescein isothiocyanate/propidium iodide (PI) apoptosis detection kit (Beijing Biosea Biotechnology Co., Ltd., Beijing, China). The treated A549 cells were washed twice with cold PBS and resuspended in 1X binding buffer. The adherent and floating cells were combined and were treated according to the manufacturer's protocol. Cells were then measured using a flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) to differentiate apoptotic cells (Annexin-V^+ and PI^-) from necrotic cells (Annexin-V^+ and PI^+).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from transfected cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was treated with DNase I (Promega Corporation, Madison, WI, USA). RT was performed using the Multiscribe RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and random hexamers or oligo(dT). The RT conditions were as follows: 10 min at 25˚C, 30 min at 48˚C and a final step at 95˚C for 5 min (18). The expression of RhoA, Wnt3a, β-catenin, Wnt5a, Bcl-2, pro-caspase-3 and cleaved caspase-3 were conducted by qRT-PCR using the SYBR Green Master Mix (Takara, Dalian, China) according to manufacturer's protocols. The PCR primers were obtained from Invitrogen (Thermo Fisher Scientific, Inc.), and the reaction conditions included the following steps: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The sequences of the primers used for qPCR were as follows: RhoA (forward: 5'-CTCATAGTCTTCAGCAAGGACCAGTT-3', reverse: 5'-ATCATTCCGAAGATCCTTCTTATT-3'), Wnt3a (forward: 5'-GCAGTTGCGAAGTGAAGACC-3', reverse: 5'-TGTGGG CACCTTGAAGTATGT-3'), β-catenin (forward: 5'-GAT TAACTATCAGGATGACGCG-3', reverse: 5'-TCCATCCCT TCCTGCTTAGTC-3'), Bcl-2 (forward: 5'-GAGTGGGAT GCGGGAGATGTG-3', reverse: 5'-AGCGGCGGGAGA AGTCGTC-3'), Wnt5a (forward: 5'-CGAAGACAGGCA TCAAAGAA-3', reverse: 5'-GCAAAGCGGTAGCCATAG TC-3'), Pro-caspase-3 (forward: 5'-CTGGACTGTGGCATT GAG-3', reverse: 5'-GCTTGTCGGCATACTGTT-3'), Cleaved caspase-3 (forward: 5'-TGGTTCATCCAGTCGCTTTG-3', reverse: 5'-ATTCTGTTGCCACCTTTCGG-3') and GAPDH (forward 5'-GCACCGTCAAGGCTGAGAAC-3', reverse: 5'-TGGTGAAGACGCCAGTGGA-3'). The mRNA of GAPDH level was used as an internal control and relative expression changes were calculated using the $2^{-\Delta\Delta Cq}$ method (19).

Western blot analysis. Protein was extracted using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche Diagnostics, Shanghai, China), and was quantified using the Bicinchoninic Acid Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Electrophoresis was conducted using a Bio-Rad Bis-Tris Gel system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. Denatured protein (30 μg) was loaded onto 15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), electrophoresed, and then transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% BSA for 2 h at room temperature. RhoA antibody (sc-179) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and GAPDH antibody (G8795) was purchased from Sigma-Aldrich;

Merck KGaA. Other primary antibodies were: anti-Wnt3a (2721), anti-β-catenin (8480), anti-Cleaved caspase 3 (9662), anti-Wnt5a (2530) and anti-Bcl-2 (4223) purchased from Cell Signaling Technology (Danvers, MA, USA). The primary antibodies were prepared in 5% BSA at a dilution of 1:1,000. The polyvinylidene fluoride membranes were incubated with the primary antibodies at 4˚C overnight, followed by washing with TBST (0.1% Tween) and incubation with a goat anti-rabbit IgG secondary antibody HRP conjugated (sc-2004; 1:5,000; Santa Cruz Biotechnology, Inc.) or a goat anti-mouse IgG secondary antibody HRP conjugated (sc-2005; 1:5,000; Santa Cruz Biotechnology, Inc.) in 5% BSA for 1 h at room temperature. Following rinsing, the blots were transferred to a Bio-Rad ChemiDoc™ XRS system (Bio-Rad Laboratories, Inc.) and 200 μl Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore) was added to cover the membrane surface. The signals were captured and analyzed using Image Lab™ software version 4.0 (Bio-Rad Laboratories, Inc.).

Statistical analysis. The results of multiple experiments are presented as the mean ± standard deviation of three independent determinations. Statistical analyses were performed using SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA). P-values were calculated using a one-way analysis of variance followed by Tukey's multiple comparison tests. P<0.05 was considered to indicate a statistically significant difference.

Results

RhoA is upregulated by LPS treatment in A549 cells. A549 cells were treated with LPS to stimulate inflammatory injury. As shown in Fig. 1A and B, LPS increased the mRNA and protein expression levels of RhoA.

si-RhoA successfully inhibits RhoA expression. The expression of RhoA in A549 cells was knocked-down by siRNA. The results of RT-qPCR and western blotting confirmed that transfection with si-RhoA significantly decreased RhoA expression (P<0.05; Fig. 2A and B).

RhoA knockdown protects cells against LPS injury. The MTT assay was used to determine the effects of RhoA on cell viability. The experimental group was treated with LPS and transfected with si-RhoA. LPS decreased cell viability; however, A549 cells treated with LPS and si-RhoA exhibited increased cell viability compared with the LPS + siNC group (Fig. 3).

RhoA knockdown prevents LPS-induced ROS increase. The present study also aimed to determine the role of RhoA in ROS activity. A549 cells were treated with LPS and were then transfected with si-RhoA. Compared with in the control group, ROS activity was significantly increased (P<0.01) in cells exposed to LPS. However, ROS activity was decreased following si-RhoA transfection. These findings suggested that RhoA knockdown may prevent LPS-induced increases in ROS activity (Fig. 4).

RhoA knockdown inhibits LPS-induced cell apoptosis. The effects of RhoA knockdown on cell apoptosis were determined by flow cytometry. The results demonstrated that LPS significantly

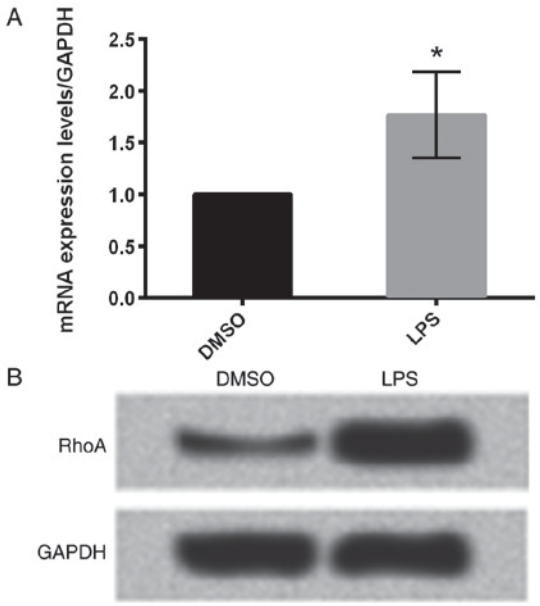

Figure 1. Expression of RhoA in LPS-treated cells. The expression levels of RhoA were analyzed by (A) reverse transcription-quantitative polymerase chain reaction and (B) western blotting. Data are presented as the mean ± standard deviation. *P<0.05 vs. the DMSO group. DMSO, dimethyl sulfoxide; LPS, lipopolysaccharide; RhoA, Ras homolog family member A.

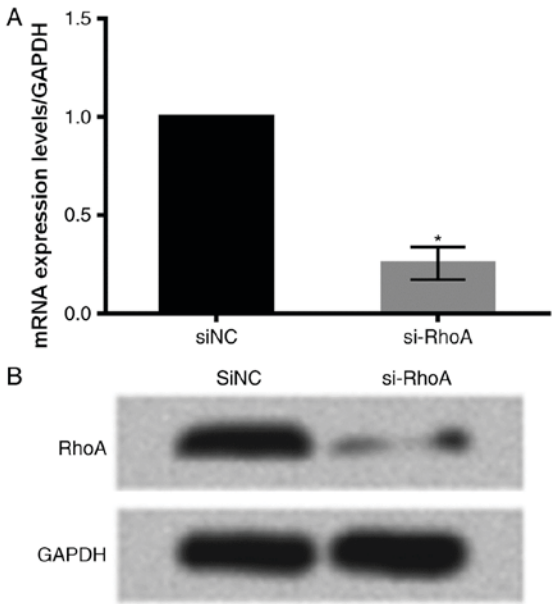

Figure 2. si-RhoA was used to knockdown RhoA expression. The expression levels of RhoA were detected by (A) reverse transcription-quantitative polymerase chain reaction and (B) western blotting. Data are presented as the mean ± standard deviation. *P<0.05 vs. the siNC group. NC, negative control; RhoA, Ras homolog family member A; si, small interfering RNA.

increased cell apoptosis (P<0.01), whereas RhoA knockdown protected cells from LPS-induced apoptosis (Fig. 5). Therefore, it may be hypothesized that inhibition of RhoA protects A549 cells from cell injury by inhibiting apoptosis.

RhoA activates the downstream Wnt/β-catenin signaling pathway and promotes cell apoptosis. As presented in Fig. 6, the mRNA and protein expression levels of Wnt3a, β-catenin,

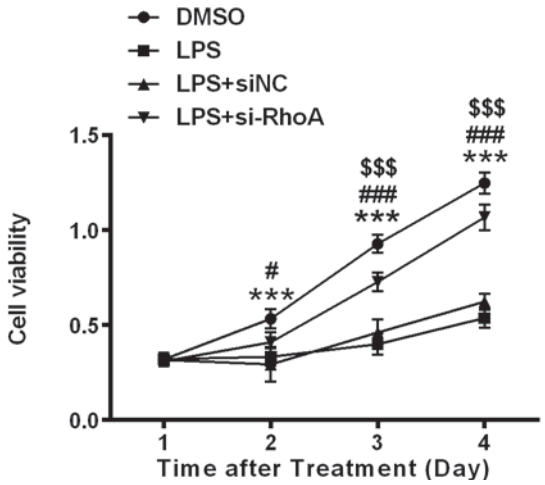

Figure 3. Effects of RhoA on cell viability. Data are presented as the mean ± standard deviation. ***P<0.0001 LPS group vs. the DMSO group; #P<0.05 LPS group vs. the LPS + si-RhoA group; ###P<0.001 LPS group vs. the LPS + si-RhoA group; $$$P<0.0001 LPS + siNC group vs. the LPS + si-RhoA group. DMSO, dimethyl sulfoxide; LPS, lipopolysaccharide; NC, negative control; RhoA, Ras homolog family member A; si, small interfering RNA.

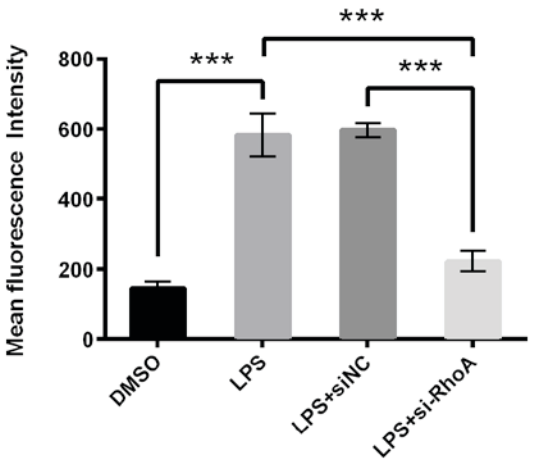

Figure 4. Effects of RhoA knockdown on ROS activity. Flow cytometry was used to measure ROS levels. Data are presented as the mean ± standard deviation. ***P<0.001 vs. the DMSO group. DMSO, dimethyl sulfoxide; LPS, lipopolysaccharide; NC, negative control; RhoA, Ras homolog family member A; si, small interfering RNA.

Wnt5a, Bcl-2, pro-caspase-3 and cleaved caspase-3 were detected. The results demonstrated that RhoA knockdown may inhibit the activation of the downstream Wnt/β-catenin signaling pathway and inhibit the expression of apoptotic factors. Therefore, knockdown of RhoA may protect cells from LPS-induced damage via the Wnt/β-catenin signaling pathway.

Discussion

ALI markedly affects patient survival, and is considered a main cause of mortality in children and adults (20). To date, numerous studies have aimed to investigate the pathogenesis of ALI, for which some hypotheses have been proposed (21,22). Niu *et al* (22) indicated that isofraxidin exerted protective

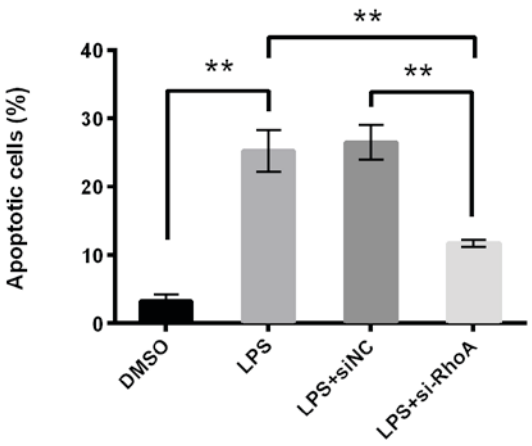

Figure 5. Effects of RhoA knockdown on cell apoptosis. The apoptotic rates of A549 cells were analyzed by flow cytometry. Data are presented as the mean ± standard deviation. **P<0.01 vs. the DMSO group. DMSO, dimethyl sulfoxide; LPS, lipopolysaccharide; NC, negative control; RhoA, Ras homolog family member A; si, small interfering RNA.

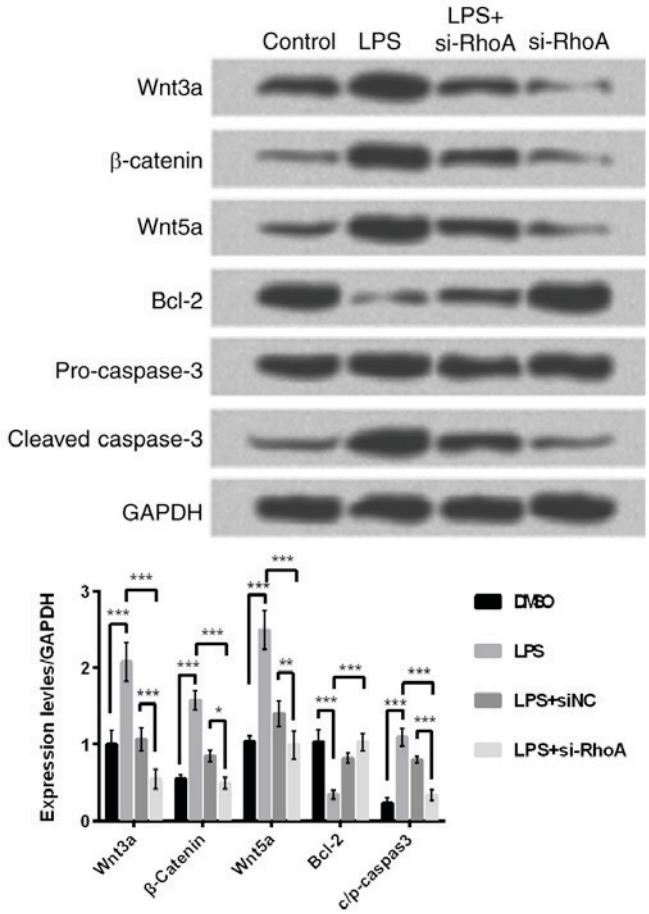

Figure 6. RhoA regulates A549 cells via the Wnt/β-catenin signaling pathway. The protein expression levels and mRNA of Wnt3a, β-catenin, Wnt5a, Bcl-2, pro-caspase-3 and cleaved caspase-3 were detected by western blotting and qRT-PCR respectively. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001 vs. the blank group. Bcl-2, B-cell lymphoma 2; c-, cleaved; LPS, lipopolysaccharide; NC, negative control; p-, pro; RhoA, Ras homolog family member A; si, small interfering RNA.

effects against LPS-induced ALI due to its antibacterial, antioxidant and anti-inflammatory activities. In addition,

veratric acid has been reported to inhibit nuclear factor-κB signaling pathways to attenuate LPS-induced inflammatory injury due to its anti-inflammatory activity (23). Although knowledge regarding ALI has improved, the underlying pathophysiological mechanisms associated with this condition are complex, and treatment of ALI remains a challenge for clinical practitioners and scientists (24).

RhoA has been reported to be involved in numerous biological processes, including transcription, cell migration and invasion (25). As a member of the Rho family of small GTPases, which are implicated in various cell functions including proliferation and survival, numerous diseases and disorders have been reported to be regulated by or associated with RhoA activation (26-28). Furthermore, research regarding the effects of podoplanin on the promotion of tumor formation has indicated that enhanced RhoA activity may result in the promotion of tumor formation, thus suggesting that it may serve important roles in tumor progression (29).

The present study used LPS to induce an ALI model in A549 cells, in order to investigate the association between RhoA and ALI. The results demonstrated that LPS treatment promoted the expression of RhoA. Therefore, si-RhoA was used to inhibit RhoA expression, in order to determine whether RhoA inactivation could serve a role in ALI. The MTT assay was used to investigate the effects of RhoA inactivation on cell viability; the results confirmed that knockdown of RhoA was able to promote proliferation. Subsequently, using flow cytometry, it was demonstrated that inhibition of RhoA could decrease cell apoptosis. Furthermore, ROS activity levels were detected in A549 cells following inactivation of RhoA; the results revealed that ROS activity was down-regulated following LPS treatment and si-RhoA transfection. These findings suggested that RhoA knockdown may inhibit ROS activity.

The Wnt/β-catenin signaling pathway has a key role in embryonic development and homeostasis, and exerts effects on cell differentiation and proliferation, and cellular and molecular mechanisms of biological processes (30,31). Activation of Wnt/β-catenin signaling inhibits apoptosis induced by activating the intrinsic or the extracellular pathway. In addition, blocking the Wnt/β-catenin pathway prevents cell proliferation and induce cell apoptosis or necrosis (32,33). As RhoA knockdown was found to inhibit apoptotic cell rate and ROS production, it was further investigated whether RhoA knockdown regulation was associated with the Wnt/β-catenin signaling pathway in A549 cells. The expressions of Wnt/β-catenin pathway- and apoptosis pathway-related core factors were detected. It demonstrated that the expressions of Wnt/β-catenin pathway-related factors (Wnt3a, β-catenin and Wnt5) were increased in A549 after LPS treatment, that Bcl-2 was decreased, and cleaved caspase-3 was increased. However, RhoA knockdown reversed the regulation of Wnt/β-catenin pathway and the apoptosis-associated factors. The results demonstrated that RhoA knockdown was able to inhibit the Wnt/β-catenin signaling pathway, and inhibit the expression of apoptosis-associated factors. These results suggested that LPS may stimulate ALI by upregulating RhoA expression and activating the downstream Wnt/β-catenin signaling pathway. Therefore, it may be hypothesized that RhoA knockdown protects against ALI via the Wnt/β-catenin signaling pathway.

In conclusion, the present study is the first, to the best of our knowledge, to identify a previously unrecognized role of RhoA in ALI. These findings may provide evidence regarding potential therapeutic targets for ALI treatment.

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