MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2018; 24: 3212-3219
DOI: 10.12659/MSM.910184

Received: 2018.03.24
Accepted: 2018.04.19
Published: 2018.05.16

Ripasudil Attenuates Lipopolysaccharide (LPS)-Mediated Apoptosis and Inflammation in Pulmonary Microvascular Endothelial Cells via ROCK2/eNOS Signaling

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Source of support: Departmental sources

Background: Microvascular endothelial inflammation and apoptosis are responsible for septic acute lung injury (ALI). Ripasudil is a novel Rho/Rho kinase (ROCK) inhibitor which shows therapeutic effects on several vascular diseases. The aim of this study was to investigate the protective effects and correlated molecular mechanisms of ripasudil on lipopolysaccharide- induced inflammation and apoptosis of pulmonary microvascular endothelial cells (PMVECs).

Material/Methods: Cultured PMVECs were exposed to lipopolysaccharide (LPS). Ripasudil at various concentrations was used to treat the cells. Several cells were also co-administrated with the endothelial nitric oxide synthase (eNOS) inhibitor N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME). Cell viability was assessed by MTT assay. Terminal dUTP transferase nick-end labeling (TUNEL) assay was used to detect the apoptosis. The colorimetric method was used to measure the activity of eNOS and ROCK2. Protein phosphorylation and expression were assessed by Western blotting.

Results: Ripasudil attenuated the LPS-induced inflammation and apoptosis in PMVECs, which was reversed by L-NAME. Ripasudil suppressed ROCK2 activity and further increased the eNOS activity. Ripasudil treatment increased the phosphorylation of eNOS, increased the expression level of Bcl2, and decreased the expression level of active caspase3 in LPS-treated PMVECs. Moreover, the ripasudil treatment also inhibited the nuclear translocation of NF-κB and further suppressed the levels of interleukin (IL) 6 and tumor necrosis factor (TNF) α. The co-treatment with L-NAME, however, impaired the anti-apoptotic and anti-inflammatory effects of ripasudil on PMVECs without affecting ROCK2.

Conclusions: The novel ROCK2 inhibitor ripasudil suppressed LPS-induced apoptosis and inflammation in PMVECs by regulating the ROCK2/eNOS signaling pathway.

MeSH Keywords: Acute Lung Injury • Apoptosis • Inflammation • rho-Associated Kinases

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/910184

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Background

Caused by various factors, including trauma, shock, and sepsis, acute lung injury (ALI) is a common cause of mortality and morbidity in intensive care units. It was reported that the mortality rate of ALI was 30–60% [1]. Although the mechanism of ALI is very complicated, severe ventilation-perfusion imbalance plays a critical role. Formed by pulmonary microvascular endothelial cells (PMVECs), the pulmonary alveolar-capillary unit is the fundamental structure for maintaining the function of ventilation-perfusion balance and is very vulnerable to harmful external stimuli [2]. Lipopolysaccharide (LPS) is one of the major toxic components of gram-negative bacteria outer membranes, causing cell and tissue damage in systemic inflammatory response syndrome (SIRS) and multiple-organ dysfunction syndrome (MODS) [3]. According to previous studies, LPS is one of the pathogenic factors leading to microcirculatory abnormalities of ALI [4].

Rho/Rho kinase (ROCK)-regulated signaling pathways are associated with many vascular diseases, including pulmonary artery hypertension, coronary spasm, stroke, and diabetic retinopathy [5]. The activity of ROCK is regulated by the small GTPase Ras homolog gene family member A (RhoA) and they form the RhoA/ROCK pathway. This signaling pathway participates in multiple cell activities such as cell migration, motility, and contraction [6]. Previous studies have confirmed that activation of the RhoA/ROCK pathway plays a role in impairing vascular function by suppressing eNOS, which further reduces the production of nitric oxide (NO) [4]. The deactivation of eNOS leads to reduction of Bcl2 expression, facilitating cell death [8]. Moreover, eNOS was reported to attenuate inflammation by suppressing NF-κB activation [9]. Thus, we hypothesized that LPS induces activation of the RhoA/ROCK signaling pathway, which further reduces eNOS activity in PMVECs. An agent blocking RhoA/ROCK activation would be helpful in improving microvascular permeability and contractility.

Investigators have been focusing on the protein kinase inhibitors for decades due to their potent pharmacological activities that confers their clinical significance. Chloromangiferamide, for instance, is an active component extracted from *Mangifera zeylanica* Hook. f. A previous study identified its anti-cancer activity on breast cancer by inhibiting tyrosine kinase [10]. Other examples include coumestrol, which is a haspin kinase inhibitor, and emodin, which was identified as a protein kinase CK2 inhibitor [11,12]. More and more synthetic kinase inhibitors also attracted attention from researchers due to their flexible availability. Ripasudil, which is also known as K-115, is a novel and potent selective ROCK inhibitor. Like other ROCK inhibitors that share similar structures, ripasudil inhibits ROCK activity by suppressing transfer of the terminal phosphate from ATP to their substrates. The anti-inflammatory and anti-fibrotic activities of ripasudil were reported in previous studies [9]. The inhibitory effect of ripasudil was higher than that of other ROCK inhibitors such as fasudil and Y-27632. This high selectivity contributes to the good biosafety profile of ripasudil [13]. Based on the above evidence, we hypothesized that ripasudil exerts endothelial-protective effects. In this study, cultured PMVECs were incubated with LPS and then administrated with ripasudil. The production of NO and apoptosis were examined. The eNOS inhibitor L-NAME was also used to further test our hypothesis. To the best of our knowledge, the present study provides novel information concerning the endothelial protective activity and possible molecular mechanism of ripasudil. We believe that results from the present study will add to understanding of the mechanisms of ALI and provide a theoretical basis for potential clinical use of ripasudil in ALI/ARDS.

Material and Methods

Cell line and treatment

Human PMVECs (HPMVECs) were provided by the Cell Bank of the Chinese Academy of Science and maintained in RPMI1640 medium (Hyclone) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin (Sigma), and 100 μg/ml streptomycin in a cell incubator providing a humidified environment, 5% CO_2 and 95% fresh air at 37°C. Subsequent experiments were implemented when cells reached 80% confluence. Cells were exposed to 100 ng/ml LPS (Invitrogen) for 24 h [2]. Cells were treated with ripasudil (Kowa Company) at concentrations of 25, 50, and 75μmol/l for 24 h. Several cells were also treated with 5 mmol/l of the eNOS inhibitor N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma) for 24 h. The selection of ripasudil and L-NAME concentrations was based on the results from our pre-experiments. According to the treatments, equal amounts of cells were assigned into 5 different groups: Control (LPS at 0ng/ml; ripasudil at 0 μmol/l, L-NAME at 0 mmol/l), LPS (LPS at 100 ng/ml; ripasudil at 0 μmol/l, L-NAME at 0 mmol/l), LPS+LRip (LPS at 100 ng/ml; ripasudil at 25 μmol/l, L-NAME at 0 mmol/l), LPS+MRip (LPS at 100 ng/ml; ripasudil at 50 μmol/l, L-NAME at 0 mmol/l), LPS+HRip (LPS at 100 ng/ml; ripasudil at 75 μmol/l, L-NAME at 0 mmol/l), and LPS+HRip+L-NAME (LPS at 100 ng/ml; ripasudil at 75 μmol/l, L-NAME at 5 mmol/l).

Cell proliferation assessment

Cell proliferation was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Cultured HPMVECs at density of 6×10^3 were seeded into a 96-well plate. Control wells were set. Cells were washed by PBS and incubated with 5 mg/ml MTT (Invitrogen) for 4 h. Then, 0.2% DMSO (Sigma) was added into the wells to dissolve the

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formazan crystals and the absorbance at 490 nm (A490) was detected with a plate reader (Bio-Rad). The detected A490 of the experimental group/A490 of Control group was considered as the cell viability.

Cell apoptosis assay

Terminal dUTP transferase nick-end labeling (TUNEL) assay was used to detect the apoptosis of cultured HPMVECs. After the cells were fixed with 4% paraformaldehyde, a TUNEL assay kit (Roche) was used to detect the apoptotic cells according to the instructions provided by the manufacturer. An inverted fluorescence microscope was used to capture the fluorescent images, which were then analyzed using software Image J (version 1.38, NIH).

NO production measurements

The generation of nitrite, which is the stable metabolite of NO, was measured to indicate the content of NO in culture medium. The concentration of nitrite in cell culture supernatants was examined using an NO assay kit (Beyotime) by following the protocol provided by the manufacturer.

Inflammation evaluation

The cell culture medium supernatants were collected. The concentrations of IL-6 and TNF-α were determined with the enzyme-linked immunosorbent assay (ELISA) with Human IL-6 Quantikine ELISA Kit (R&D Systems) and Human TNF-α Quantikine ELISA Kit (R&D Systems) per the manufacturer's instructions.

eNOS and ROCK2 activity assay

The enzymatic activity of eNOS and ROCK2 were assessed by a colorimetric method by using an NOS activity assay kit (BioVision) and ROCK2 activity assay (Abcam) in cell lysate. The experiment was carried out according to the protocol provided by the manufacturers.

Western blotting

Cultured HPMVECs were homogenized with the cell lysis buffer system (Santa Cruz) on dry ice. The protein was extracted using protein extraction reagents (Beyotime) per the manufacturer's instructions. After centrifugation of homogenates, the concentration of protein was measured with BCA method by using a BCA assay kit (Invitrogen). Protein samples were separated by SDS-PAGE and then transferred to the PVDF membranes electronically. The membranes were then incubated with the blocking buffer (Santa Cruz) to eliminate the non-specific binding sites. Then, the membranes were incubated with primary antibodies against ROCK2 (Cell Signaling Tech, 1: 2000), eNOS, (Cell Signaling Tech, 1: 2000) p-eNOS (Cell Signaling Tech, 1: 2000), Bcl2 (Santa Cruz, 1: 2000), active caspase3 (Abcam, 1: 2000), NF-κB (Abcam, 1: 1000), IL-6 (Abcam, 1: 2000), TNF-α (Abcam, 1: 1000), GAPDH (Abcam, 1: 5000), and Histone-H3 (Abcam, 1: 2000) at 4°C for 12 h. After washing in TBST, membranes were further incubated with secondary antibodies conjugated with horseradish peroxidase (Abcam) at room temperature for 2 h. The immunoblots were visualized with an enhanced chemiluminescence Western blotting detection system (ECL, Fisher Scientific).

Statistics

Data are presented as mean ±SD and were analyzed using SPSS software (ver16.0, SPSS). Differences between groups were analyzed by *t* tests and one-way ANOVA. NSK tests were carried out as post hoc testing. Differences were considered statistically significant at $p<0.05$.

Results

Ripasudil recovered the NO production and cell viability of HPMVECs, which were reversed by L-NAME co-administration (Figure 1). LPS exposure significantly decreased cell viability and NO production. The treatment of ripasudil dramatically recovered cell viability and NO production in a concentration-dependent manner. The attenuating effects of ripasudil on proliferation and NO production of HPMVECs were impaired by co-administration of L-NAME.

Ripasudil reduced LPS-induced HPMVECs apoptosis in a concentration-dependent manner, which was reversed by L-NAME treatment (Figure 2). LPS exposure significantly increased the apoptosis in HPMVECs compared with the Control group. The administration of ripasudil, however, alleviated the apoptosis in a concentration-dependent manner. Co-administration with the eNOS-specific inhibitor L-NAME impaired the anti-apoptotic effect of ripasudil.

Ripasudil inhibited inflammation of HPMVECs exposed to LPS, which was impaired by L-NAME treatment (Figure 3). Inflammation was assessed by measuring the concentrations of IL6 and TNFα in supernatants from cell culture medium. LPS exposure significantly increased the concentration of IL6 and TNFα. The ripasudil treatment dramatically decreased the concentrations of IL6 and TNFα in LPS-exposed HPMVECs in a concentration-dependent manner. However, the L-NAME co-treatment significantly impaired the anti-inflammatory effect of ripasudil on HPMVECs.

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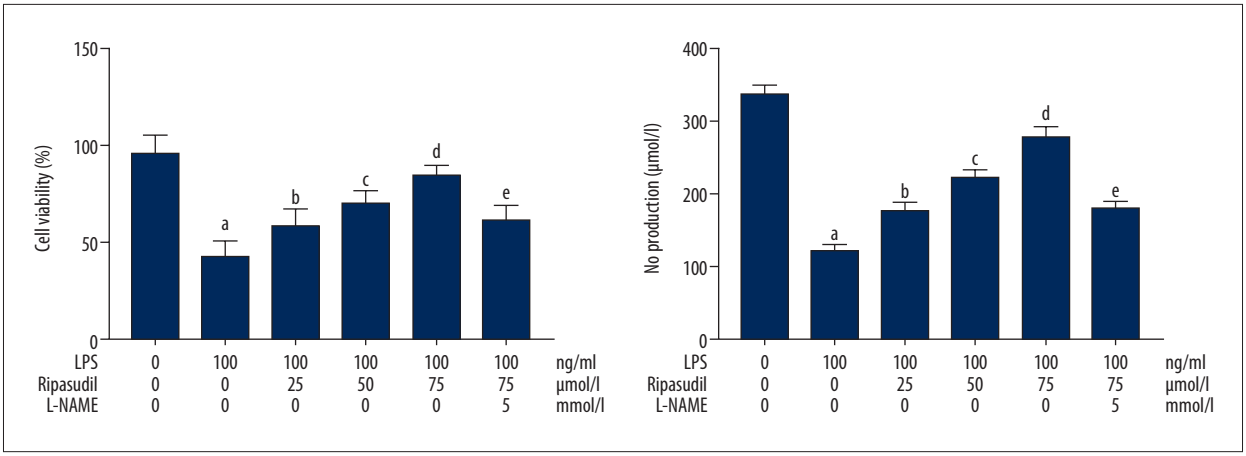

Figure 1. Columns in the upper panel indicate the cell viability of PMVECs treated with LPS, ripasudil, and/or L-NAME. Columns in the lower panel indicate the calculated NO production in PMVECs treated with LPS, ripasudil, and/or L-NAME [[a] differences were significant when compared with Control ($p<0.05$), [b] differences were significant when compared with LPS+LRip ($p<0.05$), [c] differences were significant when compared with LPS+MRip ($p<0.05$), [d] differences were significant when compared with LPS+HRip ($p<0.05$), [e] differences were significant when compared with LPS+HRip+L-NAME ($p<0.05$)].

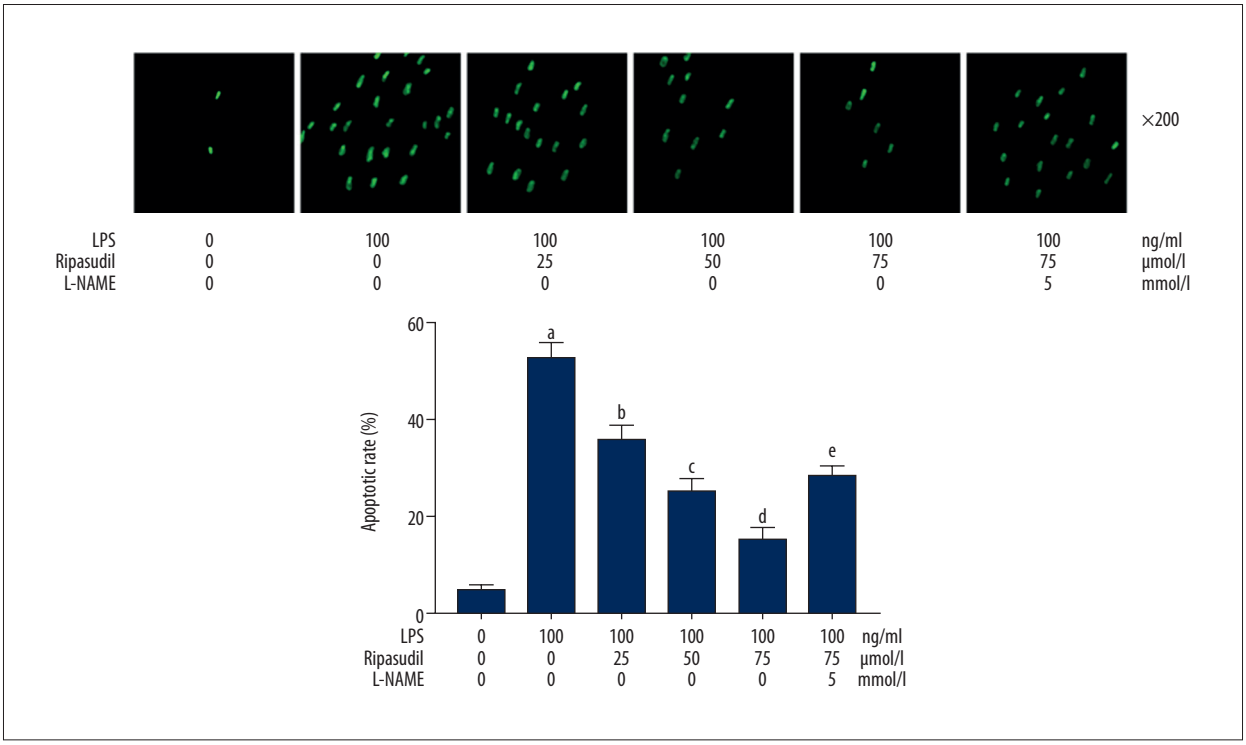

Figure 2. The upper panel demonstrates the captured images of TUNEL assay of PMVECs treated with LPS, ripasudil, and/or L-NAME. TUNEL-positive cells were tagged with green fluorescence. Columns in the lower panel indicate the apoptotic rate of PMVECs treated with LPS, ripasudil, and/or L-NAME [[a] differences were significant when compared with Control ($p<0.05$), [b] differences were significant when compared with LPS+LRip ($p<0.05$), [c] differences were significant when compared with LPS+MRip ($p<0.05$), [d] differences were significant when compared with LPS+HRip ($p<0.05$), [e] differences were significant when compared with LPS+HRip+L-NAME ($p<0.05$)].

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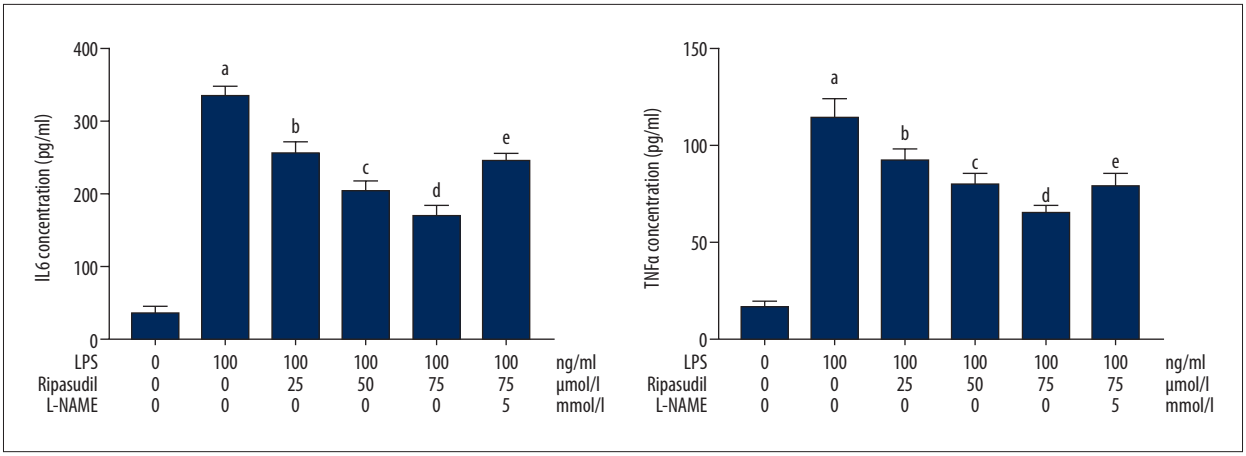

Figure 3. Columns in the upper panel indicate the IL6 concentration in the cell medium supernatant of PMVECs treated with LPS, ripasudil, and/or L-NAME. Columns in the lower panel indicate the TNFα concentration in the cell medium supernatant of PMVECs treated with LPS, ripasudil, and/or L-NAME [[a] differences were significant when compared with Control ($p<0.05$), [b] differences were significant when compared with LPS+LRip ($p<0.05$), [c] differences were significant when compared with LPS+MRip ($p<0.05$), [d] differences were significant when compared with LPS+HRip ($p<0.05$), [e] differences were significant when compared with LPS+HRip+L-NAME ($p<0.05$)].

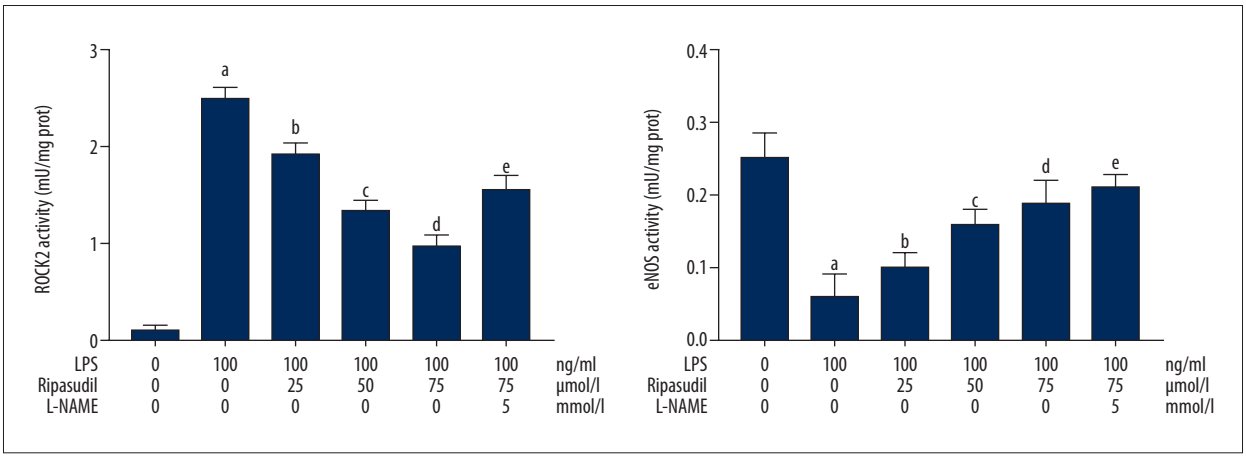

Figure 4. Columns in the upper panel indicate the ROCK2 activity in PMVECs treated with LPS, ripasudil, and/or L-NAME. Columns in the lower panel indicate the eNOS activity in PMVECs treated with LPS, ripasudil, and/or L-NAME [[a] differences were significant when compared with Control ($p<0.05$), [b] differences were significant when compared with LPS+LRip ($p<0.05$), [c] differences were significant when compared with LPS+MRip ($p<0.05$), [d] differences were significant when compared with LPS+HRip ($p<0.05$), [e] differences were significant when compared with LPS+HRip+L-NAME ($p<0.05$)].

Ripasudil depressed ROCK2 activity and recovered eNOS activity in LPS-exposed HPMVECs (Figure 4). LPS exposure significantly increased ROCK2 activity and decreased eNOS activity. The administration of ripasudil, however, increased eNOS activity and reduced ROCK2 activity in a concentration-dependent manner. The co-administration of L-NAME impaired the effects of ripasudil on eNOS activity.

Ripasudil regulated activation of the ROCK2/eNOS signaling pathway in LPS-exposed HPMVECs (Figure 5). LPS exposure significantly increased expression levels of ROCK2, TNFα, IL6, and active caspase3, as well as the nuclear translocation level of NF-κB, but decreased the expression level of Bcl2 and the phosphorylation level of eNOS in cultured HPMVECs. The administration of ripasudil dramatically increased the phosphorylation level of eNOS and the expression level of Bcl2 in a concentration-dependent manner. Administration of ripasudil significantly decreased the expression levels of active caspase3, TNFα, and IL6, as well as the nuclear translocation level of NF-κB. Moreover, the administration of L-NAME dramatically impaired the effects of ripasudil on phosphorylation level of eNOS, nuclear translocation level of NF-κB, and expression levels of Bcl2, active caspase3, TNFα, and IL6.

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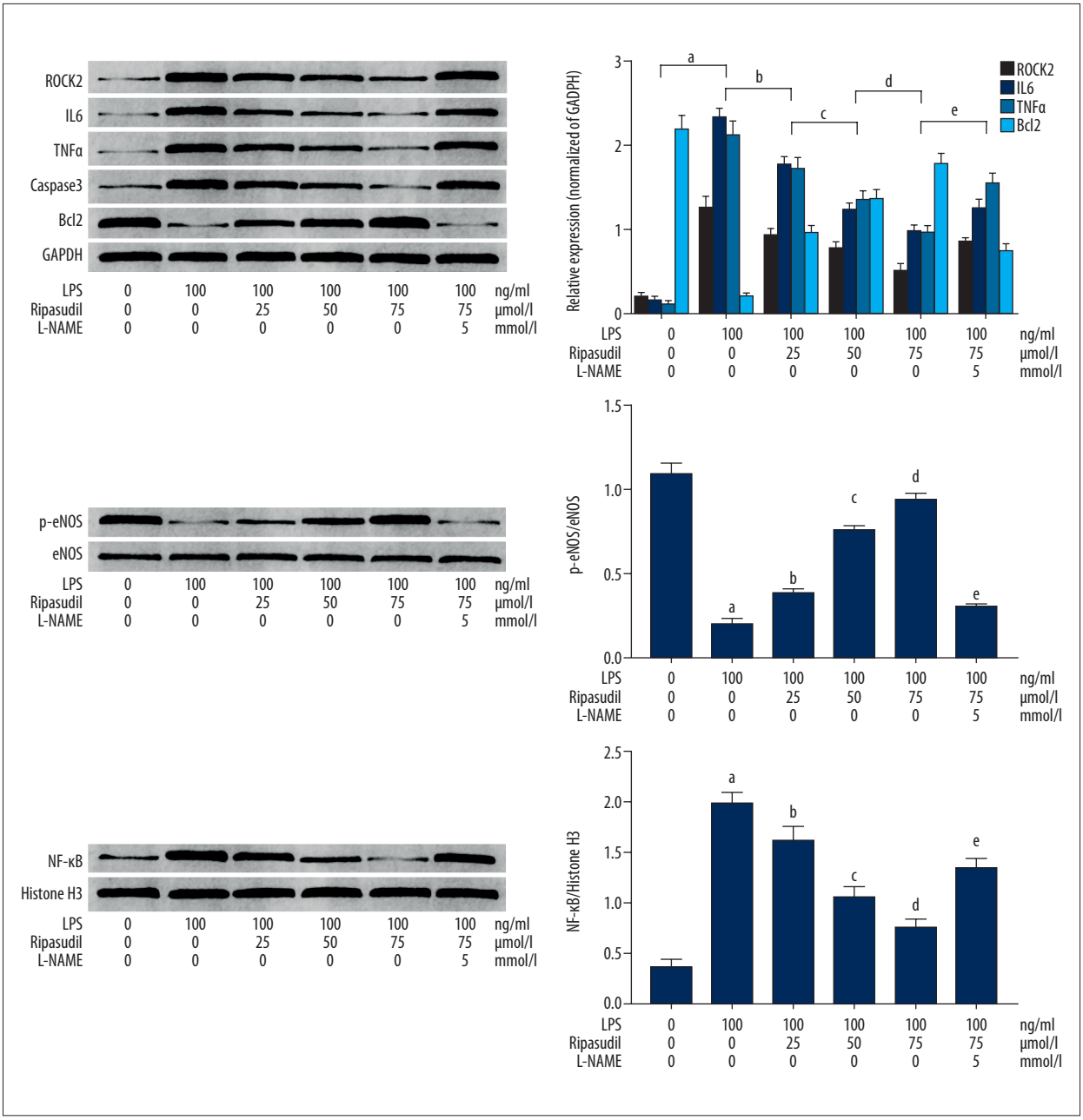

Figure 5. Immunoblots of ROCK2, IL6, TNFα, caspase3, Bcl2, and GAPDH are demonstrated in the left side of the upper panel. Columns in the right side of the upper panel indicate the relative expression levels of ROCK2, IL6, TNFα, caspase3, and Bcl2 in PMVECs treated with LPS, ripasudil, and/or L-NAME. Immunoblots of p-eNOS and eNOS are demonstrated in the left side of the middle panel. Columns in the right side of the middle panel indicate the phosphorylation level of eNOS in PMVECs treated with LPS, ripasudil, and/or L-NAME. Immunoblots of NF-κB and Histone H3 are demonstrated in the right side of the lower panel. Columns on the right side of the lower panel indicate the nuclear translocation level of NFκB in PMVECs treated with LPS, ripasudil, and/or L-NAME [[a] differences were significant when compared with Control ($p<0.05$), [b] differences were significant when compared with LPS+LRip ($p<0.05$), [c] differences were significant when compared with LPS+MRip ($p<0.05$), [d] differences were significant when compared with LPS+HRip ($p<0.05$), [e] differences were significant when compared with LPS+HRip+L-NAME ($p<0.05$)].

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Discussion

LPS is one of the major components of gram-negative bacterial outer membranes and acts as an endotoxin participating in septic ALI by triggering severe microcirculatory disorders [14]. LPS was reported to induce vascular leakage by increasing the permeability of pulmonary microcirculation, which facilitates the ventilation-perfusion imbalance [4]. The mechanism of elevation of circulatory permeability is associated with increased vascular endothelial apoptosis. Moreover, recovery of endothelial cell proliferation was reported to improve ALI [14]. In the present study, HPMVECs were exposed to LPS. The results showed that LPS induced apoptosis and thus reduced proliferation of HPMVECs. Inflammation is another vital characteristic of ALI, playing a role in initiation and progression of ALI [15]. Excessively produced inflammatory factors were found in bronchoalveolar lavage fluid of individuals with ALI [16]. These inflammatory factors recruit accumulation of immune cells such as neutrophils, which damages the structure of pulmonary alveoli and the vascular wall. In the present study, we found that LPS incubation dramatically up-regulated the synthesis and secretion of the identical inflammatory factors IL6 and TNFα in cultured HPMVECs.

As a small GTPase, Rho is activated by guanine nucleotide exchange factors (GEF). The GTP-bond RhoA subsequently activates its down-stream effector ROCKs, which further activate its substrates by phorphorylation [17]. ROCK is an important member of the cAMP-dependent protein kinase/protein kinase G/protein kinase C (AGC) kinase family, which has 2 major isoforms: ROCK1 and ROCK2. The facilitating roles of activation of RhoA/ROCK in many vascular diseases were suggested by previous studies [18]. It has been established that eNOS s one of the substrates of ROCK2 and acts as a vascular protective factor [19]. In this study, we found that in HPMVECs exposed to LPS, the expression level and activity of ROCK2 increased while the phosphorylation level of eNOS decreased significantly. Moreover, by measuring the production of NO, which is considered to be a strong marker of eNOS activity and direct eNOS activity measurements, the suppression of eNOS activity in LPS-exposed HPMVECs was confirmed.

eNOS regulates vascular endothelial function via multiple pathways. It was reported that eNOS exerts anti-apoptotic effects by increasing the transcription of several anti-apoptotic genes such as bcl2 [20]. The mechanism of the anti-inflammatory effect of eNOS was proposed to be correlated with the regulation of nuclear factor NF-κB [21]. It was reported that activated eNOS inhibits nuclear translocation of NF-κB, resulting in reduced expression of inflammatory factors [22]. In the present study, the ROCK2 inhibitor ripasudil was used to treat LPS-exposed HPMVECs. Ripasudil was developed as a more potent ROCK inhibitor and the steric affinity was enhanced by the

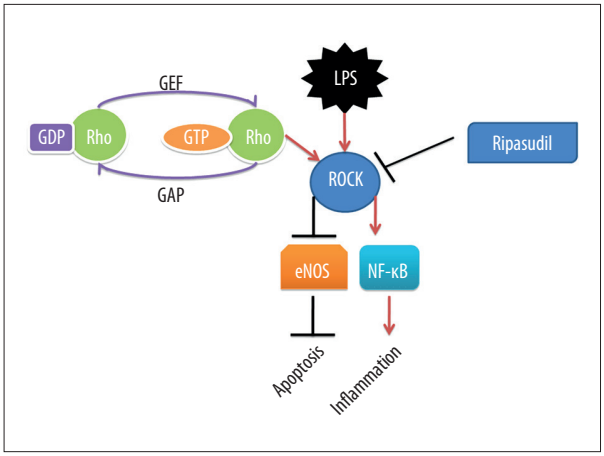

Figure 6. Diagram of protective effects of the molecular mechanism of ripasudil on LPS-induced endothelial inflammation and apoptosis.

structural alterations. Ripasudil exhibits high selectivity for ROCK inhibition, especially for ROCK2. We found that ripasudil treatment dramatically decreased ROCKs activity without altering its expression. As a result, the phosphorylation level of eNOS was recovered. The expression level anti-apoptotic protein Bcl2 was increased, which suppressed the expression of active caspase3. Moreover, the nuclear translocation of NF-κB was inhibited by ripasudil and the levels of the inflammatory cytokines IL6 and TNFα were decreased. These results show that ripasudil administration increases cell viability by reducing apoptosis and inhibiting inflammation.

To verify our conclusions, L-NAME, the inhibitor of eNOS, was co-administered with ripasudil to LPS-exposed HPMVECs, showing that co-administration of L-NAME impaired the anti-apoptosis and anti-inflammatory of ripasudil on LPS-treated HPMVECs. Further investigation of the mechanism revealed that L-NAME reduced phosphorylation and activity of eNOS without affecting ROCK2 activity. Taken together, our results show that inhibiting ROCK/eNOS signaling is highly associated with the anti-inflammatory and anti-apoptotic effects of ripasudil in LPS-treated HPMVECs. Figure 6 is a diagram of the involved molecular mechanism. The clinical application of ripasudil is limited because the evidence of its efficacy is still not sufficient.

Conclusions

The anti-inflammatory and anti-apoptotic effects of ripasudil were investigated only *in vitro* instead of *in vivo* in this study. Additionally, the information concerning the biosafety of ripasudil should be taken into consideration. We believe that results from this study will provide new evidence for the therapeutic value of ripasudil in treatment of ALI. However, more pre-clinical investigations are needed in the future.

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