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EphA2 antagonism alleviates LPS-induced acute lung injury via Nrf2/HO-1, TLR4/MyD88 and RhoA/ROCK pathways

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ABSTRACT

Eph receptor tyrosine kinases have a wide range of biological functions and have gradually been recognized increasingly as key regulators of inflammation and injury diseases. Although previous studies suggested that EphA2 receptor may be involved in the regulation of inflammation and vascular permeability in injured lung, the detailed effects of EphA2 on LPS-induced acute lung injury (ALI) are still inadequate and the underlying mechanism remains poorly understood. In this study, we detected the effects of EphA2 antagonism on inflammation, pulmonary vascular permeability and oxidative stress in LPS-induced ALI and investigate the potential mechanism. Our results showed that EphA2 antagonism markedly inhibited the cytokines release and inflammatory cells infiltration in BALF, prevented the LPS-induced elevations of MPO activity and MDA level in lung tissues. Our study also found that EphA2 antagonism significantly decreased the wet/dry ratios, reduced the Evans blue albumin extravasation in lung tissues and obviously alleviated the LPS-induced increment of pulmonary vascular permeability. Mechanistically, EphA2 antagonism significantly increased the activation of Nrf2 along with its target antioxidant enzyme HO-1 and inhibited the expressions of TLR4/MyD88 in lung tissues and A549 alveolar epithelial cells. Furthermore, EphA2 antagonism dramatically inhibited the LPS-evoked activations of RhoA/ROCK in lung tissues. In conclusion, our data indicate that EphA2 receptor plays an essential role in LPS-induced ALI and EphA2 antagonism has protective effects against LPS-induced ALI via Nrf2/HO-1, TLR4/MyD88 and RhoA/ROCK pathways. These results suggest that antagonism of EphA2 may be an effective therapeutic strategy for the treatment of ALI.

1. Introduction

Acute lung injury (ALI) and its severer form acute respiratory distress syndrome (ARDS) represent a continuum of a clinical syndrome of respiratory failure due to refractory hypoxia [1,2]. The critical pathophysiological processes of ALI are overwhelming inflammation, oxidative stress and alveolar barrier dysfunction. In the past 40 years, there have been significant advances in the treatment of ALI, including low tidal volume ventilation, restrictive fluid resuscitation and higher positive end-expiratory pressure [3]. In spite of these advances, the clinical mortality rate of ALI remains extremely high, up to 45% for severe ALI [4]. Therefore, it is very important to further clarify the pathogenesis of ALI. Novel treatment methods for ALI are urgently needed.

The Eph receptor tyrosine kinase family consists of fourteen members in mammalian systems. According to binding specificity and sequence homology of their membrane-bound ligands, the Eph receptor family are divided into EphA and EphB kinases. Previous research indicated that EphA2 receptor maybe play a crucial role in the pathogenesis of ALI. Researchers found that EphA2 expression was markedly up-regulated in the lung tissues of hypoxic infected rats and EphA2 signaling may be responsible for the regulation of vascular permeability in ALI [5,6]. Another research demonstrated that EphA2 knockout mice had the reduced permeability and less inflammatory response than wild type mice in bleomycin-induced lung injury model [7]. Recently, it has been reported that EphA2 antagonism may inhibit the PI3K-Akt pathway and attenuate inflammation [8]. Furthermore, it has been shown that EphA2 receptor serves as an unrecognized modulator of several inflammation pathways, including Src-NF-κB and PI3K-Akt-NF-κB in lung injury [9]. However, the data of EphA2 receptor for the regulation of inflammatory response, oxidative stress and pulmonary permeability in LPS-induced ALI is still limited and specially, the underlying mechanism remains poorly understood. So a better understanding of the role of EphA2 in LPS-induced ALI is mandatory to clarify the mechanisms of ALI and to develop a new therapeutic strategy for

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the treatment of ALI.

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a regulator of cellular resistance to oxidative stress. The physiological effects of Nrf2 and its downstream target antioxidant enzyme heme oxygenase-1 (HO-1) have been proved to be essential for cytoprotection against oxidant and inflammatory damage [10]. Furthermore, previous studies explored the association between Nrf2/HO-1 and some key inflammatory regulatory pathways and found activation of Nrf2/HO-1 pathway could inhibit NF-κB pathway [11]. Thus, Nrf2/HO-1 pathway plays a crucial role in the pathogenesis of ALI and serves as an essential signaling pathway during ALI [12]. As a member of Toll-like receptor family that specifically recognizes LPS, toll-like receptor 4 (TLR4) plays a principal role in the gram-negative bacteria infection-induced lung injury [13]. Binding to LPS, the activation of TLR4 leads to trigger the downstream inflammatory signaling pathways and initiate the release of inflammatory cytokines through a myeloid differentiation factor 88 (MyD88)-dependent pathway, which is responsible for the development of inflammatory cascade reaction in ALI [14]. The increment of pulmonary vascular permeability is an important landmark event in the development of ALI. Rho-kinase (ROCK) is a serine/threonine kinase, which has been identified as the first downstream target of the small GTP-binding protein RhoA. RhoA/ROCK signaling pathway has been previously shown to mediate the enhancement of pulmonary endothelial permeability and play an important pathophysiological role in ALI [15,16].

Based on these previous findings, we test the hypothesis that EphA2 receptor plays an essential role in LPS-induced ALI. The aim of this study was to explore the effects of EphA2 antagonism on inflammatory response, oxidative stress and pulmonary vascular permeability in LPS-induced ALI and investigate the potential mechanism.

2. Materials and methods

2.1. Major reagents

Lipopolysaccharide (*E. coli* 055:B5) was purchased from Sigma Chemical Company. The enzyme linked immunosorbent assay (ELISA) kits for determinations of IL-6 and TNF-α and the EphA2 monoclonal antibody were supplied by American R&D Systems. The myeloperoxidase (MPO) and the malondialdehyde (MDA) determination kits were obtained from the Jiangsu Nanjing Jiancheng Bioengineering Institute. The HE test kit was obtained from the Beyotime Biotechnology. Anti-Nrf2, anti-HO-1 and anti-MyD88 antibodies were obtained from Abcam (Cambridge, MA, USA). Anti-RhoA, anti-TLR4 and anti-p-MYPT1 antibodies were supplied by Santa Cruz Biotechnology.

2.2. Animals and experimental design

Male SD rats were obtained from Shanghai Experimental Animal Center, which were weighing approximately 250–300 g. Animals were housed at a constant room temperature with normal circadian rhythm and fed with a standard rodent water and diet. Rats were randomized into the following four groups ($n = 6$): (1) control group, (2) LPS group (LPS 5 mg/kg intratracheal instillation), (3) LPS + EphA2 monoclonal antibody (25 μg/kg) group, (4) LPS + EphA2 monoclonal antibody (50 μg/kg) group. The animal model of acute lung injury was made by intratracheal instillation of LPS (5 mg/kg) according to previous studies [17]. All animal experimental procedures were in accordance with the guidelines of the National Institutes of Health. In this study, the effects of EphA2 in the lungs were antagonized by injection of EphA2 monoclonal antibody (EphA2 Ab) according to previous studies [5,8,18]. EphA2 monoclonal antibody (25, 50 μg/kg) was subcutaneously injected 30 min before LPS challenge. Bronchial lavage and sample acquisition were performed at 7 h after LPS administration.

2.3. Cell culture and intervention

Alveolar epithelial cells (A549) were supplied by the oncology laboratory of Beijing Shijitan Hospital. A549 Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin in a cell culture incubator (5% CO_2, 37 °C). Then, cells were seeded into six-well plates and were divided into 3 groups: control group, LPS group and LPS + EphA2 Ab group. LPS group received LPS stimulation (10 μg/ml) and LPS + EphA2 Ab group was treated with EphA2 monoclonal antibody (5 μg/ml) 15 min before LPS stimulation. Six hours after LPS stimulation, cell samples were collected for the following western blot detection.

2.4. Bronchoalveolar lavage fluid collection and cell count

The rat lungs were intratracheal lavaged by 5 ml ice-cold PBS and the total collection rate was about 85%. Then, the collected fluids were centrifuged for 10 min at 1500/min 4 °C. The BCA protein assay kit was used to determinate the concentration of total protein in the supernatant. Subsequently, 50 μl PBS was used to re-suspend the cell pellet and the numbers of neutrophils, macrophages and total cells were counted using a hemocytometer.

2.5. The wet to dry lung weight ratio (W/D ratio) measurement

After rats were sacrificed, the lungs were excised and weighed immediately to record the wet weight. Then, the lungs were placed in the oven for 72 h at 80 °C to get the stable dry weight. Finally, the wet/dry ratio was calculated for the assessment of pulmonary edema.

2.6. Determination of TNF-α and IL-6 in BALF

The concentrations of inflammatory cytokines in bronchoalveolar lavage fluid were detected with the commercial enzyme linked immunosorbent assay (ELISA) kits from R&D system following the manufacturer's recommendations.

2.7. Measure of MPO activity and MDA level in lung tissues

Lung samples were homogenized in HEPES followed by thawed and centrifuged. Subsequently, the MPO activities in lung homogenates were assayed by using commercial test kits according to manufacturer's instructions. The levels of MDA in lung tissues were evaluated with the MDA detection kit supplied by Nanjing Jiancheng Corporation. Procedures were according to manufacturer's instructions.

2.8. Histopathologic evaluation

When rats were sacrificed, the lungs were recruited and fixed with buffered formalin (10%) for 48 h at 4 °C. Subsequently, the lung tissues were embedded in paraffin and cut into 5 μm sections. The sections were stained with hematoxylin and eosin (H&E) reagent and visualized with a light microscope.

2.9. RhoA activation assay

RhoA activity was assessed by a pull-down assay using the RhoA activation assay kit according to the manufacturer's guidance. As previously reported [19,20], lung tissues were lysed in MLB buffer or hypertonic. Then, immunoprecipitation was used to separate of GTP-bound RhoA from cleared lysate with glutathione S-transferase-tagged Rhotekin Rho-binding domain protein bound to glutathione agarose. Subsequently, the beads were washed and western blot analysis was used to analyze the immunoprecipitates.

2.10. Western blot analysis

We extracted proteins from lung tissues or cell samples and determined the protein concentrations of samples by BCA method. Then, equal amounts of protein were fractionated on 12% polyacrylamide SDS gel and transferred to a polyvinylidene difluoride membrane. Next, 5% nonfat dry milk was used to block the membrane at room temperature for 2 h. After that, the membrane was incubated with the primary antibodies (1:1000) in the refrigerator at 4 °C overnight. Subsequently, the membrane was incubated with HRP-conjugated secondary antibodies (1:10000) at room temperature for 2 h and visualized by the enhanced chemiluminescence detection system (ECL).

2.11. Evaluation of lung capillary leakage

In this study, we used Evans blue dye to evaluate lung capillary leakage which reflects pulmonary vascular permeability. Evans blue dye (20 mg/kg) was intravenously injected 1 h before rats were sacrificed. Then, rat lung tissues were homogenized with formamide and incubated at 37 °C for 18 h. Subsequently, the supernatant was collected and the optical density was determined at 620 nm with spectrophotometer after centrifugation. The content of Evans blue dye in lung homogenate was calculated according to a standard curve and was presented as micrograms of Evans blue dye/g of lung tissue.

2.12. Statistical analysis

All data were expressed as means ± SEM. Differences between more than two sets of data were assessed with one-way ANOVA followed by Tukey's post hoc test with SPSS11.0 and significant differences were considered if $P < 0.05$.

3. Results

3.1. Effects of EphA2 antagonism on LPS-mediated lung histopathological changes

The histopathological changes of rat lung tissues were displayed in Fig. 1. Control group showed normal structure with intact alveoli. In LPS group, LPS challenge caused obvious pathologic changes, such as thickening of alveolar wall, alveolar collapse, abundant inflammatory cell infiltration, pulmonary edema and haemorrhagia in stroma. However, these alterations and the destruction of lung structure were effectively alleviated by EphA2 antagonism in LPS + EphA2 Ab groups.

3.2. Effects of EphA2 antagonism on the lung W/D ratio

The lung W/D ratio was measured to evaluate the degree of pulmonary edema. As displayed in Fig. 2A, compared with control group, the lung W/D ratio of LPS group produced a remarkable increase. However, the increment of W/D ratio was remarkably suppressed by EphA2 antagonism. The results indicated that EphA2 antagonism was capable of reducing the pulmonary water content in LPS-induced ALI.

3.3. Effects of EphA2 antagonism on the content of total protein in BALF

In this study, the content of total protein in BALF was detected to assess LPS-induced changes of pulmonary vascular leak. As displayed in Fig. 2B, the concentration of total protein increased evidently 7 h after LPS challenge. However, relative to LPS group, EphA2 antagonism significantly reduced the level of total protein in BALF.

3.4. Regulation of EphA2 receptor on LPS-induced pulmonary vascular permeability

To further investigate the regulation of EphA2 receptor on the pulmonary vascular permeability of LPS-induced lung injury, we evaluated the Evans blue albumin extravasation from the vascular space into surrounding lung tissues. As illustrated in Fig. 2C, LPS challenge dramatically raised the accumulation of Evans blue in rat lung tissues. However, the increases of Evans blue values in lung tissues were significantly attenuated by EphA2 antagonism. These results suggested that EphA2 antagonism was able to reduce the LPS-induced enhancement of pulmonary vascular permeability.

3.5. Effects of EphA2 antagonism on the levels of TNF-α and IL-6 in BALF

Previous researches have proven that the increase of explosive inflammatory cytokines is a typical manifestation of ALI and the levels of inflammatory cytokines are closely related to the prognosis of ALI. In

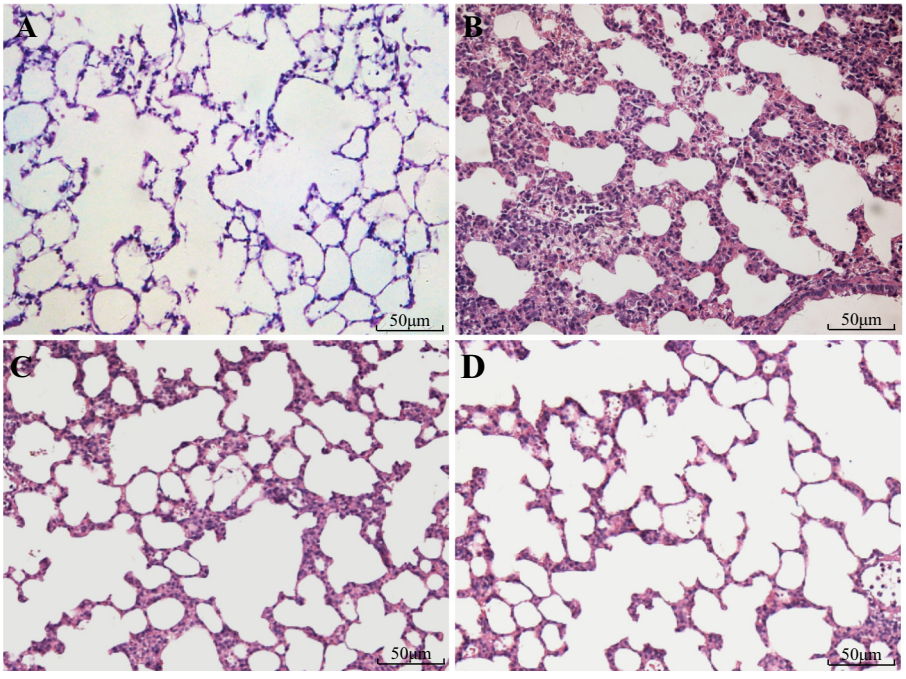


Fig. 1. Histologic assessment of the effects of EphA2 antagonism on LPS-induced ALI. Rats were given an injection of EphA2 monoclonal antibody 30 min before LPS administration. Lungs from each experimental group were processed for histological evaluation at 7 h after LPS administration. (A) Control group, (B) LPS group, (C) LPS + EphA2 Ab (25 μg/kg) group, (D) LPS + EphA2 Ab (50 μg/kg) group.

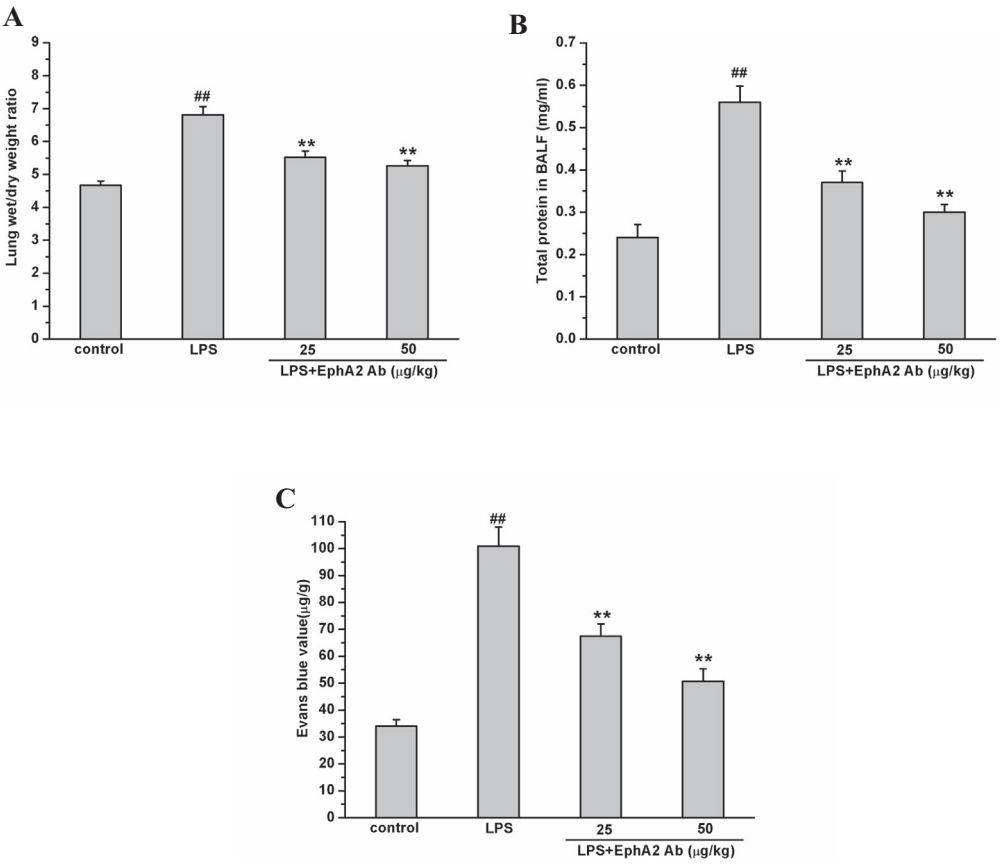


Fig. 2. Effect of EphA2 antagonism on pulmonary vascular permeability in LPS-induced ALI. Rats were given an injection of EphA2 monoclonal antibody 30 min before LPS administration. (A) The lung wet/dry weight ratio, (B) the total protein content of BALF and (C) the lung Evans blue values were determined at 7 h after LPS administration. Data are presented as means ± SEM ($n = 6$). $^{\#\#}P < 0.01$ versus control group; $^{**}P < 0.01$, versus LPS group.

the present study, we examined the classical inflammatory cytokines TNF-α and IL-6 of ALI by ELISA. The results showed that the levels of TNF-α and IL-6 markedly increased 7 h after LPS administration. However, EphA2 antagonism obviously suppressed the levels of those inflammatory cytokines in BALF (Fig. 3A and B).

3.6. Effects of EphA2 antagonism on cells counts in BALF

In the present study, cells counts (neutrophils, macrophages and total cells) in BALF were detected to examine the influences of EphA2 antagonism on pulmonary inflammatory cell infiltration. As displayed in Figs. 3, 7 h after LPS challenge, cell counts (neutrophils, macrophages and total cells) remarkably increased in BALF. Relative to LPS group, this increase was obviously reduced by EphA2 antagonism in LPS + EphA2 Ab groups.

3.7. Effects of EphA2 antagonism on MPO activity and MDA level in rat lung tissues

It is well known that MPO activity can be used as a marker of neutrophil activation. In the present study, our results demonstrated that the MPO activity of rat lung tissues was significantly enhanced by LPS challenge. However, EphA2 antagonism significantly inhibited the LPS-induced MPO activity (Fig. 4A). It has been proved that MDA is the end product of polyunsaturated fatty acids and is commonly used as a biomarker for assessing oxidative stress. In our study, LPS stimulation obviously induced the generation of MDA, whereas EphA2 antagonism effectively reduced the MDA content of rat lung tissues in LPS + EphA2 Ab groups (Fig. 4B).

3.8. Regulation of EphA2 receptor on the activity of Nrf2/HO-1 in lung tissues of LPS-challenged rats and A549 alveolar epithelial cells

As an oxidative stress sensing genetic transcription factor, Nrf2 is considered to be a major regulator of cellular responses to LPS-induced oxidative damage. In this study, to analyze the activation of Nrf2, we detected the nuclear expression of Nrf2 in lung tissues by western blot analysis. According to Fig. 5A and B, although LPS alone also induced the increase in the nuclear expression of Nrf2, EphA2 antagonism treatment evoked a more significant rise of Nrf2 nuclear expression in lung tissues. The results indicated that EphA2 antagonism may markedly enhance the activation of Nrf2 in LPS-induced ALI. On the other hand, we investigated the effects of EphA2 antagonism on HO-1 expression in lung tissues of ALI rats. As shown in Fig. 5A and C, LPS administration significantly up-regulated the expression of HO-1 in LPS group and the expressions of HO-1 were further increased by EphA2 antagonism treatment in LPS + EphA2 Ab groups.

Based on the above findings, our results suggested that EphA2 antagonism treatment could effectively activate the antioxidant pathway Nrf2/HO-1 in LPS-induced ALI. Subsequently, we confirmed these findings in vitro experiment. Similarly, as illustrated in Fig. 5E and F, the results in cell experiment demonstrated that antagonism of EphA2 receptor could significantly activate Nrf2/HO-1 pathway in LPS-stimulated A549 cells relative to LPS group.

3.9. Regulation of EphA2 receptor on the activity of TLR4/MyD88 in lung tissues of LPS-challenged rats and A549 alveolar epithelial cells

To explore whether antagonizing EphA2 regulates the expression and function of TLR4 which is the receptor for LPS, the expressions of TLR4 and MyD88 were examined in rat lung tissues. As indicated in Fig. 6B and C, western blotting results showed a significant increase of TLR4 and MyD88 protein expressions in LPS group, while EphA2

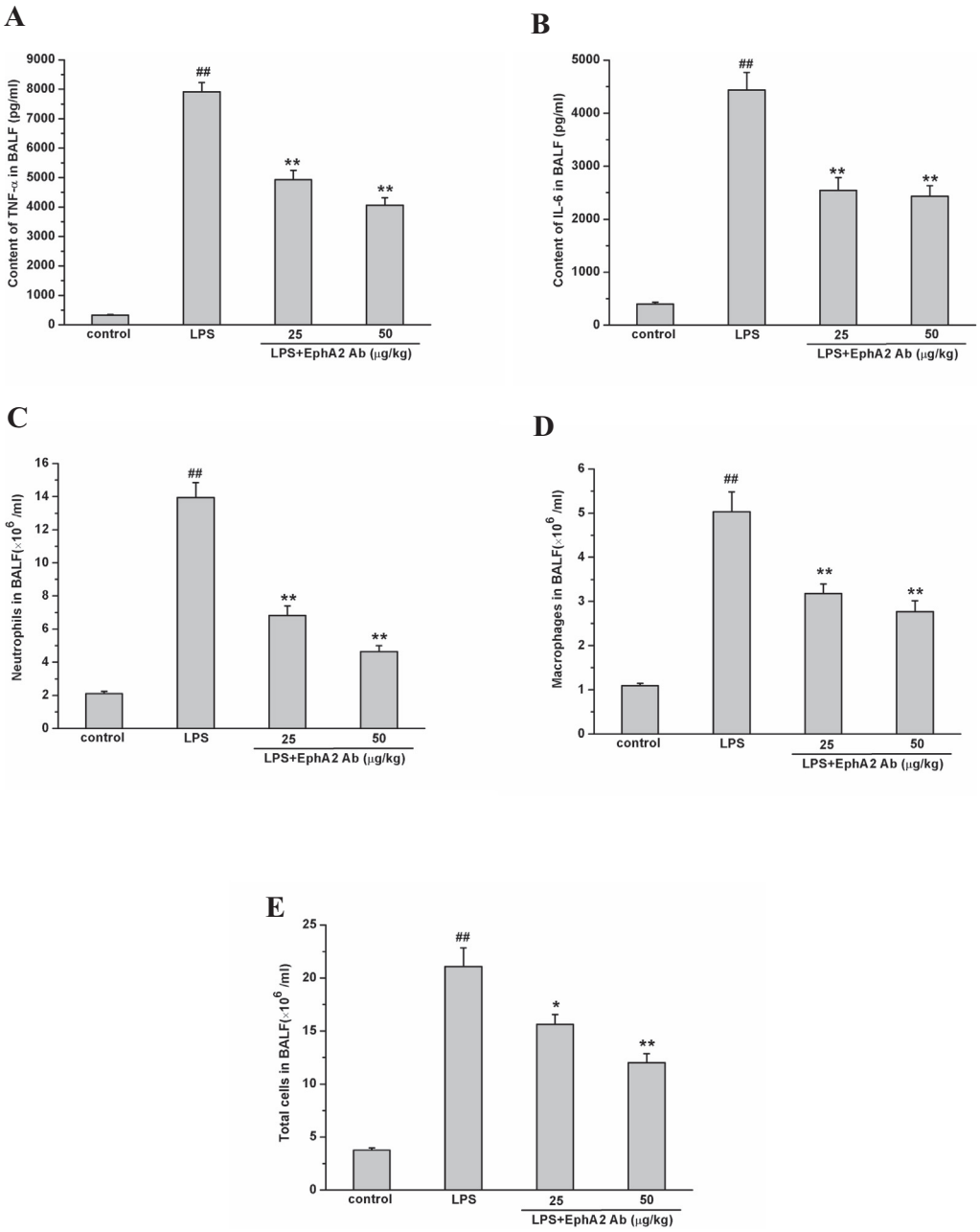


Fig. 3. Effect of EphA2 antagonism on pulmonary inflammation of LPS-induced lung injury based on analysis of BALF. Rats were given an injection of EphA2 monoclonal antibody 30 min before LPS administration. BALF was collected at 7 h after LPS administration to analyze the inflammatory cytokines (A) TNF-α and (B) IL-6. The numbers of (C) neutrophils, (D) macrophages and (E) total cells were also measured in BALF. Data are presented as means ± SEM ($n = 6$). $^{\#\#}P < 0.01$ versus control group; $^{*}P < 0.05$, versus LPS group; $^{**}P < 0.01$, versus LPS group.

antagonism markedly attenuated this increase of TLR4 and MyD88 protein expressions in LPS+ EphA2 Ab groups. As expected, similar results were observed in vitro (Fig. 6E and F). The protein levels of TLR4 and MyD88 are both increased in LPS-stimulated A549 cells but decreased under the action of EphA2 antagonism.

3.10. Regulation of EphA2 receptor on the activity RhoA/ROCK pathway in lung tissues of LPS-challenged rats

To identify the activation of RhoA/ROCK pathway in lung tissues of LPS-challenged rats, the expression of RhoA-GTP were measured which represents the activation of RhoA. As shown in Fig. 7A and B, relative to control group, the activity of RhoA was significantly enhanced after LPS administration. However, EphA2 antagonism apparently inhibited the activation of RhoA in LPS-challenged rats. On the other hand, to investigate the activity of ROCK, we assessed the protein level of phosphorylated-myosin phosphatase target subunit 1 (p-MYPT1) which is the specific substrate of ROCK and represents the activation of ROCK. Relative to control group, the protein level of p-MYPT1 in lung tissues was significantly up-regulated by LPS challenge. However, as shown in Fig. 7A and C, EphA2 antagonism remarkably attenuated the increase of p-MYPT1 protein induced by LPS.

4. Discussion

The Eph receptor tyrosine kinases and their ephrin ligands are cell surface molecules with extensive biological functions that regulate cell behavior during both adult life and embryogenesis [21]. Emerging

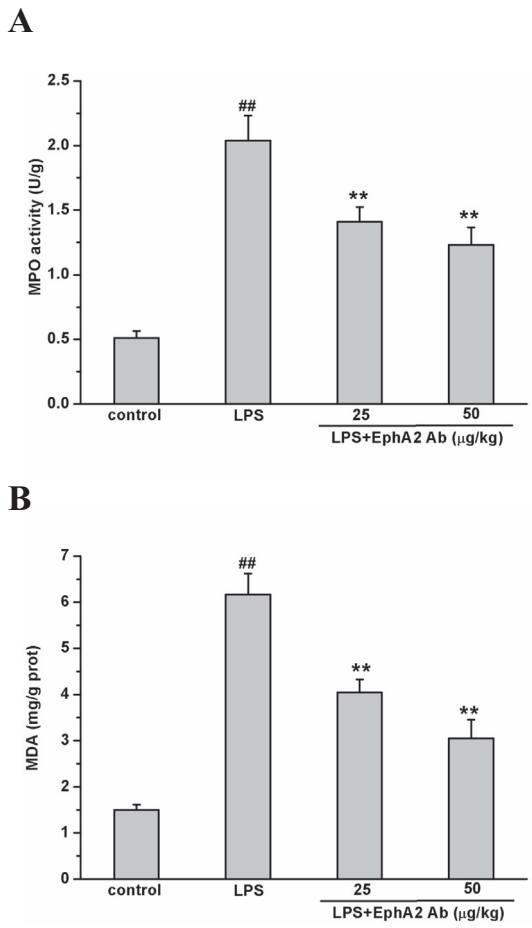


Fig. 4. Effect of EphA2 antagonism on MPO activity and MDA level in lung tissues. Rats were given an injection of EphA2 monoclonal antibody 30 min before LPS administration. (A) MPO activity and (B) MDA level were determined at 7 h after LPS administration. Data are presented as means ± SEM ($n = 6$). $^{\#\#}P < 0.01$ versus control group; $^{**}P < 0.01$, versus LPS group.

evidence suggests that EphA2 receptor plays important roles in a range of inflammatory diseases [22,23]. The aim of this present work was to investigate the role of EphA2 receptor on the regulation of the pulmonary inflammation, oxidative stress and vascular permeability in LPS-induced ALI and reveal the underlying molecular mechanism. Our results illustrated that EphA2 antagonism decreased the cytokines release, inflammatory cells activation and vascular permeability, alleviated LPS-induced lung damage and pulmonary edema. Moreover, EphA2 antagonism could effectively increase the activation of Nrf2/HO-1 and inhibit the activation of TLR4/MyD88 and RhoA/ROCK in LPS-challenged rats. Subsequently, the results of in vitro studies further confirmed the regulation of the EphA2 receptor on Nrf2/HO-1 and TLR4/MyD88 pathways. These data demonstrate that EphA2 receptor contributes to the pathophysiology of LPS-induced acute lung injury and regulates inflammation, oxidative stress and vascular permeability in injured lungs via Nrf2/HO-1, TLR4/MyD88 and RhoA/ROCK pathways. Our study for the first time reported the regulation of the EphA2 receptor on the Nrf2/HO-1, TLR4/MyD88 and RhoA/ROCK pathways in ALI. Our results suggested that EphA2 antagonism could attenuate the increase in RhoA-dependent pulmonary vascular permeability in LPS-induced ALI. It is meaningful for elucidating the molecular mechanism of EphA2 receptor regulation of pulmonary vascular permeability and future studies should continue to focus on this point.

Excessive inflammatory cells activation and accumulation into the alveolar space is the characteristic of ALI. Specially, neutrophil transmigration is closely related to pulmonary vascular permeability and endothelial barrier integrity [24]. Based on our findings, the numbers of macrophages and neutrophils in BALF were markedly reduced by EphA2 antagonism. MPO is an enzyme located mainly in the primary granules of neutrophils and the level of MPO is usually detected in lung tissues to assess the activation and pulmonary infiltration of neutrophils in ALI [25]. In the present study, treatment with EphA2 monoclonal antibody could obvious decrease the MPO activity in lung tissues after LPS administration which suggests that inhibition of EphA2 receptor may inhibit the LPS-induced activation of neutrophils in lung tissues.

To further assess the regulation of EphA2 antagonism on pulmonary inflammation, the changes of inflammatory cytokines were examined in BALF. Over-expressed inflammatory cytokines induced by LPS, such as TNF-α and IL-6, have been proved to participate in the development of inflammation and play an essential role in the pathogenesis of ALI [3,26]. These excessive cytokines not only amplify the inflammatory cascade and cause severe lung tissue damages, but also are closely associated with the increase of MPO activity and the recruitment of neutrophils to the lungs. It was reported that the persistent elevation of inflammatory cytokines during ALI/ARDS was a key cause of more severe outcomes [27]. In this study, antagonism of EphA2 receptor obviously inhibited the release of pulmonary inflammatory cytokines. The results suggested that the protective effects of EphA2 antagonism on injured lungs may be related to its inhibition of inflammatory mediators release and limitation of inflammatory response.

Hypoxemia caused by severe pulmonary edema is an important cause of death in patients with acute lung injury [28]. Endothelial injury associated increased vascular permeability was considered as the main reason of pulmonary edema in ALI [29]. Previous studies indicated that control of pulmonary vascular permeability is a critical factor in the clinical treatment of ALI, but the present therapy for regulating vascular permeability is still limited. In this study, the effects of EphA2 antagonism on lung permeability were assessed by four means. Firstly, pathological examination visually showed the reduction of pulmonary edema by EphA2 antagonism compared with LPS group. Secondly, we detected the lung W/D ratio and the protein extravasation. The results showed that the lung W/D ratio and the total protein in BALF were obviously decreased by EphA2 antagonism. This results further demonstrated that EphA2 receptor was involved in the regulation of pulmonary permeability in this ALI model. Thirdly, to further investigate and identify the effect of EphA2 antagonism on pulmonary vascular permeability, we directly measured the lung Evans blue albumin extravasation from the vascular space into surrounding lung tissue. Our data showed that the lung Evans blue values were significantly reduced by EphA2 antagonism relative to LPS group. Collectively, these results supported that EphA2 antagonism could effectively decrease pulmonary vascular permeability and thus alleviate pulmonary edema in LPS-induced ALI.

As above, the increment of pulmonary vascular permeability is a key event in the development of ALI/ARDS. Previous researches have confirmed that RhoA/ROCK pathway plays an essential pathophysiological role in the regulation of pulmonary vascular permeability [15,30,31]. The small GTP-binding protein RhoA and its downstream target Rho kinase (ROCK) regulate cellular proliferation, adherence and migration through control of cell contraction and actin–cytoskeletal assembly. It is well known that abnormal activation of RhoA/ROCK pathway could elevate vascular tone through unbalancing the production of vasoconstricting and vasodilating substances [32]. Therefore, inhibition of RhoA/ROCK pathway could decrease vascular permeability in a variety of pathological conditions. In the present study, the activation of RhoA and ROCK was investigated in lung tissues of LPS-treated rats. Our data showed that EphA2 antagonism could obviously inhibit the LPS-induced activation of RhoA and ROCK in lung tissues. The present finding is suggestive that EphA2 antagonism may decrease the increment of pulmonary vascular permeability via RhoA/ROCK pathway in LPS-induced ALI.

There are clear evidences that excessive oxidative stress plays a vital

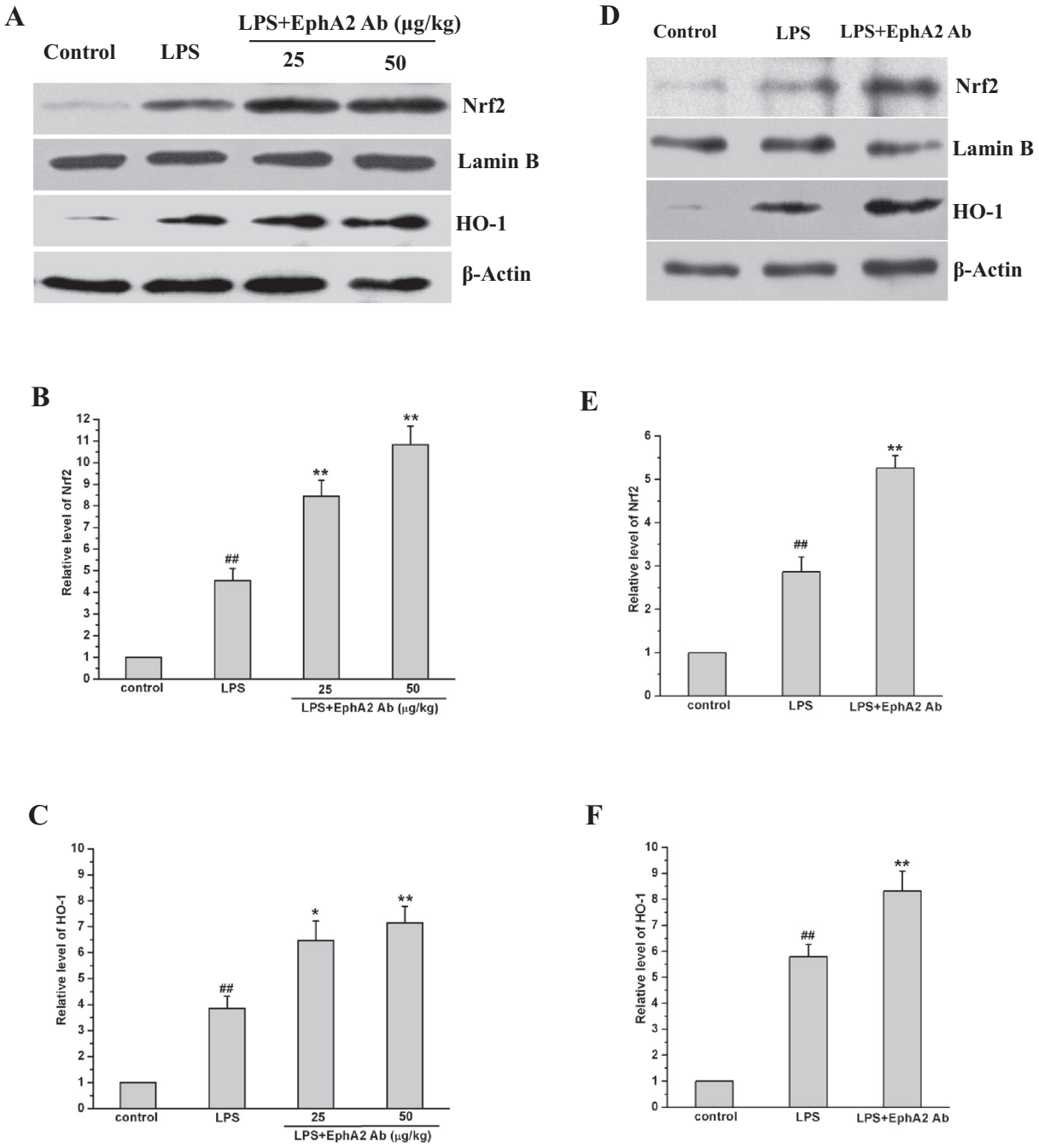


Fig. 5. Effect of EphA2 antagonism on the activation of Nrf2/HO-1 pathway in rat lung tissues and A549 alveolar epithelial cells. Rats were given an injection of EphA2 monoclonal antibody 30 min before LPS administration. A549 cells were treated with EphA2 monoclonal antibody (5 μg/ml) 15 min before LPS (10 μg/ml) stimulation. (A) Representative gels for Nrf2 and HO-1 in lung tissues, (B C) Quantitative analysis for Nrf2 and HO-1 in lung tissues, (D) Representative gels for Nrf2 and HO-1 in A549 cells, (E F) Quantitative analysis for Nrf2 and HO-1 in A549 cells. Data are presented as means ± SEM ($n = 6$). $^{\#\#}P < 0.01$ versus control group; $^{*}P < 0.05$, versus LPS group; $^{**}P < 0.01$, versus LPS group.

role in the pathogenesis of ALI [33,34]. Excessive oxidative stress could stimulate pulmonary inflammatory cells activation and mediates damages to alveolar epithelial cells and pulmonary vascular endothelial cells, leading to severe lung injury. As a member of the cap-N-collar family, Nrf2 is believed to be a major transcription factor that regulates the expression of antioxidant enzymes mediated by antioxidant response elements. Nrf2 plays an important regulatory role in the induction of a variety of cyto-protective genes. Among these, HO-1 is one of the genes regulated by Nrf2 and the induction of HO-1 can exert obvious protective effects against oxidative stress [35]. It is well documented that Nrf2/HO-1 signaling pathway also plays an important role in regulating oxidative stress and inflammation and served as a critical signaling pathway during ALI [36]. In our study, the activation of Nrf2/HO-1 signaling pathway was analyzed in lung tissues and A549 cells by western blotting. The results showed that LPS alone induced increase in the activation of Nrf2 and HO-1, while EphA2 antagonism treatment evoked a more significant activation of Nrf2 and HO-1. The results suggested that antagonism of EphA2 receptor may activate Nrf2 to enhance the expression of Nrf2 dependent genes, contributing to the protective effects of EphA2 antagonism on LPS-induced ALI.

TLR4 has been well documented as a pattern recognition receptor in acute infection-induced lung injury [37]. As an important receptor for

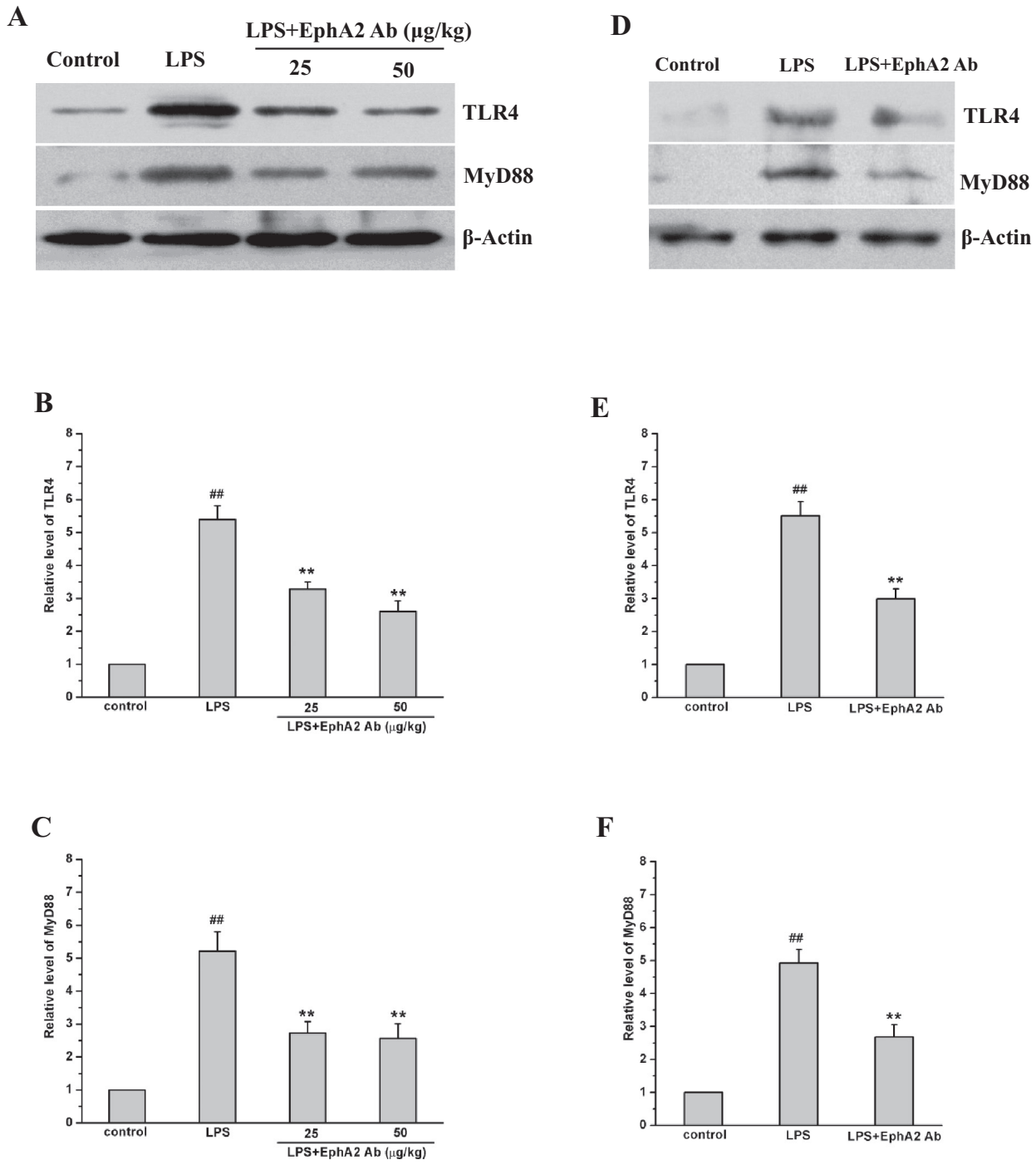
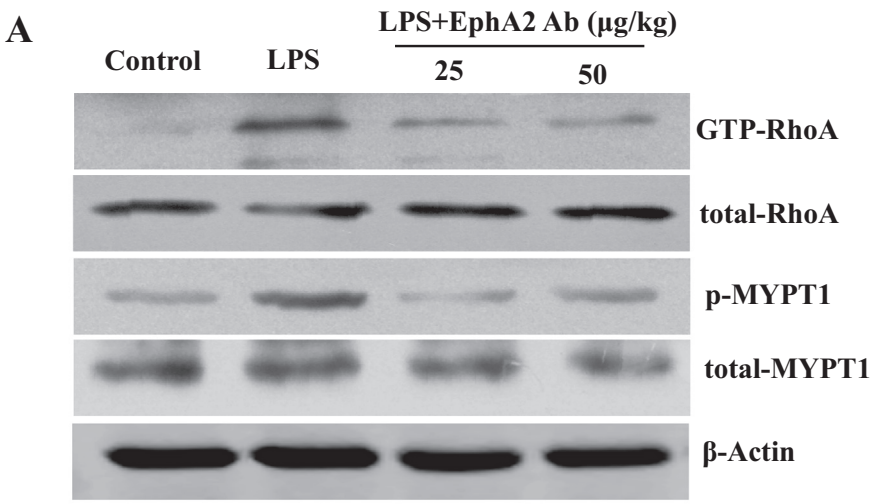


Fig. 6. EphA2 antagonism inhibited the expressions of TLR4 and MyD88 in rat lung tissues and A549 alveolar epithelial cells. Rats were given an injection of EphA2 monoclonal antibody 30 min before LPS administration. A549 cells were treated with EphA2 monoclonal antibody (5 μg/ml) 15 min before LPS (10 μg/ml) stimulation. (A) Representative gels for TLR4 and MyD88 in lung tissues, (B C) Quantitative analysis for TLR4 and MyD88 in lung tissues, (D) Representative gels for TLR4 and MyD88 in A549 cells, (E F) Quantitative analysis for TLR4 and MyD88 in A549 cells. Data are presented as means ± SEM ($n = 6$). $^{\#\#}P < 0.01$ versus control group; $^{**}P < 0.01$, versus LPS group.

LPS, the activation of TLR4 may trigger several inflammatory regulatory pathways including NF-κB and MAPK through a MyD88-dependent pathway, which eventually leads to the development of inflammatory cascade in LPS-induced ALI [14]. In this study, to confirm the impact of EphA2 receptor on the activation of TLR4/MyD88 pathway, the protein expressions of TLR4 and MyD88 were detected in vivo and in vitro. Our data showed that EphA2 antagonism could effectively attenuate the LPS-induced increase of TLR4 and MyD88 protein expressions in both lung tissues and A549 cells. These results clearly demonstrated the regulatory effects of EphA2 receptor on the TLR4/MyD88 pathway in LPS-induced ALI and our results supported that LPS receptor maybe an important target for EphA2 antagonism alleviating LPS-induced lung injury.

In summary, our results suggest that EphA2 antagonism may alleviate inflammation, oxidative stress and pulmonary vascular permeability and produce beneficial actions in LPS-induced acute lung injury. Specially, evidence from our study reveals novel potential mechanisms that EphA2 receptor may regulate Nrf2/HO-1, TLR4/MyD88 and



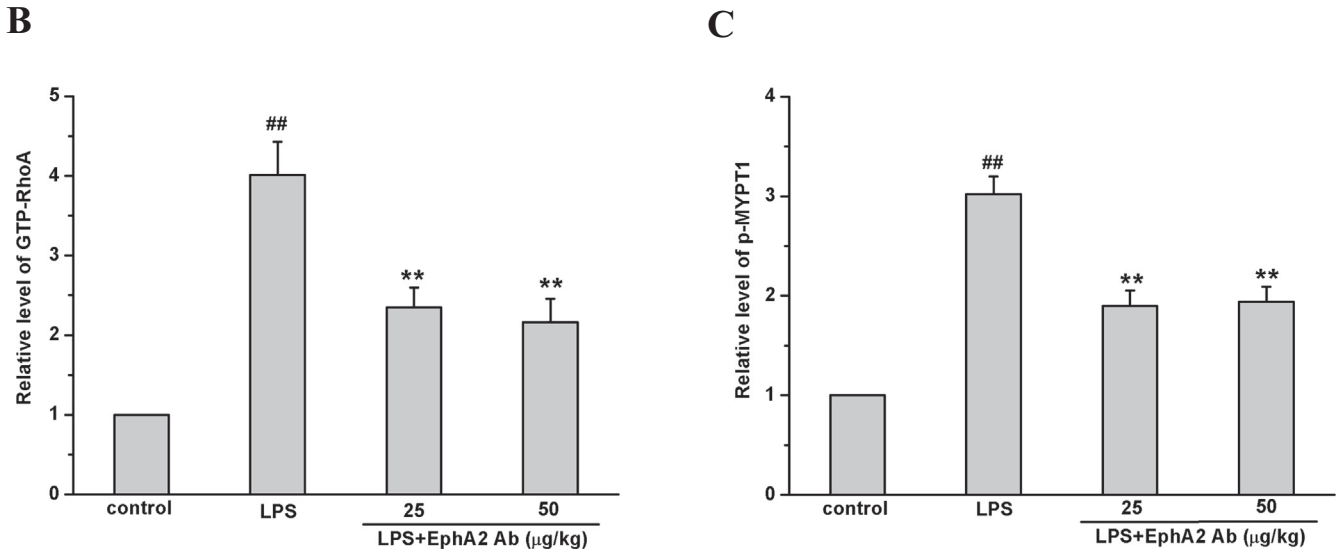


Fig. 7. EphA2 antagonism inhibited the activation of RhoA/ROCK pathway in lung tissues of LPS-challenged rats. Rats were given an injection of EphA2 monoclonal antibody 30 min before LPS administration. The expressions of (B) GTP-RhoA and (C) p-MYPT1 were detected by Western blotting. Data are presented as means ± SEM ($n = 6$). $^{\#\#}P < 0.01$ versus control group; $^{**}P < 0.01$, versus LPS group.

RhoA/ROCK pathways in this model. Therefore, EphA2 antagonism may be an effective therapeutic strategy for the treatment of LPS-induced ALI.

Conflicts of interest

All authors declare that they have no conflict of interest.

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