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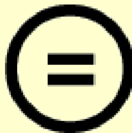
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The role of EphA2 signaling in Lipopolysaccharide-induced lung injury

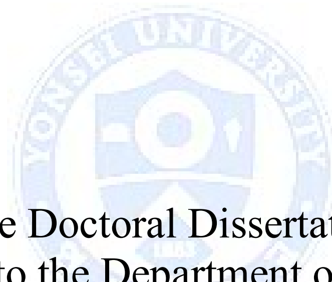
Ji Young Hong

Department of Medicine

The Graduate School, Yonsei University

The role of EphA2 signaling in Lipopolysaccharide-induced lung injury

Directed by Professor Young-Sam Kim



The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the
degree of Doctor of Philosophy

Ji Young Hong

June 2015

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ABSTRACT

The role of EphA2 signaling in Lipopolysaccharide-induced lung injury

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The Graduate School, Yonsei University

(Directed by Professor Young-Sam Kim)

Purpose: Eph-Ephrin signaling mediates various cellular processes including vasculogenesis, angiogenesis, cell migration, axon guidance, fluid homeostasis and repair after injury. Although previous studies demonstrate that stimulation of EphA receptor induces increased vascular permeability and inflammatory response in lung injury, the detailed mechanisms of EphA2 signaling are unknown. The aim of this study is to evaluate the role and related signal pathways of EphA2 signaling in the lipopolysaccharide (LPS)-induced lung injury model.

Materials and Methods: We studied three experimental mice groups. These were PBS + IgG (IgG instillation after PBS exposure), LPS + IgG group (IgG instillation after LPS exposure) and LPS+ EphA2 mAb group (EphA2 monoclonal antibody instillation postreatment after LPS exposure). The cell numbers and protein concentration in the bronchoalveolar lavage fluid (BALF), changes in histopathology and the expression of several signal pathway proteins

including PI3K-Akt-NFkB,Src, Erk, E-cadherin and mTOR signaling were compared among three groups.

Results: We report that acute LPS exposure significantly upregulated EphA2 and EphrinA1 expression. Inhibiting EphA2 receptor by intranasal EphA2 mAb instillation attenuated lung injury and reduced cell counts and protein concentration of BALF (all, $P < 0.05$). EphA2 mAb posttreatment downregulated the expression of PI3K 110γ, phospho-Akt, phosphor-NFkB, Erk1/Erk2, phospho-Src and phospho-S6K. In addition, inhibiting EphA2 receptor augmented the expression of E- cadherin protein related to cell –cell adhesions.

Conclusion: The present data suggest that EphA2 receptor may be an unrecognized contributor modulating several signal pathways including PI3K-Akt-NFkB, Src-NFkB, E-cadherin in cell-cell adhesions and mTOR in LPS-induced lung injury. Further studies are needed to verify the potential of EphA2 receptor inhibitor as a novel therapeutic agent in LPS-induced lung injury.

Key Words : EphA2; EphrinA1; lipopolysaccharide

The role of EphA2 signaling in Lipopolysaccharide-induced lung injury

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Ⅰ. INTRODUCTION

Eph tyrosine kinase receptor and Ephrin ligand are cell surface-bound and are involved in cell to cell communication.[1,2] The influence of Eph-Ephrin activation differs depending on the cell types and environments. In addition to bidirectional signaling, Eph receptor and Ephrin ligand function independent of each other or in convert with other cell surface communication system. Eph-Ephrin signaling contributes to several functions including vasculogenesis, angiogenesis, cell migration, axon guidance, fluid homeostasis and repair after injury.[1-3] Several researches have been focused on complex role of Eph and Ephrin in malignancy.[4,5] According to several studies, Eph receptor and Ephrin ligand affect multiple oncogenic signaling pathways such as MAPK/ERK, PI3K, E-cadherin and integrin/FAK/paxillin.[4,6-8] Recently, Eph-Ephrin signaling is found to

contribute inflammation by the mechanism that phenotypic change to the vascular endothelium allows the movement of inflammatory cells into the injured tissue.[3]

The research is needed to explore mechanism and to discover the novel therapeutic approaches in the acute lung injury (ALI) because there are currently no effective pharmacological therapies except general supportive cares.[9] Some studies are reported about the role of EphA2 signaling in the lung injury and inflammation. In bleomycin- induced lung injury model, EphA2 knockout mice showed reduced permeability and less inflammatory response than wild type mice.[10] Similarly, in lung injury due to viral infection and hypoxia, EphA2 antagonism with EphA2/Fc and anti-EphA2 antibody reduced vascular leakage and albumin extravasation.[11] However, data is limited regarding the detailed mechanism of EphA2 signaling in the LPS induced lung injury.

Given the previous studies, we hypothesize that 1) the expression of EphA2 and EphrinA1increases in the LPS induced lung injury, 2) inhibition of EphA2 signaling even after established endotoxemia is of therapeutic utility in lung injury and 3) The crosstalk exists between EphA2 signaling and other signal pathways in the LPS induced lung injury.

Ⅱ. MATERIALS AND METHODS

1. Study Subjects

All animal protocols were approved by the institutional animal care committee of the Medical College of Yonsei University. All animal experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All human study protocols were approved by Severance Hospital Institutional Review Board (4-2008-0099). All study subjects gave informed consent to the use of the samples by flexible bronchoscopy with bronchoalveolar lavage (BAL) using standard operating procedures.

2. LPS induced lung injury model in mice

Wild type male C57BL/6J mice, 8~10 weeks of age and weighing 20~24 g were purchased from Orient Bio (Sungnam, Republic of Korea). All animals were supplied with food and water and were subjected to a similar day and night light cycle.

Twelve mice were randomly divided to three groups: (A) control group with IgG posttreatment, (B) LPS induced lung injury group with IgG posttreatment, (C) LPS induced lung injury group with EphA2 monoclonal antibody (mAb) posttreatment

The experiment was performed by intranasal administration. Mice were lightly anesthetized by inhalation of isoflurane (Abbott Laboratories).The mouse is held in a supine position with the head elevated. The administration solution was gradually released into the nostril with the help of microsyringe (Hamilton Company cat# 7637-01). We adjusted the rate of release so as to allow the mouse to inhale the solution without trying to form bubbles.

Except for the control group, *Escherichia coli LPS* (E.coli 0127: B8; Sigma, St Louis, MO, USA) 40 μg/g in 50 μl phosphate-buffered saline (PBS) was administered by intranasal instillation. As posttreatment, 4 μg of either mouse Immunoglobulin-G (IgG) (Abcam, Cat# ab37355) or monoclonal EphA2 antibody (R&D Systems, Cat# MAB639) were intranasally administered 6 and 12 hours after LPS treatment. The control group was administered with 50 μl of sterile PBS followed by two doses of IgG (at 6 and 12 hours after PBS treatment, i.n., 4 μg).

A. **Analysis of bronchoalveolar lavage fluid (BALF)**

24 hours after LPS/PBS inoculation, all mice were humanely euthanized by lethal overdose of ketamine and xylazine. BAL was performed through a tracheal cannula using with a two 1 ml aliquots of sterile saline. The BAL fluid was centrifuged (4˚C, 1500~5000 rpm, 10 min) and the supernatant was stored at 80°C for further analysis.

The cell pellet was reconstituted in 100 μl PBS and used for quantitative

and qualitative cell counts. Total cell numbers were counted from each sample using a hemocytometer (Marienfield, Germany) according to the manufacturer's protocol. The slide chambers were prepared by inserting slide into frame with Poly-L lysine coating up and clamping with clips on either side. 90 μl aliquot of each sample was transferred into the slide chambers that were inserted into cytospin with the slide facing outward. Spinning was done at 600 rpm for 6 minutes. The slides were removed from cytocentrifuge and dried prior to staining. Diff Quick (Sysmex corporation) staining was used. The slides were immersed in three Diff Quickfluid (Fixative, Solution I, Solution II) for 5 seconds and rinsed with purified water.

The protein content of the BAL supernatant was measured using Coomassie Brilliant Blue G-250 technique (Quick StartTM Bradford Protein Assay, US). 25 μl of each sample and 200 μl of working reagent were pipetted into a microplate well and mixed thoroughly on a plate shaker for 30 seconds. After incubation for 30 min at 37°C, the plate was cooled and read at 595 nm by spectrophotometer.

B. Lung tissue harvest and histologic examination

The right lung was isolated and stored at -80°C prior to protein extraction, after flushing the pulmonary vasculature with saline under low pressure. The left lung was inflated via the tracheotomy with low-melting point

agarose (4%) in PBS at 25 cm H_2O pressure and until the pleural margins became sharp. The lungs were then excised and fixed overnight in 10% formaldehyde in PBS and embedded in paraffin for sectioning at 5-μm thickness. Left lung sections were stained with H&E and subjectively evaluated under light microscopy. The histopathology was reviewed in a blinded manner by two qualified investigators. Five easily identifiable pathologic processes were scored by a weighted scale presented in the official ATS workshop report[12]**.** Lung sections were processed for immunohistochemistry using anti rabbit EphA2 (Thermo Fisher Scientific, Germany), and anti-goat EphrinA1 (Thermo Fisher Scientific, Germany) antibody. A peroxidase-based assay was performed using diaminobenzamide (DAB) as the chromagen.

C. Western blotting

Frozen right lung were mechanically disrupted using a homogenizer in homogenization buffer, PRO-PREP™ Extraction solution (iNtRON BIOTECHNOLOGY cat# 17081). The amount of solution is 600 μl per 10 mg tissue. Cell lysis was induced by incubation for 20-30 min on ice or freezer at -20˚**C.** The samples were centrifuged at 13,000 x g for 30 min at 4˚**C.** Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membrane before immunoblotting with primary Abs as indicated. Membranes were incubated with anti-rabbit or

anti-mouse IgG HRP conjugated Abs and developed using Super-Signal West Pico chemiluminescence detection kit (Pierce). The band images were quantified by Alpha Ease FC software (Innotech, version 4.1.0).

The antibodies used in this study included rabbit EphA2 (Thermo Fisher Scientific, Germany), goat EphrinA1 (Thermo Fisher Scientific, Germany), rabbit PI3 Kinase 110γ (Cell Signaling Technologies, Beverly, MA), rabbit α-tubulin (Cell Signaling Technologies, Beverly, MA), rabbit phosphate–Akt (Cell Signaling Technologies, Beverly, MA), rabbit Akt (Cell Signaling Technologies, Beverly, MA), rabbit phosphate–Src (Cell Signaling Technologies, Beverly, MA), rabbit Src (Cell Signaling Technologies, Beverly, MA), rabbit phosphate–p65 NF-κB (Thermo Fisher Scientific, Germany), rabbit p65 NF-κB (Thermo Fisher Scientific, Germany),mouse phosphate–Erk (Cell Signaling Technologies, Beverly, MA), mouse Erk (Cell Signaling Technologies, Beverly, MA), rabbit phosphate–p70 S6 kinase (Cell Signaling Technologies, Beverly, MA), rabbit p70 S6 kinase (Cell Signaling Technologies, Beverly, MA), and rabbit E-cadherin(Cell Signaling Technologies, Beverly, MA). The image densities were measured with NIH Image J for semiquantitative comparison.

D. Immunofluorescence staining

Mouse paraffin –embedded lung samples were frozen sectioned (7 μm), fixed in PFA 4%, blocked with PBS containing 1% donkey serum and 3% BSA, and then permeabilized with PBS/Triton 0.01%. Sections were incubated with E-cadherin antibody (Cell Signaling Technologies, Beverly, MA) and then with species-specific secondary antibodies conjugated fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology, Santa Cruz, CA). The slides were visualized using confocal laser scanning microscope [Zeiss LSM 510 (Axicovert 100/m), Zeiss, Thornwood, NY], as reported earlier.[13]

3. Human bronchoalveolar lavage fluid (BALF) collection

This retrospective study included 60 consecutive patients [Control group (n=5), interstitial lung disease (ILD) group (n=35) and pulmonary infection group (n=20)] who had a bronchoalveolar lavage (BAL) performed at the Severance Hospital between March 2008 and December 2009.

The subjects with single granuloma that turned out to be not malignancy, were used as control group. In control group, the BALF for study was recruited in opposite bronchus from granuloma. The diagnosis of ILD was based on pathological, clinical, and radiological findings, according to the 2010 American Thoracic Society (ATS) guideline.[14] The pulmonary infection group included the participants who had suspected or known

pulmonary infection with systemic inflammatory response syndrome (SIRS) according to the 2001SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference guidelines.[15]

Each participant received the injection of midazolam and fentanyl, followed by local anesthesia with lidocaine. A flexible bronchofiberscope (OLYMPUS, Tokyo, Japan) was inserted orally to pour 50 mL of 0.9% saline (37°C) into the bronchus (Control: opposite bronchus from granuloma, ILD and infection: bronchus of pulmonary lesion) and the BALF was recovered. BALF was centrifuged (10 min; 5,000 rpm) and the supernatant was cryopreserved at −80°C until use. The EphA2 and EphrinA1 contents of supernatant were measured using an enzyme linked immunosorbent assay (ELISA) kits (CusabioBiotech, Newark, NJ, USA), according to the manufacturer's directions.

4. Statistical analysis

Statistical analysis was performed using Prism 5.0 (Graphpad Software, Durham, NC). The group comparisons were performed with an unpaired student t test or ANOVA with Bonferroni multiple comparisons tests. Data are expressed as means ± SD for each group. Differences were considered significant at $P < 0.05$.

Ⅲ. RESULTS

1. LPS upregulates EphA2 receptor and its ligand Ephrin A1 expression.

As measured by Western blotting, the expression of EphA2 protein and EphrinA1 protein in lung tissue was increased after LPS treatment (EphA2:13.54-fold, EphrinA1: 8.35-fold), compared with PBS treatment. Increased expressions of EphA2 and EphrinA1 in the lung were inhibited by EphA2 mAb posttreatment (Figure 1).

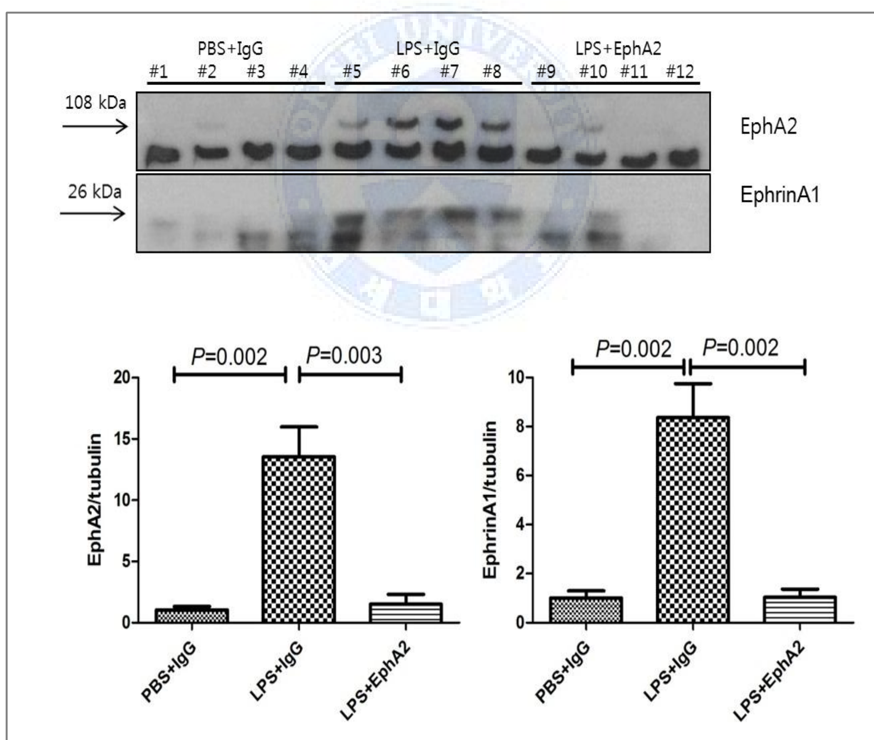


Figure 1. The expression of EphA2 and EphrinA1 protein in lung lysates, as shown by Western blots and densitometry. N=4 per group

To determine where in the lung EphA2 and EphrinA1 were expressed, immunohistochemistry was performed. Immunostaining for EphA2 and EphrinA1 in lung tissue from PBS instilled control animals showed weakly detectable expression in alveolar epithelium, as well as brighter staining in alveolar septum. On the contrary, LPS injured mice demonstrated marked increases in EphA2 and EphrinA1 staining in type II pneumocytes and alveolar macrophages around inflamed areas with thickened septae (Figure 2).

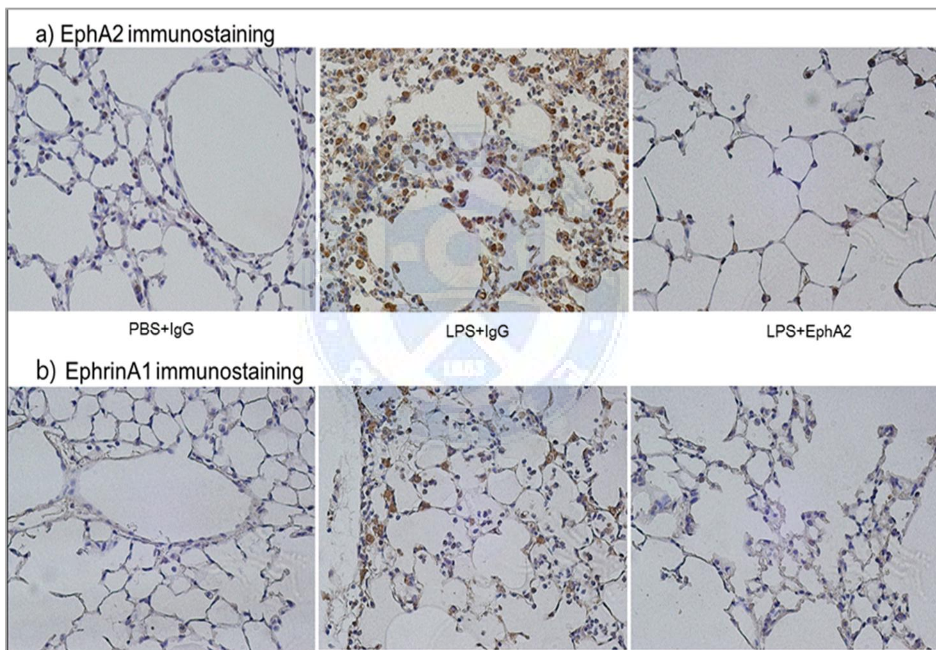


Figure 2. The immunostaining of EphA2 and EphrinA1 in mice lung

(a) The increased EphA2 immunostaining after LPS exposure, compared with control group (PBS+IgG) is inhibited by EphA2 mAb posttreatment. (b) The increased EphrinA1 immunostaining after LPS exposure, is reduced by EphA2 mAb posttreatment

2. EphA2 antagonism attenuates LPS induced lung injury.

After finding that LPS induced lung injury is associated with the increased expression of EphA2 and EphrinA1 in the lung, we sought to determine whether EphA2 contributes to permeability and inflammation by verifying the effects of EphA2 mAb posttreatment.

As shown in Figure 3, LPS caused lung injury and edema, as demonstrated by a significant increase in concentration of total protein (LPS+IgG: 1.13 ± 0.26 mg/ml, PBS +IgG: 0.16 ± 0.06 mg/ml, $P<0.001$) and total cell counts in BALF (LPS+IgG: $5117 \times 10^4 \pm 2880 \times 10^4$, PBS+IgG: $23 \times 10^4 \pm 16 \times 10^4$, $P=0.012$). The EphA2 mAb posttreatment resulted in a significant reduction in concentration of total protein (LPS + EphA2 mAb: 0.12 ± 0.08 mg/ml, LPS + IgG: 1.13 ± 0.26 mg/ml, $P<0.001$) and total cell counts in BALF (LPS+EphA2 mAb: $180 \times 10^4 \pm 87 \times 10^4$, LPS + IgG: $5117 \times 10^4 \pm 2880 \times 10^4$, $P=0.014$) compared with IgG posttreatment after LPS instillation. Also, histologic examination of the lungs of mice that received EphA2 mAb in addition to LPS instillation revealed a significant decrease in lung injury score compared with mice that received LPS and IgG posttreatment (LPS+EphA2 mAb: 35.9 ± 10.6, LPS+IgG: 70.2 ± 7.5 , $P=0.002$).

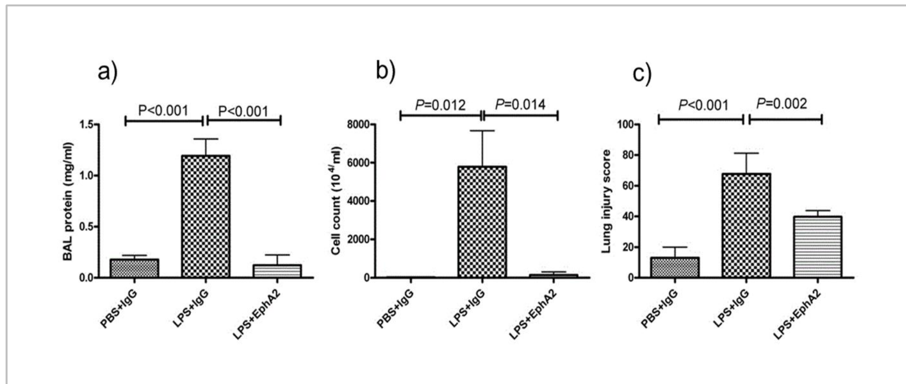


Figure 3. EphA2 monoclonal antibody posttreatment attenuates LPS induced lung injury. (a) Total bronchoalveolar fluid (BALF) protein concentration. (b) Total BALF cell counts. (c) The lung injury scores

3. LPS upregulates PI3K-Akt-NFkB signaling and Src-NFkB signaling via EphA2 activation.

Given the protective effect of EphA2 antagonism in LPS induced lung injury, we wondered whether inhibiting the EphA2-EphrinA1 signaling had an effect on the existing signal pathways. To answer this question, the expression of various proteins in lung tissue was measured in PBS+ IgG group, LPS+IgG group and LPS+EphA2 mAb group.

As shown Figure 4, compared to the PBS+IgG group, the LPS+IgG group showed significantly increased PI3K 110γ and phosphorylation of Akt (PI3K 110γ: 14.77- fold, phopho-Akt:1.7-fold). After EphA2 mAb posttreatment, the expression of PI3K 110γ and phosphorylation of Akt by LPS challenge were reduced, showing that EphA2 signaling is involved in LPS induced activation of Akt through a PI3K-γ dependent step.

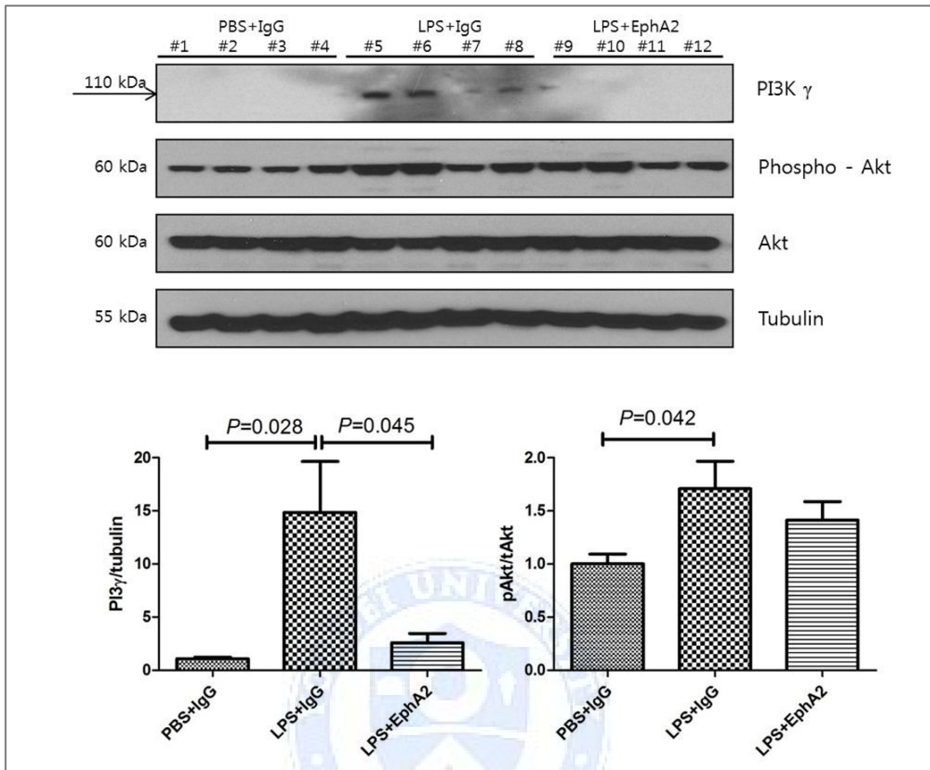


Figure 4. The expression of PI3γ and Akt protein in lung lysates, as shown by Western blots and densitometry.

Also, LPS induced 1.6 fold increase in Src phosphorylation in the lung that was inhibited by EphA2 mAb (twofold decrease, Figure 5). The phosphorylation of NFkB p65 increased significantly in lung after LPS exposure, but very little change in EphA2 antagonism compared with control group (Figure 5). Our finding from this present study indicates that EphA2 may play a critical role in activating downstream signaling pathway such as PI3K γ- Akt-NFkB signaling and Src-NFkB signaling.

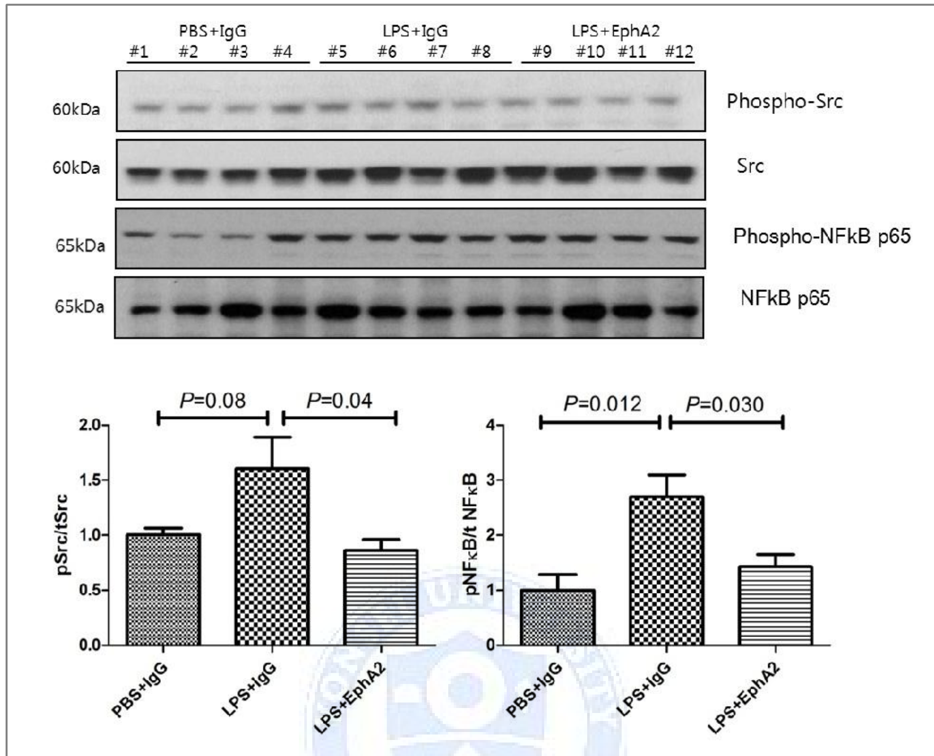


Figure 5. The expression of Src and NFkB protein in lung lysates, as shown by Western blots and densitometry.

4. LPS upregulates Erk signaling and mTOR signaling via EphA2 activation.

While LPS exposure induced the phosphorylation of Erk1/Erk2, EphA2 mAb posttreatment attenuated the LPS induced phosphorylation of Erk1/Erk2 (Figure 6).

Also, the phosphorylation of S6, a major target of mTOR, had a 2.66-fold increase in lung tissue after LPS exposure, but the increase was inhibited by EphA2 mAb posttreatment (Figure 6).

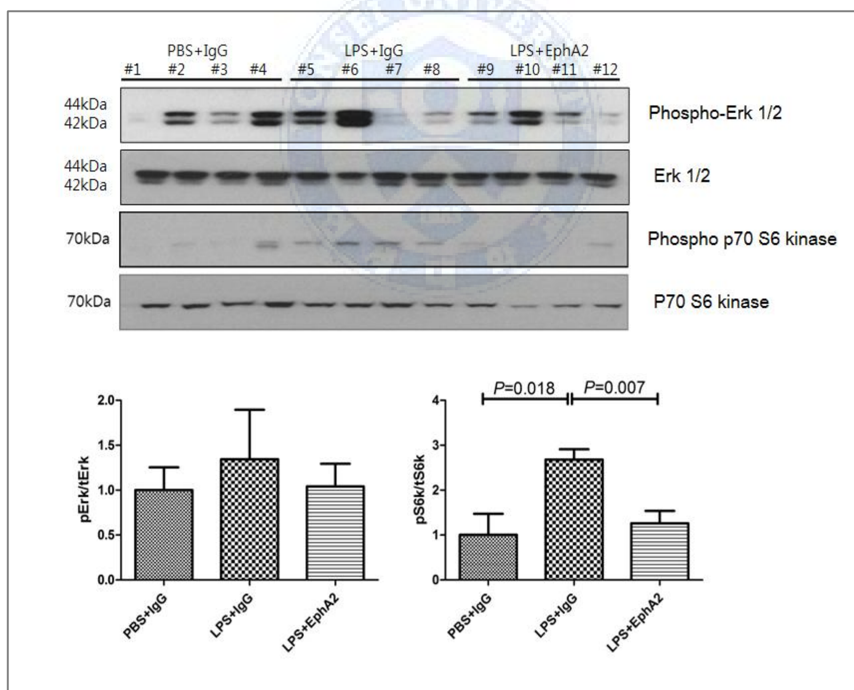
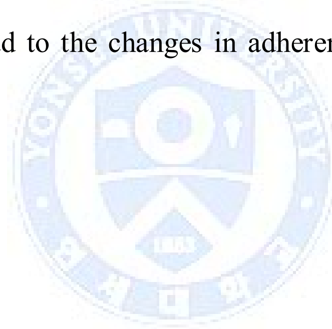


Figure 6. The expression of Erk and S6K protein in lung lysates, as shown by Western blots and densitometry.

5. LPS downregulates E-cadherin via EphA2 activation.

LPS exposure reduced the expression of E-cadherin and EphA2 mAb posttreatment restored E-cadherin protein decreased by LPS exposure (Figure 7a). We confirmed E-cadherin expression by immunofluorescence analysis (Figure 7b). While E-cadherin expression was strong in the control group, after LPS exposure E-cadherin expression was downregulated. Inhibiting EphA2 signaling by EphA2 mAb, blocked LPS induced downregulation of E-cadherin expression in lung tissue. These results demonstrate that LPS induced EphA2 expression may regulate the expression of E-cadherin and lead to the changes in adherens junction and epithelial hyperpermeability.



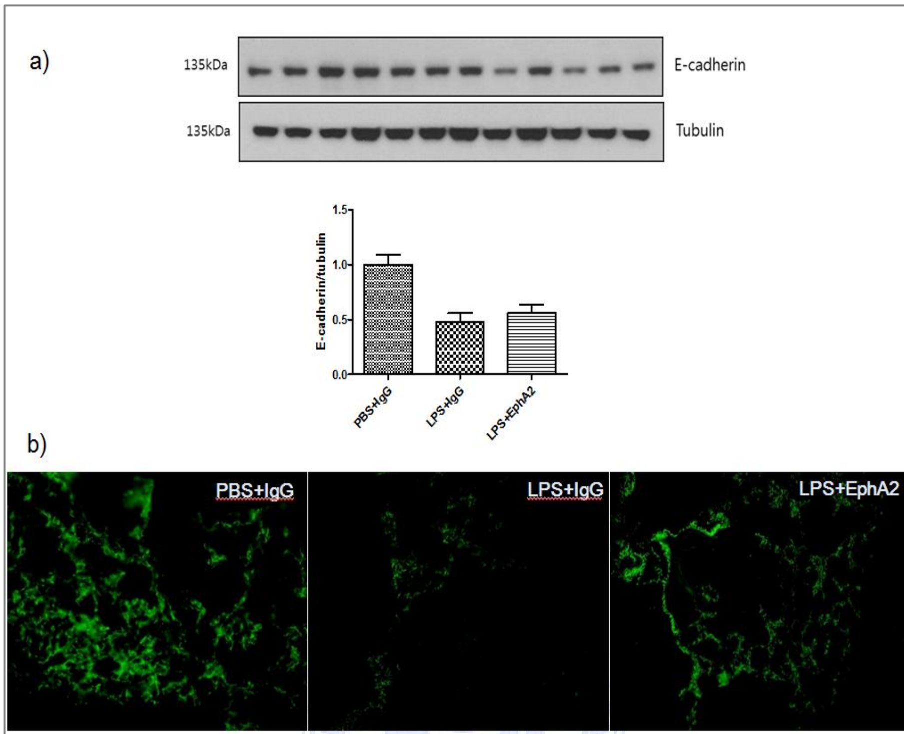


Figure 7. The expression of E-cadherin protein in lung lysates that is confirmed by western blotting and immunofluorescence staining.

The lung slides were labeled with anti E-cadherin antibody and fluorescein isothiocyanate (FITC) conjugated secondary antibody (Figure 7b). Magnification is 400X

6. The expression of EphA2 and Ephrin levels in Human BALF is increased in pulmonary infection group

We measured EphA2 and EphrinA1 protein in BALF of human adults. EphA2 levels were significantly elevated in pulmonary infection group, when compared with the control subjects and patients with interstitial lung disease (ILD) (control: 0.03 ± 0.02 ng/ml, ILD: 0.06 ± 0.06 ng/ml, pulmonary infection: 0.14 ± 0.16 ng/ml, Figure 8). EphrinA1 levels of pulmonary infection group were higher than other two groups and EphrinA1 levels of adults with ILD were higher than the control subjects (control: 0.01 ± 0.01 ng/ml, ILD: 0.13 ± 0.10 ng/ml, pulmonary infection: 0.63 ± 0.67 ng/ml).

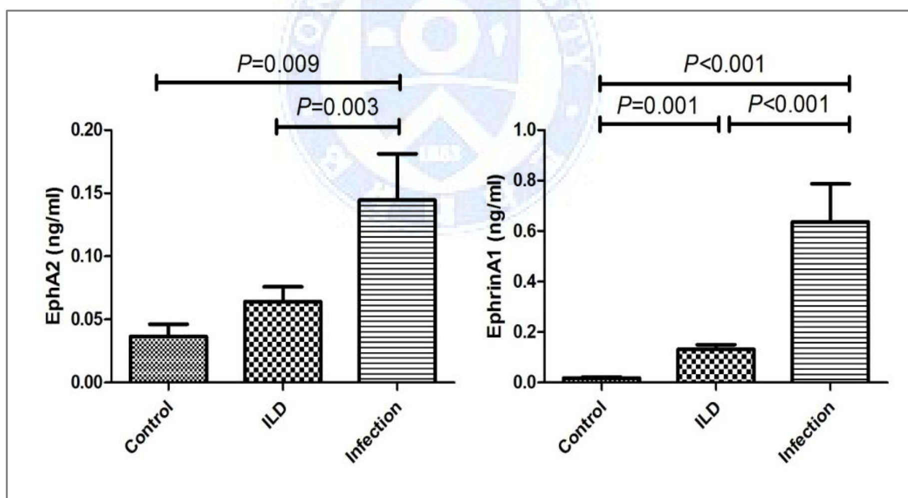


Figure 8. The EphA2 and EphrinA1 levels in bronchoalveolar fluid (BALF) of healthy controls and adult patients with ILD or pulmonary infection. ILD, interstitial lung disease, Control (n=5), ILD (n=35) and infection (n=20)

Ⅳ. DISCUSSION

EphA2 signaling has been studied in angiogenesis, cell migration, fluid homeostasis and vascular assembly during early stages of development.[1,2] The interaction between Eph receptor and Ephrin ligand results in modification of cytoskeletal proteins and cell surface receptors.[1,2] The previous studies found that actin cytoskeleton rearrangement may be a key preceding event during the regulation of inflammatory responses in various cell populations.[16-19] The disruption of endothelical cell junction by EphA2 signaling, allows the passage of fluid, protein and inflammatory cells into an injured tissue.[3,20] The various evidences suggest that EphA2 signaling may an important mediator in inflammation and injury.[10,11,21,22]

Our studies demonstrate that EphA2 contributes to the pathogenesis in LPS induced lung injury. Both the expressions of EphA2 receptor and the ligand EphrinA1, were increased in the LPS induced lung injury. Also, blocking the activation of EphA2 receptor by EphA2 mAb ameliorates permeability and inflammatory changes associated with lung injury.

These results are consistent with previous studies. In rats exposed to viral respiratory infection and hypoxia, EphA2 expression is markedly upregulated and EphA2 antagonism reduced vascular leakage in injured lung injury.[11] Similarly, in bleomycin induced lung injury, EphA2 KO mice were protected from protein extravasation and inflammatory responses.[10] Both studies

demonstrated that ligand EphrinA1 stimulation of lung endothelial EphA2 receptor leads to disruption of endothelial adherens junctions and increased permeability. Our results suggest the increased ligand mediated activation of EphA2 in the LPS induced lung injury.

However, Ivanov *et al.* demonstrated counter-directed changes in expressional regulation of the EphA2 receptor and Ephrin ligand in lung tissue in phase 2 (90 min post-LPS) of LPS injection, contrary to our data.[23] The reasons for different results may be the difference of experiment time interval. The sacrifice time of our study was 24 hours after LPS exposure, while it was 90 min after LPS exposure in the study of Ivanov *et al.* Different temporal expressions of EphA2 and EphrinA1 may be involved in regulation of cellular events underlying stages of systemic inflammation.

The cascade of multiple signaling pathways in the mechanism of acute lung injury (ALI) is complicated and remains unclear. Despite of some advances in research about ALI, it accounts for significant morbidity and mortality in critically ill patients.[24,25] Therefore, investigating the molecular and cellular signaling pathway that mediates ALI is important for the development of specific effective therapies.

We found several potential mechanisms by EphA2 signaling may contribute to the development of LPS induced lung inflammation and injury (Figure 9).

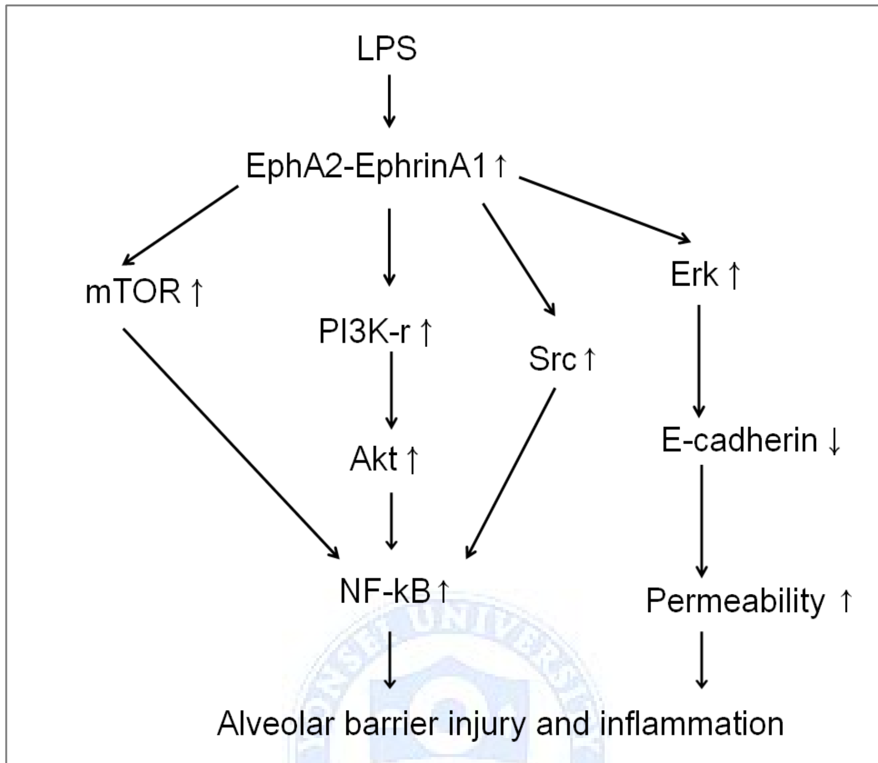


Figure 9. The potential mechanisms through which EphA2 may contribute to the development of LPS-induced ALI.

Activation of Akt through PI3K dependent pathway leads to increased nuclear translocation of NFκB that regulates the proinflammatory cytokine production in endotoxemia associated ALI.[26] The role of PI3K-Akt pathway in modulating of NFκB activation was proven in numerous cell populations including neutrophils, epithelial cells and fibroblasts.[27-30] In the present study, antagonizing EphA2 expression with EphA2 mAb posttreatment after LPS exposure, downregulated the expression of PI3K-Akt-NFκB compared with

group exposed with LPS only. The results showed LPS induced upregulation of PI3K-Akt-NFκB pathway was mediated through EphA2 signaling.

Lee et al and Severgnini et al have reported that Src tyrosine kinases mediates activation of NF-kB in LPS induced lung injury and selective Src TK inhibitor may provide a therapeutic agent.[31,32] Our data showed EphA2 antagonism significantly reduced Src phosphorylation and NF-kB activation. These finding suggests the possible involvement of EphA2 signaling in LPS induced NF-kB activation via Src tyrosine kinase as an upstream pathway. The previous study by Holen et al indicated that Ephrin A signaling initiated by interaction with EphA2 receptor leads to phosphorylation of several proteins including the Src-family kinases and Akt in the T cell lines.[33]

Intratracheally instilled LPS induces epithelial injury and barrier integrity dysfunction in a murine model of ALI.[34-36] He et al reported that LPS induced epithelial barrier dysfunction through regulation of E-cadherin intracellular trafficking.[37] Nasreen et al demonstrates that induction of EphA2 and EphrinA1 in the bronchial airway epithelial cells exposed to tobacco smoking, may be an important preceding event leading to downregulated E-cadherin expression and induced hyperpermeability in MAPK dependent manner.[38]

Our study provides evidence that EphA2 mAb may enhance pulmonary epithelial barrier integrity through E-cadherin accumulation in LPS induced lung injury. Further studies are needed to find whether reduced Erk phosphorylation after EphA2 mAb posttreatment is associated with upregulated

E-cadherin expression and reduced protein leakage like the study of Nasreen et al.[38]

The previous studies showed that LPS exposure activated mTOR signaling in inflammatory cells and lung tissues.[39,40] Our results showed that the phosphorylation of S6, downstream target of mTOR, increased after inhaled endotoxin administration and reduced after inhibiting EphA2 signaling.

There are several studies to use rapamycin to dissect the role of mTOR in ALI.[40-42] Feilhaber et al showed that inactivation of mTOR attenuates MyD88-dependent processes (i.e., NFkB, TNF-α, neutrophil recruitment, but enhances MyD88-independent signaling (i.e., STAT1, apoptosis) leading to lung injury and apoptosis.[41] Similarly, Lan et al reported that rapamycin reduced the level of inflammatory mediator but did not change the permeability and mortality in LPS induced lung injury.[42] Considering that EphA2 contributes not just to changes in inflammatory responses but also to permeability in our results, different types of signal transduction pathway besides mTOR signaling, may constitute a complex network around EphA2 signaling.

The mammalian target of rapamycin (mTOR) is known to serve as a negative regulator of autophagy, and initiation of autophagy is largely regulated by release of mTOR inhibition[43]. Further detailed studies are needed to investigate the relationship between EphA2 antagonism and the autophagic response including the inflammasome activation.

In human data, adult patients with pulmonary infection had higher EphA2 and

EphrinA1 levels in the BAL fluid when compared to adults with ILD and control group. The result is consistent with the mice experiment that LPS exposure leads to elevation in EphA2 and EphrinA1. However, the sample size is small and the causality cannot be proven. The clinical significance of elevated EphA2 and EphrinA1 levels of adults with pulmonary infection remains unclear. Since the EphA2 receptor is reported to be oncogenic and to promote metastasis in several cancers,[44] clinical trials inhibiting the EphA2 receptor as a potential therapeutic target have been ongoing in the oncology field.[45,46] Similarly, clinical studies are required to clarify the possible role of EphA2 as a therapeutic target for ALI in human studies, based on the several animal experiments.

Ⅴ. CONCLUSION

In conclusion, we demonstrate that EphA2 signaling contributes permeability and inflammation in the LPS induced lung injury, and that it may regulate the several signal pathways including PI3K-Akt-NFκB pathway, E-cadherin and mTOR pathway. In mice experiment, the inhibition of EphA2 expression by EphA2 mAb instillation attenuates the lung injury. The implementation of an inhibitor for EphA2 signaling into clinical practice deserves attention in further research.

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ABSTRACT(IN KOREAN)

LPS 유도 폐손상 쥐모델에서 EphA2 신호전달의 역할

The role of EphA2 signaling in LPS induced lung injury

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배경: Eph 수용체-Ephrin 리간드 신호체제는 혈관형성, 발달, 신경축삭돌기의 유도, 세포이동, 체액의 항상성 유지, 손상 후 회복 등의 다양한 세포 과정을 담당한다. 이전의 연구결과에서 폐손상에서 EphA 수용체을 자극시키면 혈관 투과성을 증가시키고 염증 반응을 유도된다는 사실이 입증되었으나 EphA2 신호전달의 자세한 기전은 알려지지 않았다. 이 연구의 목적은 LPS 유도 폐손상 쥐모델에서 EphA2 신호전달의 역할과 관련 신호전달을 평가하는 것이다.

방법: 세 가지 쥐 실험 모델을 연구하였다. PBS+IgG 군 (PBS 노출 후 IgG 흡입 군), LPS+ IgG 군(LPS 노출 후 IgG 흡입 군), LPS+EphA2 mAb 군(LPS 노출 후 EphA2 단클론성 항체 흡입 군)이 비교되었다.

세 군간의 기관지 폐포 세척액의 세포 수와 단백질 농도,

조직학적 변화 차이를 비교하였으며 PI3K-Akt-NFkB,Src, Erk, E-cadherin and mTOR signaling 의 발현도 비교하였다.

결과: 급성 LPS 노출은 EphA2 와 Ephrin A1 의 단백질 발현을 증가시켰다. 비강으로 EphA2 단클론 항체를 흡입함으로써 EphA2 수용체를 억제하는 것은 폐손상을 호전시키고 세포 수와 단백질 농도를 감소시켰다 (P < 0.05). EphA2 단클론 항체 치료는 PI3K 110r, phospho-Akt, phosphor-NFkB, Erk1/Erk2, phospho-Src phospho-S6K의 발현을 억제하였다. 또한 EphA2 수용체 억제군에서 LPS 노출 군에 비해 세포 간의 부착과 관련된 E-cadherin 단백질 발현이 증가하였다.

결론: 본 연구는 LPS 유도 폐손상에서 EphA2 수용체가 PI3K-Akt-NFkB, Src-NFkB 신호체제, 세포간의 접촉과 관련된 E-cadherin, mTOR 신호전달 등의 여러 신호체제를 조절하는 주요 기여 인자임을 시사한다. LPS 유도 폐손상에서 EphA2 수용체 억제제가 새로운 약제로서의 잠재력을 확인하기 위해 추가적인 연구가 필요하다.

핵심되는 말: EphA2; EphrinA1; lipopolysaccharide