Differential roles of p38 MAPK and ERK1/2 in angiopoietin-2-mediated rat pulmonary microvascular endothelial cell apoptosis induced by lipopolysaccharide

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Abstract. Angiopoietin-2 (Ang-2) is a Tie-2 ligand that destabilizes vascular structures, enhances vascular permeability and induces vascular regression and endothelial cell apoptosis. Although there is evidence for the involvement of the Ang/Tie2 axis in acute lung injury (ALI), the underlying mechanisms involved in Ang-2-induced cell apoptosis are not well understood. In this study, whether Ang-2 contributes to microvascular endothelial cell injury and mediates lipopolysaccharide (LPS)-induced endothelial cell apoptosis and its associated signaling pathways was investigated. Exposure of rat pulmonary microvascular endothelial cells (RPMVECs) to LPS, Ang-2 and related inhibitors was performed to measure the expression levels of Ang-2, the activation of mitogen-activated protein kinases (MAPKs), the phosphorylation of extracellular signal-regulated kinase (ERK)1/2, and expression of the apoptosis-related proteins Bax and Bcl-2 using western blotting, reverse transcription-quantitative polymerase chain reaction, flow cytometry and fluorescence microscopy. The expression of Ang-2 in the RPMVECs was increased by LPS independent of time. The phosphorylation of p38 MAPK and ERK1/2 was significantly upregulated and the activation of apoptosis-related proteins Bax and Bcl was mediated by Ang-2. In addition, inhibition of the p38 pathway by SB203580 attenuated the Ang-2-mediated cell apoptosis, but inhibition of the ERK1/2 pathway by PD98059 exerted an anti-apoptotic effect against Ang-2. In conclusion, LPS-induced apoptosis is partly mediated via stimulation of p38 and ERK1/2 signaling pathways, where Ang-2 acts an inflammation-related factor to participate in the course of cell apoptosis in RPMVECs.

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Introduction

Acute lung injury (ALI) or acute respiratory distress syndrome (ARDS), as a clinically common lethal disease, is characterized by extensive neutrophilic influx into the lungs, the expression of pro-inflammatory mediators and damage of the lung epithelium and endothelium, which result in pulmonary edema and the deterioration of gas exchange (1,2). The pathogenesis of ALI is considered to involve lung inflammation and cell apoptosis, characterized by the accumulation of inflammatory cells, the aberrant release of proteases, reactive oxygen species (ROS) and proinflammatory cytokines (3,4). Lipopolysaccharide (LPS) of gram-negative bacteria has been suggested to be an important etiological factor responsible for lung diseases, characterized by the presence of apoptosis in the endothelium (5). Apoptosis of pulmonary microvascular endothelial cells (PMVECs) damages the barrier function of the pulmonary microvascular endothelium. Thus, inhibition of the apoptosis of PMVECs is a crucial intervention measure to prevent the occurrence of ALI (6,7).

Angiopoietins (Ang) are Tie2 receptor ligands that play important roles in vascular development, vessel remodeling and angiogenesis (8). Ang-1 and Ang-2 are the most specific ligands of Tie2, and Ang-2 has been shown to be a competitive antagonist for Ang-1 at the receptor tyrosine kinase Tie2 in endothelial cells (9). Ang-2, a secreted oligomeric glycoprotein, stimulates endothelial cells and increases vascular inflammation (10). *In vitro* experiments have confirmed that Ang-2, under certain circumstances, induces the phosphorylation of Tie-2 receptors, protein kinase B (also known as Akt), extracellular signal-related kinase (ERK)1/2 and p38 members of the mitogen-activated protein kinase family (11). *In vivo*, Ang-2-deficient mice did not exhibit any vascular inflammatory responses in LPS-induced sepsis experiments (12). Exceptionally high levels of circulating Ang-2 have been observed in human sepsis and correlated with mortality (10).

In addition, Harfouche *et al* (13) demonstrated that ERK1/2 and p38 are activated by Ang-1 in endothelial cells. Harfouche and Hussain (11) also reported that Ang-2 evokes p38 phosphorylation as strongly as that elicited by Ang-1. The finding that Ang-2 promotes endothelial cell survival through the phosphoinositide 3-kinase (PI3K) and ERK1/2 pathways

suggests that Ang-2 exerts qualitatively similar anti-apoptotic effects to those elicited by Ang-1 (13,14).

However, little is known about the possible impact of Ang-2 mediation on the LPS-induced apoptosis of rat PMVECs (RPMVECs) and the mechanisms by which the mitogen-activated protein kinase (MAPK) signaling pathway contributes to lung injury. Therefore, in the present study, the LPS-induced expression of Ang-2 was examined and the role of the p38 and ERK1/2 signaling pathway in the apoptotic damage of PMVECs was investigated. The study also focused on the possible influence of MAPK inhibitors on PMVEC apoptosis. Moreover, p38 and ERK1/2 signaling pathways were examined to elucidate whether Ang-2 acts as an upstream factor of MAPK pathways in the modulation of the LPS-induced apoptosis of PMVECs.

Materials and methods

Materials. RPMVECs were obtained from Shanghai Biomart Technology Co., Ltd. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA), Antibodies to p38 (no. 14451), p-p38 (no. 4511), ERK1/2 (no. 4695), p-ERK1/2 (no. 8544), Bcl-2 (no. 15071) and Bax (no. 5023) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Ang-2 antibody (no. ab155106) was purchased from Abcam (Shanghai, China). β-actin antibody (no. sc-2357 and sc-2005), SB203580 and PD98059 were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Recombinant human Ang-2 was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). LPS (no. sc-3535) was obtained from Santa Cruz Biotechnology, Inc. The Annexin V-fluorescein isothiocyanate (FITC) kit was acquired from BestBio Co. (Shanghai, China). TRIzol reagent was purchased from Invitrogen (Thermo Fisher Scientific, Inc.), and the RevertAid First Strand cDNA Synthesis kit and Maxima SYBR Green/ROX qPCR Master Mix were obtained from Thermo Fisher Scientific, Inc.

Cell culture and treatment. RPMVECs were cultured in DMEM with 10% FBS, 1% penicillin-streptomycin (Lonza, Cologne, Germany) and 1.5 g/l glucose at 37˚C in a humidified atmosphere containing 5% CO_2. Cells from passages 5-8 were used for all experiments, and harvested with trypsin (0.25%) and EDTA (0.03%) when the cells had reached exponential growth. RPMVECs were incubated with LPS (10 μg/ml) and Ang-2 (300 ng/ml) for 6, 12, 24 h respectively, or were incubated with Ang-2-alone, Ang-2 plus SB203580 (300 ng/ml + 10 μM), and Ang-2 plus PD98059 (300 ng/ml + 30 μM) for 24 h prior to analysis by western blot, flow cytometry and RT-qPCR.

Observation and detection of apoptosis by fluorescence microscopy and flow cytometry. Cells were harvested and the percentage of apoptosis was measured by flow cytometry using an Annexin V-FITC kit according to the manufacturer's instructions. Following the treatment, the adherent and floating cells were collected, washed twice with cold PBS (4˚C) and resuspended in 400 μl binding buffer. Cells were first incubated with 5 μl Annexin V-FITC at room temperature in the dark for 15 min and then with 10 μl propidium iodide (PI; 40 μg/ml) at room temperature in the dark for 5 min. Cells were observed under fluorescence microscopy and cell suspensions were transferred to test tubes and detected by flow cytometry. Cells with no drug treatment were used as a control. Data were analyzed using CellQuest software version 3.3 (BD Biosciences, San Jose, CA, USA).

Western blot analysis. Cells were washed with ice-cold PBS solution twice and incubated for 1 h at 4˚C in lysis buffer comprised of the following: 150 mM NaCl, 50 mmol/l Tris HCl pH 8.0, 1% TritonX-100, 100 μg/ml phenylmethane sulfonyl fluoride. The lysates were centrifuged at 13,380 x g for 30 min at 4˚C and the supernatant was collected. A total of 10 μG protein was loaded in each lane, separated by 10% SDS-PAGE and transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA) by electroblotting for 2 h at 100 V. Membranes were blocked for 1 h in 5% non-fat milk in PBS and then incubated with primary antibodies (Ang-2, p38, p-p38, ERK1/2, p-ERK1/2, Bcl-2 and Bax; 1:1,000 dilution) at 4˚C overnight. The membrane was washed with PBS solution and then incubated with peroxidase-conjugated anti-rabbit (no. sc-2357; Santa Cruz Biotechnology, Inc.) or anti-mouse (no. sc-2005; Santa Cruz Biotechnology, Inc.) secondary antibody (1:4,000 dilution) for 1 h at room temperature. The blots were assayed by chemiluminescence (EMD Millipore) on X-ray film. Finally, the bands were analyzed using ImageJ 1.43 software (National Institutes of Health, Bethesda, MA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the RPMVECs using TRIzol reagent. Complementary DNA (cDNA) was synthesized from 2 μg total RNA and 1 μl oligo (dT), diluted to a volume of 8 μl in DEPC-treated water, and heated at 70˚C for 5 min. Then, 4 μl 5X Reverse Transcription buffer, 2 μl Superscript reverse transcriptase, 2 μl 2.5 mM dNTP mix, 1 μl RNase inhibitor and DEPC-treated water were added to 20 μl. The mixture was gently incubated at 37˚C for 5 min, incubated at 42˚C for 60 min to synthesize cDNA and then heated at 70˚C for 10 min to stop cDNA synthesis. The cDNA was stored at -20˚C. qPCR was performed using the SYBR Green qPCR Master mix in the ABI 7300 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction volume was 20 μl, and the reaction mixture comprised 10 μl SYBR-Green qPCR Master mix, 1 μl cDNA, 0.5 μl each of sense and antisense primers, and water to 20 μl. The amplification profile was as follows: Initial denaturation at 95˚C for 10 min, followed by 45 cycles at 95˚C for 15 sec and 60˚C for 30 sec. Primers for Ang-2, Bcl-2, Bax and β-actin were designed with Primer Premier 5.0 software for the rat (Invitrogen; Thermo Fisher Scientific, Inc.) as follows: Ang-2, sense 5'-CTGAAGATCCAGCTGAAG-3' and antisense 5'-ATTGTCCGAATCCTTTGT-3'; Bcl-2, sense 5'-ATGTGT GTGGAGAGCGTCAACC-3' and antisense 5'-CCAGGA GAAATCAAACAGAGGC-3'; Bax, sense 5'-GCGATGAAC TGGACAACAACAT-3' and antisense 5'-TAGCAAAGTAGA AAAGGGCAACC-3'; β-actin, sense 5'-TCATGAAGTGTG ACGTTGACATCCGT-3' and antisense 5'-CCTAGAAGC ATTTGCGGTGCAGGATG-3'. Relative gene expression data were calculated from the formula: $2^{-\Delta\Delta Cq}$, where Cq represents

the fractional cycle number at which the amount of target reaches a fixed threshold (15).

Statistical analysis. All data are expressed as mean ± standard deviation. Data were analyzed by repeated measures, one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference. Data were analyzed using the commercially available SPSS software package, version 18.0 (SPSS, Inc., Chicago, IL, USA). All data were obtained from three separate experiments.

Results

LPS time-dependently induces Ang-2 expression in RPMVECs. In the present study, RPMVECs were used to investigate LPS-induced Ang-2 expression. The RPMVECs were cultured in DMEM and 10% FBS medium containing 10 μg/ml LPS for 0, 6, 12 and 24 h respectively, which resulted in a time-dependent increase of Ang-2 expression. Western blot analysis showed that expression level of Ang-2 was significantly higher in cells incubated for 6, 12 and 24 h with LPS compared with cells incubated without LPS (P<0.01). Following 24 h of exposure to LPS, the expression levels of Ang-2 exhibited a >6-fold increase compared with the control group (P<0.01; Fig. 1A). The relative quantity of Ang-2 mRNA was also determined and was >3-fold higher at 24 h following LPS induction compared with that in the control cultures (P<0.01; Fig. 1B).

Ang-2 evokes MAPK activation and its inhibitors modulate the expression of MAPKs. To confirm whether the MAPK pathway is involved in the LPS-induced Ang-2-mediated apoptosis of RPMVECs, the expression levels of p38 and ERK1/2 and their phosphorylation levels when RPMVECs were treated with Ang-2 were first investigated, and then selective inhibitors of MAPKs were used to further confirm the roles of the p38 and ERK1/2 pathways. RPMVECs were incubated in DMEM and 10% FBS medium containing 300 ng/ml Ang-2 for 0-2 h. A significant time-dependent increase was observed in the expression levels of p-P38 and p-ERK1/2 compared with that prior to treatment (P<0.01; Fig. 2A). To further clarify whether Ang-2 induced the phosphorylation of MAPKs, RPMVECs were cultured in DMEM and 10% FBS medium and treated with SB203580 (10 μM) and PD98059 (30 μM) for 2 h followed by Ang-2 (300 ng/ml) treatment for 1 h. Western blotting results revealed that pre-incubation with these inhibitors significantly prevented the phosphorylation of p38 and ERK1/2 compared with that in the RPMVECs treated with Ang-2 alone (P<0.01; Fig. 2B).

Ang-2 time-dependently mediates the apoptosis of RPMVECs. To demonstrate how Ang-2 induces cell apoptosis, flow cytometry with Annexin V-FITC and PI double staining was performed. The results demonstrated a significant increase in the numbers of apoptotic and necrotic cells in the Ang-2 group compared with the control group (Fig. 3A). RPMVECs treated with Ang-2 demonstrated a time-dependent increase in apoptosis rate compared with the control group (P<0.01).

To investigate the expression levels of apoptosis-related proteins, the downstream molecules Bax and Bcl-2 were investigated. In Ang-2-treated RPMVECs, activation of MAPK pathways led to a time-dependent downregulation of antiapoptotic Bcl-2 expression levels, whereas proapoptotic Bax expression levels were time-dependently upregulated (Fig. 3B and C). The expression of Bax and Bcl-2 protein and mRNA was significantly changed by exposure to Ang-2 compared with that in the control group (P<0.01).

p38 and ERK1/2 signaling pathways are involved in the Ang-2-induced apoptosis of RPMVECs. To further investigate the effects of p38 and ERK1/2 on Ang-2 induced cell apoptosis, RPMVECs were incubated with vehicle control, Ang-2, Ang-2 + SB203580, or Ang-2 + PD98059 for 24 h. Electron microscopy revealed morphological changes following treatment, including cells turning round and detaching from the neighboring cells. In particular, PD98059 had an obvious effect on cell morphology. Changes in the RPMVECs included emitting light-green and light-red fluorescence, as observed using PI staining and fluorescence microscopy; changes were most notable in the PD98059-treated group (Fig. 4A). Analysis of the cell apoptosis rate demonstrated that Ang-2 treatment increased early and late apoptotic cell rates compared with those in the control group (P<0.01), while SB203580 pre-incubation significantly attenuated the cell apoptosis rate compared with that in the Ang-2-alone group (P<0.01). However, the effect of PD98059 on RPMVEC apoptosis was opposite to that of SB203580 (Fig. 4B).

In addition, the expression levels of apoptosis-related Bax and Bcl-2 were investigated. The expression levels of Bax and Bcl-2 were significantly altered by Ang-2 compared with that in the control group (P<0.01); while SB203580 pretreatment attenuated Bax protein (P<0.01) and mRNA expression levels (P<0.05), it increased the expression levels of Bcl-2 protein (P<0.01) and mRNA (P<0.05) compared with those in the group treated with Ang-2 alone. However, PD98059 pretreatment increased Bax protein (P<0.01) and mRNA expression (P<0.05) and attenuated the expression levels of Bcl-2 protein (P<0.01) and mRNA (P<0.05) compared with those in the group treated with Ang-2 alone (Fig. 4C and D).

Discussion

The results of the current study indicate demonstrate that Ang-2, which is induced by LPS, mediates RPMVEC apoptosis via the MAPK signaling pathway. The main observations of the study include: i) LPS at a concentration of 10 μg/ml significantly promoted Ang-2 expression by endothelial cells in a time-dependent manner. ii) Ang-2 at a concentration of 300 ng/ml significantly increased the phosphorylation levels of p38 and ERK1/2, elicited morphological changes in endothelial cells and induced apoptosis-related protein expression. iii) Activation of the p38 pathway by Ang-2 induced endothelial cell apoptosis since the selective inhibition of this pathway by SB203580 attenuated cell apoptosis and decreased the expression of apoptosis-related proteins. iv) The anti-apoptotic effect of Ang-2 was mediated through the ERK1/2 pathway because selective suppression of this pathway by PD98059 promoted endothelial cell apoptosis and apoptosis-related protein activation.

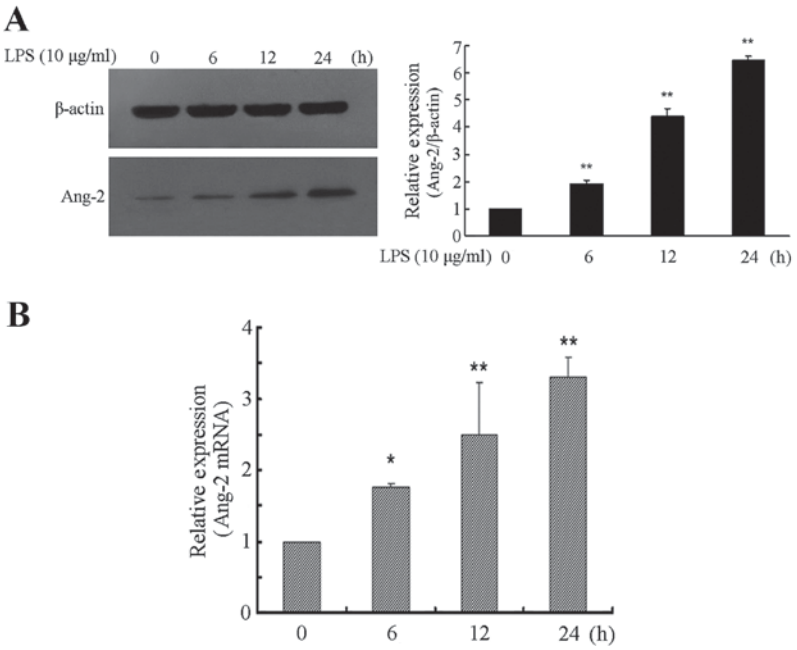

Figure 1. Rat pulmonary microvascular endothelial cells were cultured with LPS (10 μg/ml) for 0, 6, 12 and 24 h. (A) Protein expression of Ang-2 was assessed by western blot analysis. β-actin was used as loading control. (B) Expression of Ang-2 mRNA was evaluated by reverse transcription-quantitative polymerase chain reaction. The relative Ang-2 mRNA expression is shown using β-actin as a reference. *P<0.05, **P<0.01 vs. the control group (0 h). LPS, lipopolysaccharide; Ang-2, angiopoietin 2.

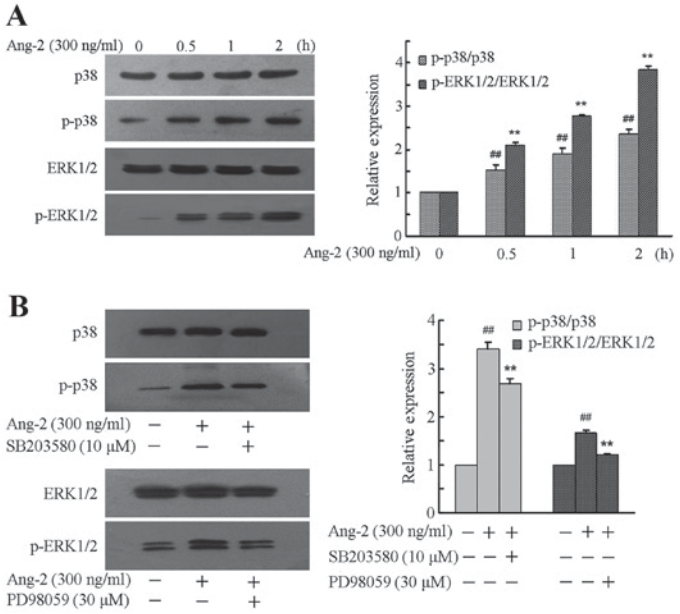

Figure 2. Activation of p38 mitogen-activated protein kinase and ERK1/2 determined in Ang-2-induced RPMVECs. (A) RPMVECs were cultured with Ang-2 (300 ng/ml) for 0, 0.5, 1 and 2 h. Proteins of downstream signaling pathways were assessed by western blot analysis. $^{\#\#}$P<0.01 vs. the control group (0 h p-P38); **P<0.01 vs. the control group (0 h p-ERK1/2). (B) RPMVECs were preincubated with or without SB203580 (10 μM) or PD98059 (30 μM) for 2 h, and then cultured with Ang-2 (300 ng/ml) for 1 h. The phosphorylation of p38 and ERK1/2 was detected by western blot analysis. $^{\#\#}$P<0.01 vs. the untreated control group; **P<0.01 vs. the Ang-2-alone group. RPMVECs, rat pulmonary microvascular endothelial cells; ERK, extracellular signal-regulated kinase; Ang-2, angiopoietin 2.

There is increasing evidence indicate that Ang proteins are associated with the inflammatory response, as the over-expression of Ang-1 has been shown to promote survival and homodynamic functions and reduce the expression of adhesion molecules in mice with LPS-induced ALI (16). However, studies of Ang-2 have found that high levels disrupt the functional architecture of lung endothelial cells, and that the barrier can be rescued with administration of Ang-1, indicating that Ang-2 may promote inflammation (16,17). Genetic studies have identified polymorphisms in the ANGPT2 gene that are associated with an increased risk of developing ALI (18). An early increase of Ang-2 levels indicates the importance of early endothelial injury and vascular permeability in the pathogenesis of ALI (19). Whether Ang-3 and Ang-4 regulate

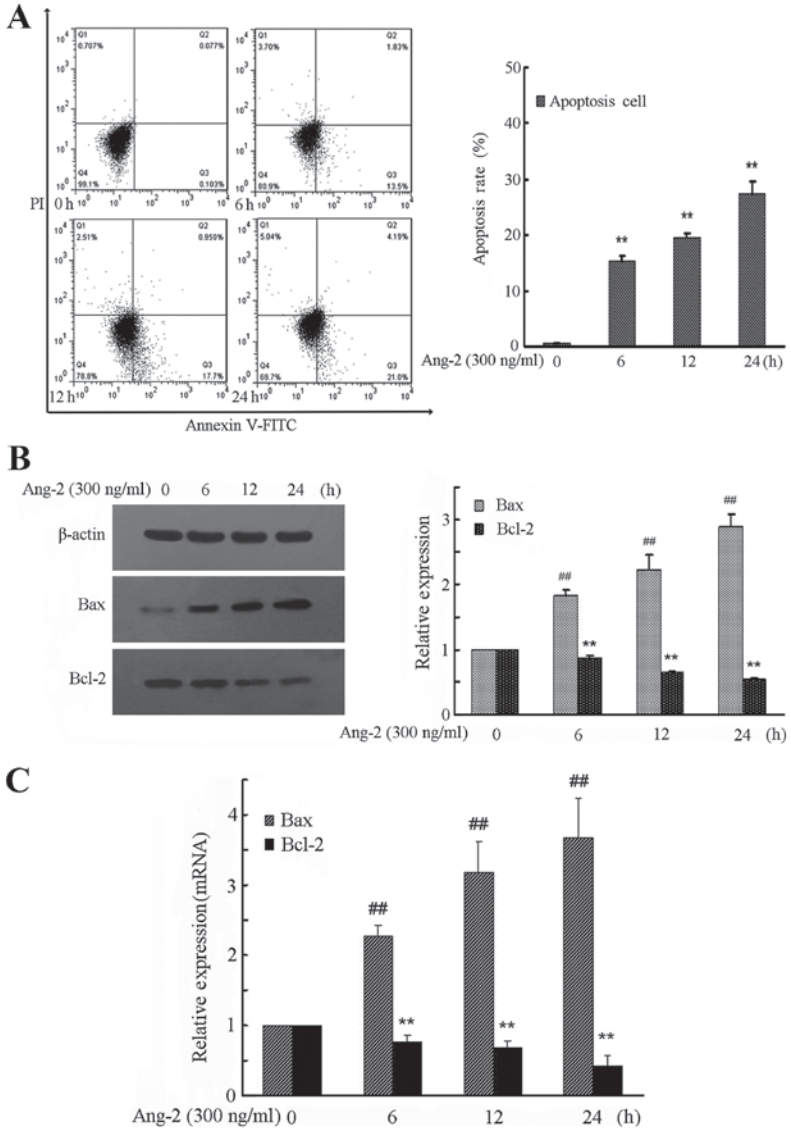

Figure 3. Effect of Ang-2 on cell apoptosis. (A) The apoptosis of RPMVECs was detected by An/PI staining and the mean percentage of cells in early and late apoptosis was analyzed by flow cytometry. Upper left quadrants (An^-/PI^+), necrotic cells; upper right quadrants (An^+/PI^+), late apoptotic cells; lower left quadrant (An^-/PI^-), live cells; and lower right quadrant (An^+/PI^-), early apoptotic cells. $^{**}P<0.01$ vs. the control group (0 h). (B) Expression of apoptosis-related proteins Bax and Bcl-2 was detected by western blotting. β-actin was used for normalization. $^{\#\#}P<0.01$ vs. the control group (0 h Bax); $^{**}P<0.01$ vs. the control group (0 h Bcl-2). (C) Expression of Bax and Bcl-2 mRNA in Ang-2 treated RPMVECs was assessed by reverse transcription-quantitative polymerase chain reaction. $^{\#\#}P<0.01$ vs. the control group (0 h Bax); $^{**}P<0.01$ vs. the control group (0 h Bcl-2). Ang-2, angiopoietin 2; RPMVECs, rat pulmonary microvascular endothelial cells; An, Annexin V; PI, propidium iodide.

inflammation has not yet been elucidated; however, Ang-3 and Ang-4 have both been observed to activate Tie-2 receptors, suggesting that they may induce anti-inflammatory effects similar to those of Ang-1 (20).

Despite evidence indicating the important role of Ang in the regulation of inflammatory reaction, little is known regarding the stimulation of endogenous Ang production by LPS. High concentrations of LPS have been shown to stimulate numerous endothelial responses, including the induction of apoptosis, which may impair vascular integrity and increase the permeability of the endothelium (21). However, the underlying mechanism by which Ang-2 mediates LPS-induced microvascular endothelial cell apoptosis is not fully known. Although several studies have shown that p38 and ERK1/2 signaling pathways are involved in the apoptosis of microvascular endothelial cells, it is unclear whether the apoptotic effect of Ang-2 is mediated through p38 and ERK1/2 pathways (22,23). In the present study, the roles of p38 and ERK1/2 in the Ang-2-induced apoptosis of RPMVECs were investigated. It was found that Ang-2 activates the phosphorylation of p38 and ERK1/2. These findings indicate that elevated activity of p38 and ERK1/2 might be involved in the apoptosis of endothelial cells. Therefore, inhibition of p38 MAPK and ERK1/2 pathways might ameliorate or aggravate microvascular endothelial cell damage.

To explore the mechanisms through which Ang-2 mediates LPS-induced endothelial cell apoptosis, the role of MAPK activation was examined by assessing the expression of the phosphorylation of P38 and ERK1/2. Previous studies have shown that MAPK pathways are associated with vascular inflammatory reactions modulated by ROS (24), and activation of MAPK proteins is vital in the cellular responses associated with inflammatory stimuli such as LPS (25).

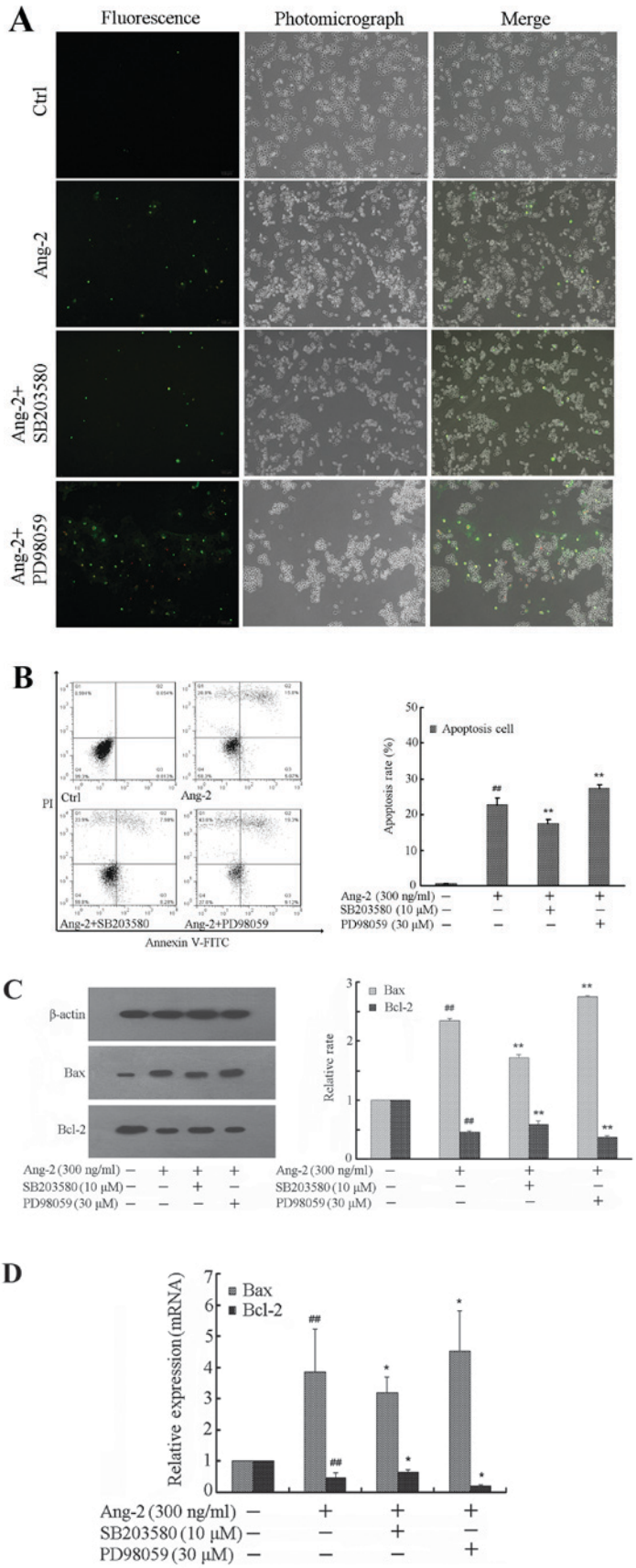

Figure 4. Effects of SB203580 and PD98059 on the apoptosis of RPMVECs induced by Ang-2. RPMVECs were incubated with vehicle control, Ang-2 (300 ng/ml), Ang-2 + 10 μM SB203580 and Ang-2 + 30 μM PD98059 for 24 h. (A) Apoptosis of RPMVECs was observed by inverted system microscopy and fluorescence microscopy. Original magnification, x200. (B) Apoptosis of RPMVECs was determined by Annexin V/PI staining and flow cytometric analysis. $^{\#\#}$P<0.01 vs. the untreated control group; **P<0.01 vs. the Ang-2-alone group. (C) Expression levels of apoptosis-related proteins Bax and Bcl-2 were evaluated by western blot analysis. β-actin was used as loading control. $^{\#\#}$P<0.01 vs. the untreated control group; **P<0.01 vs. the Ang-2-alone group. (D) Expression of Bax and Bcl-2 mRNA was detected by reverse transcription-quantitative polymerase chain reaction. $^{\#\#}$P<0.01 vs. the untreated control group; *P<0.05 vs. the Ang-2-alone group. RPMVECs, rat pulmonary microvascular endothelial cells; Ang-2, angiopoietin 2; PI, propidium iodide.

Previous studies have demonstrated that inactivation of ERK1/2 and activation of p38 are involved in the induction of mitochondrial-mediated apoptosis in cancer cells (26,27). The present study investigated whether endothelial cells are affected similarly. The results revealed that LPS (10 μg/ml) time-dependently increased Ang-2 protein and mRNA expression. Furthermore, cells incubated with Ang-2 (300 ng/ml) exhibited significantly elevated expression levels of p-p38 and p-ERK1/2. A study by Harfouche and Hussain indicated that Ang-2 at concentrations of 50-300 ng/ml activates Tie-2 receptors and increases phosphorylation in the Akt, ERK1/2 and p38 MAPK pathways while significantly inhibiting the JNK/SAPK pathway (11). In the present study, following the selective regulation of p38 and ERK1/2 pathways, Ang-2 significantly increased Bax expression levels and suppressed Bcl-2 expression levels. Thus, it may be concluded that the apoptosis-related proteins Bax and Bcl-2 are downstream target proteins of p38 and ERK1/2 pathways. These results are similar to those of other studies, which indicated that inactivation of ERK1/2 and activation of p38 could mediate the upregulation of Bax and downregulation of Bcl-2, and finally trigger the apoptotic pathway (28,29).

To further demonstrate the involvement of MAPK signaling pathways in Ang-2-mediated apoptosis, inhibitors of p38 (SB203580) and ERK1/2 (PD98059) were used to explore the association of P38 MAPK and ERK1/2 signaling pathways with the modulation of RPMVEC apoptosis. Results indicated that the apoptosis of RPMVECs induced by Ang-2 was significantly inhibited by SB203580 and markedly increased by PD098059, suggesting that p38 and/or ERK1/2 may play important roles in modulating the Ang-2-mediated apoptosis of RPMVECs. Further assessment of the expression of apoptosis-related proteins Bax and Bcl-2 was conducted by western blot and RT-qPCR analysis following pre-incubation for 2 h with SB203580 (10 μM) or PD098059 (30 μM). The p38 inhibitor significantly decreased the expression of Bax and increased the expression of Bcl-2 mediated by Ang-2, while the apoptotic effect of the ERK1/2 inhibitor opposes that of the p38 inhibitor. These results indicate that inhibition of the p38 pathway prevented the Ang-2-mediated apoptosis of RPMVECs, while inhibition of the ERK1/2 pathway resulted in a pro-apoptotic effect. This is consistent with a previous study showing that activation of the p38 pathway by Ang-2 promotes endothelial cell apoptosis, where selective inhibition of this signaling pathway improved endothelial cell survival and attenuated caspase-3 and -9 activation (11). Also consistent with this, inhibition of either p38 or ERK1/2 has been shown to prevent TNF-α-induced increases in the permeability of human lung microvascular endothelial cells, suggesting crucial roles for both p38 and ERK1/2 in the microvascular endothelium (30). However, in the present study, the reason why activation of MAPK signaling pathways by Ang-2 resulted in cell apoptosis rather than an anti-apoptotic effect may be the involvement of an additional cell signaling pathway, such as PI3K/Akt, c-Jun N-terminal kinase and nuclear factor-κB, and the cross-talk between them.

There are several limitations of the current study. Firstly, downstream signaling from the MAPKs that may act to cause cell apoptosis were not explored. Secondly, cross-talk among MAPKs was not analyzed, although the activity of one MAPK can be influenced by another or there may be interplay between NF-κB and MAPK signaling pathways (31,32). Thirdly, only a single RPMVEC cell line was investigated. It remains unclear whether human pulmonary microvascular endothelial cells would function in the same way as the RPMVECs used in the current study.

In summary, it may be concluded that Ang-2, as the downstream factor of LPS, could increase the LPS-induced effects on RPMVECs. The activation of p38 MAPK and ERK1/2 plays an important role in the Ang-2-mediated apoptosis of RPMVECs. Inhibition of the p38 MAPK pathway exerts an anti-apoptotic effect on endothelial cells, while inhibition of the ERK1/2 pathway exhibits a pro-apoptotic effect. These findings imply that Ang-2 may act as an inflammatory factor in the inflammatory injury of the microvascular endothelium in ALI.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SL was involved in data collection and analysis and contributed to writing the manuscript. MZ designed and performed the experiments. YY performed experiments and provided guidance. LZ contributed to experimental design and data collection.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that there are no competing interests.

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