The role of erythropoietin-producing hepatoma receptor tyrosine kinase A2 on ventilator-induced lung injury

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Directed by Professor **Young Sam Kim**

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It is impossible to convey the flavor of my grateful feelings toward my great-hearted family.

To my wife, Hyo Jeong Lee, and my son, I dedicate this doctoral dissertation in token of my affection and gratitude.

<TABLE OF CONTENTS>

LIST OF FIGURES

ABSTRACT

The role of erythropoietin-producing hepatoma receptor tyrosine kinase A2 on ventilator-induced lung injury

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Background: The erythropoietin-producing hepatoma (Eph) receptor tyrosine kinases and their ligand ephrins have diverse biological functions and influence cellular behaviors during embryogenesis and adult life. Among these, EphA2 receptor and the ligand ephrinA1 play a pivotal role in inflammation and tissue injury by modulating epithelial and endothelial barrier integrity. However, the role of EphA2/ephrinA1 in the process of ventilator-induced lung injury (VILI) remains unclear. Therefore, we hypothesized that the blockade of EphA2/ephrinA1 signaling using an EphA2 receptor antibody (Ab) would result in decreased inflammation and injury in VILI model.

Methods: Wild type male C57BL/6J mice were randomly assigned to four experimental groups (n = 4/group); (1) non-ventilated mice as a control group, (2) immunoglobulin G (IgG) (1 hour before mechanical ventilation) + high tidal volume (HTV) ventilation group, (3) EphA2 receptor Ab (1 hour before mechanical ventilation) + HTV ventilation group, and (4) HTV ventilation +

EphA2 receptor Ab (2 hours after mechanical ventilation) group. Injury was assessed by bronchoalveolar lavage fluid analysis, lung injury scoring, and transmission electron micrographic evaluation. Western blotting for EphA2/ephrinA1/phosphoinositide 3-kinase γ (PI3Kγ)/protein kinase B (Akt)/NF-κB/P70S6 kinase and enzyme-linked immunoassay for interleukin (IL)-1β/IL-6/keratinocyte chemoattractant (KC/CXCL1) were performed on lung lysates.

Results: EphA2/EphrinA1 expression was higher in HTV ventilation group rather than in control group and decreased by pre- or post-treatment with EphA2 receptor Ab. EphA2 receptor Ab also ameliorated the extent of lung injury and downregulated the expression of PI3Kγ/Akt/NF-κB and mammalian target of rapamycin (mTOR). EphA2 receptor Ab did not influence on the proinflammatory cytokine/chemokine levels.

Conclusion: These findings suggest that EphA2/ephrinA1 may be involved in the pathogenesis of VILI, partly through PI3Kγ/Akt/NF-κB signaling pathway and possibly via mTOR pathway. Our data provide an evidence for EphA2/ehprinA1 as a promising therapeutic target for modulating VILI.

Key words: ephA2, ephrinA1, ventilator-induced lung injury, PI3kγ, Akt, NF-κB, mTOR

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I. INTRODUCTION

Mechanical ventilation remains an important life supportive modality in intensive care setting. This therapy, however, can itself cause or exacerbate lung damage by a variety of mechanisms referred to as ventilator-induced lung injury (VILI).[1] In addition to barotrauma, volutrauma, and atelectrauma by cyclic overdistension of lung architecture at a tissue level, injurious mechanical forces can trigger biotrauma at a cellular level, which is characterized by an influx of inflammatory cells, epithelial and endothelial cell damage, impaired edema clearance, and apoptosis.[2]

VILI can propagate injury to extra-pulmonary organs, resulting in systemic inflammatory response and multiple system organ dysfunction, which can in turn increase morbidity and mortality.[3] Although protective ventilation strategies have been proven effective for reducing mortality associated with VILI in patients with acute respiratory distress syndrome (ARDS),[4] some patients with preinjured lungs are vulnerable to the development of VILI even in the setting of protective ventilation strategies.[5-7] Therefore, it is clinically important to explore the related mechanism of

VILI and to discover novel therapeutic targets.

The erythropoietin-producing hepatoma (Eph) receptor tyrosine kinases and their ephrin ligands are the largest family of receptor tyrosine kinases in the mammalian genome and are differentially expressed in most cell types.[8] They are cell surface molecules with diverse biological functions including axonal guidance, angiogenesis, hematopoiesis, cell positioning/migration, tissue morphogenesis/repair, and oncogenesis, thus influencing cell behavior and cell-to-cell communication during embryogenesis and adult life.[8-10]

Both EphA2 receptor and its ligand ephrinA1 are expressed in distal normal lung tissue and play a crucial role in cell-cell adhesion in epithelial and endothelial cells.[9] Moreover, EphA2 receptor is closely related to epithelial or endothelial barrier integrity through tight and adherence junction molecules such as E-cadherin, VE-cadherin, ZO-1, and Claudin-5.[11-14] In response to tumor necrosis factor (TNF)-α, interleukin (IL)-1β, thrombin, and various other inflammatory mediators, EphA2/ehprinA1 may increase vascular permeability by modulating the integrity of vascular endothelial junctions and cytoskeletal structures.[12,15,16] In practice, previous in vivo or in vitro studies with various animal models demonstrated that EphA2 receptor and ephrinA1 contribute to lung injury and inflammation by increasing epithelial/endothelial permeability.[13,14,17-19] The exact role of EphA2/ephrinA1 in the process that converts mechanical lung strain into increased epithelial/endothelial permeability during injurious mechanical ventilation, however, has not yet been elucidated in detail.

Therefore, we hypothesized that EphA2/ephrinA1 signaling would play a crucial role in inflammation and tissue injury in VILI, and that EphA2 antagonism would result in attenuation of VILI. To examine our hypothesis, we investigated the pattern of EphA2/ephrinA1 expression and the protective effect of EphA2 receptor antibody (Ab) in VILI model. In addition, we evaluated the association between EphA2 receptor and downstream signaling cascades of VILI, including

phosphoinositide 3-kinase γ (PI3Kγ),[20-22] protein kinase B (Akt),[21-23] nuclear factor κB (NF-κB),[21,24] and mammalian target of rapamycin (mTOR).[25]

II. MATERIALS AND METHODS

1. Experimental animals and patients

Wild type male C57BL/6J mice, 8～10 weeks of age and weighing 20~24g (Orient Bio, Sungnam, Korea) were used in our experiments. All animals were kept in microisolator cages under specific pathogen-free conditions, fed with autoclaved food and water, and were subjected to a similar day-and-night light cycle.

2. Study design (Experimental protocol)

Animals were randomly assigned to four experimental groups (n = 4/group); (1) non-ventilated mice as a control group, (2) immunoglobulin G (IgG) (1 hour before mechanical ventilation) + HTV ventilation group, (3) EphA2 receptor Ab (1 hour before mechanical ventilation) + HTV ventilation group, and (4) HTV ventilation + EphA2 receptor Ab (2 hours after mechanical ventilation) group. In ventilated group, mice were injected with 200 g/kg of mouse IgG (Abcam, Cambridge, MA, USA) or EphA2 receptor Ab (R&D Systems, Minneapolis, MN, USA) 1 hour before or 2 hours after HTV mechanical ventilation.

3. Ventilator-induced lung injury model

Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) mixture before tracheostomy and half dose every 20～30 minutes for maintenance during mechanical ventilation. Mice were then tracheostomized and attached to a Harvard small rodent ventilator, model 683 (Harvard apparatus, Holliston, MA, USA) in a supine position. Air flows and pressures in ventilator circuit as well as rectal temperature were monitored

continuously. If needed, vecuronium (0.3 mg/kg) was used for controlling spontaneous breaths. Then, mice were ventilated with a high tidal volume of 24 ml/kg and a frequency of 100 breaths/min for 5 hours. All experiments were conducted with 0 cm H_2O end-expiratory pressure and inspired oxygen fraction of 0.21. Body temperature was maintained between 37 and 38℃ with a heating lamp. The mice received 0.2 ml of 0.9% normal saline after initiating mechanical ventilation, followed by 0.1 ml of normal saline every 30 minutes during mechanical ventilation. Peripheral oxygen saturation and pulse rate were measured continuously using MouseOx (STARR Life Sciences Corp., Oakmont, PA, USA).

4. Bronchoalveolar lavage fluid (BALF) analysis

After 5 hours of mechanical ventilation, mice were euthanized by lethal overdose of ketamine and xylazine mixture and lung tissue was harvested for further evaluation. Bronchoalveolar lavage (BAL) was performed in the left lung with two 1-mL aliquots of sterile saline through the tracheal cannula for BAL fluid (BALF) analysis. The BALF was briefly centrifuged (4˚C, 1200rpm, 10min) and the supernatant was stored at 80°C for further analysis. The cell pellet was reconstituted in 100 l PBS and differential cell counts in each sample were measured by a hemocytometer (Marienfield, Lauda-Königshofen, Germany). For cytospin, the slides were inserted into the frame coated with poly-L lysine and a 90 l aliquot of each sample was transferred into the slide chambers. These slides were then centrifuged at 600 rpm for 6 minutes and removed from the cytocentrifuge for special staining (Diff Quick, Sysmex, Singapore). As a marker of alveolar capillary permeability, the protein concentration in the supernatants was determined using Quick StartTM Bradford Protein Assay (Bio Rad, Hemel Hempstead, UK) according to the manufacturer's protocol based on the Bradford method.[26]

5. Tissue preparation

After washing out the pulmonary vasculature with heparinized saline, the right lung was isolated and stored at -80°C prior to protein extraction for subsequent analysis. Left lung was inflated with low-melting point agarose (4%) in 10% formaldehyde in PBS at 25 cmH_2O pressure for sharpening the pleural margins. The lungs were then removed and preserved in 10% formaldehyde in PBS overnight. Formaldehyde-fixed tissue was washed with PBS, dehydrated in 70% ethanol, and then embedded in paraffin for sectioning into 5- m thickness. Sections were stained with hematoxylin & eosin (H&E) for light microscopic examination.

6. Histopathologic grading of lung injury

The severity and extent of lung injury on the H&E stained lung sections was measured according to the official ATS workshop report about the features and measurements of experimental acute lung injury in animals.[27] Briefly, five easily identifiable pathologic processes were scored by a differently weighted scale: *1)* neutrophils in the alveolar space, *2)* neutrophils in the interstitial space, *3)* hyaline membranes, *4)* proteinaceous debris filling the airspaces, *5)* alveolar septal thickening. Each item was graded on a scale from 0 (minimal damage) to 2 (maximal damage). The degree of lung injury was quantified by summation of the scores of items in 5 random high-power fields (×400 magnification) and then the average sum of each field was calculated as a mean ± SD of the mean for group comparisons. The histopathology was reviewed by two pathologists in a blinded manner with regard to the experimental conditions.

7. Transmission electron microscopy

Lung damage was assessed in alveolar capillary membrane, endothelial cells,

epithelial cells, and type 2 pneumocytes using electron microscopy. In detail, samples were fixed for 12 hours in 2% Glutaraldehyde - Paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and were washed in 0.1 M phosphate buffer. They were then postfixed with 1% OsO4 dissolved in 0.1 M PB for 2 hrs and were dehydrated in ascending gradual series (50 ~ 100%) of ethanol and infiltrated with propylene oxide. Specimens were embedded by special kit (Poly/Bed 812 kit, Polysciences, Washington, USA) after pure fresh resin embedding and polymerization at 65℃ electron microscope oven (TD-700, DOSAKA, Japan) for 24 hrs. Thick sections (about 200~250 nm) were initially cut and stained with toluidine blue (T3260, Sigma-Aldrich, MO, USA) for light microscope. For contrast staining, thin sections (70 nm) were double stained with 6% uranyl acetate (EMS 22400, Electron Microscopy Science, PA, USA) for 20 mins and lead citrate (AC413061000, Thermo Fisher Scientific, Bremen, Germany) for 10 mins. These sections were cut by ultramicrotome (LEICA EM UC-7, Leica Microsystems, Austria) with a diamond knife (Diatome, Switzerland) and transferred on copper and nickel grids. All of the thin sections were observed by transmission electron microscopy (JEM-1011, JEOL, Japan) at the acceleration voltage of 80kV.

8. Western blotting and enzyme-linked immunoassays

The frozen right lung tissue was homogenated in a lysis buffer (iNtRON BIOTECHNOLOGY, Seongnam, Korea) according to the manufacturer's protocol. Equal amounts of protein from each lysate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) and were blocked in nitrocellulose membranes. The membranes were then incubated overnight at 4℃ with relevant primary antibodies for immunoblotting. Afterward, the membranes were washed and incubated with goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase for 60 minutes. The protein bands were detected using Super-Signal West Pico chemiluminescence detection kit (Pierce, Rockford, IL, USA). Relative band

densities of the proteins were quantified with the Alpha Ease FC software version 4.1.0 (Alpha Innotech, San Leandro, CA, USA).

The primary antibodies used in Western blot analysis were as follows: EphA2 (Thermo Fisher Scientific), ephrinA1 (Thermo Fisher Scientific), PI3kγ p110γ (Cell Signaling Technology, Beverly, MA, USA), Akt (Cell Signaling Technology), rabbit phosphorylated Akt (Cell Signaling Technology), NF-κB p65 (Cell Signaling Technology), phosphorylated NF-κB p65 (Cell Signaling Technology), IκBα (Cell Signaling Technology), phosphorylated IκBα (Cell Signaling Technology), P70S6 kinase (Cell Signaling Technology), phosphorylated P70S6 kinase (Cell Signaling Technology), and Tubulin α (Thermo Fisher Scientific). Semi-quantitative comparisons of image densities were performed using NIH Image J.

Levels of IL-1β, IL-6, and keratinocyte chemoattractant (KC/CXCL1) were measured on the aliquots of lung homogenates using a MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

9. Human bronchoalveolar lavage fluid collection

To determine the degree of EphA2/ephrinA1 expression in human, we also performed bronchoalveolar lavage in 67 consecutive patients who admitted at the Severance Hospital between March 2008 and December 2010 (n = 15 in control group, n = 14 in patients diagnosed as having pneumonia without mechanical ventilatory support, and n = 38 in patients diagnosed as having pneumonia with mechanical ventilatory support). Subjects diagnosed as having non-malignant granuloma were used as the control group. The pneumonia groups included patients who had sepsis with suspected or proven respiratory infection according to the 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference guidelines.[28]

After lidocaine spray, fentanyl and midazolam were applied consequently. Then, a flexible bronchofiberscope (OLYMPUS, Tokyo, Japan) was inserted via intra-oral or -nasal route to inject 50 ml of normal saline (37°C) into the appropriate site (the opposite site from the lesions in the control group; the same site with the primary lesions in the pneumonia group), and then BALF samples were collected as much as possible. The collected BALF was briefly centrifuged (1200 rpm, 10 min) and the supernatant was cryopreserved at −80°C for further analysis. The amount of EphA2/EphrinA1 in the supernatant was determined by an enzyme-linked immunosorbent assay kits (Cusabio Biotech, Newark, NJ, USA) according to the manufacturer's instructions.

10. Statistical analysis

Data are presented as mean ± SD of the mean unless otherwise noted. Comparisons between groups were performed using Two-way ANOVA with Bonferroni's correction, as needed. Statistical analysis was performed using Prism version 5.0 (GraphPad Software, Durham, NC, USA). A value of $p < 0.05$ was considered significant. Significance levels are expressed in the figures as $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$.

11. Study approval

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Yonsei University College of Medicine and were conducted in accordance with the National Institutes of Health Guidelines for the care and use of laboratory animals. All human studies were approved by Severance Hospital Institutional Review Board (4-2008-0099). All study participants were given written informed consent to the use of bronchoalveolar lavage samples obtained by flexible bronchoscopy under standard operating procedures.

III. RESULTS

1. High tidal volume mechanical ventilation increases the expression of EphA2 and ephrinA1 in lung tissue

Western blot analysis was performed to evaluate the effect of HTV mechanical ventilation and EphA2 receptor Ab on EphA2/ephrinA1 expression. As shown in Figure 1, HTV mechanical ventilation induced a significant increase in EphA2/ephrinA1 expression compared with control group ($p < 0.05$), whereas pre- or post-treatment with EphA2 receptor Ab prevented the HTV-induced increase in EphA2/ephrinA1 expression ($p < 0.01$).

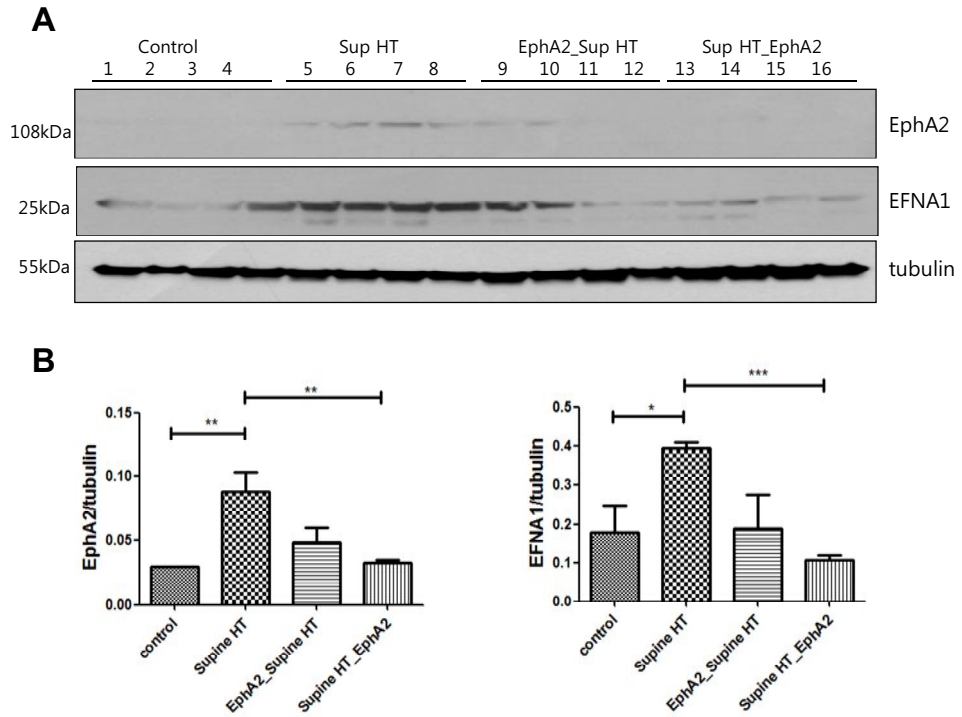


Figure 1. High tidal volume (HTV) mechanical ventilation induced a significant

increase in EphA2/ephrinA1 expression in lung tissue compared with control group, whereas pre- or post-treatment with EphA2 receptor Ab decreased the expression of EphA2/ephrinA1, as shown by Western blots (A) and densitometry (B) of EphA2/ephrinA1 bands normalized to α-tubulin (n = 4/group). *Control*, non-ventilated group; *Supine HT*, IgG injection 1 hour before mechanical ventilation + HTV ventilation; *EphA2_Supine HT*, EphA2 Ab injection 1 hour before mechanical ventilation + HTV ventilation; *Supine HT_EphA2*, HTV ventilation + EphA2 Ab injection 2 hours after mechanical ventilation. *$p < 0.05$ and **$p < 0.01$ by two-way ANOVA and *post hoc* analysis with Bonferroni's correction. EFNA1, ephrinA1

2. Blockade of EphA2 receptor ameliorates ventilator-induced lung injury

To evaluate the protective effect of EphA2 receptor Ab treatment before or after HTV mechanical ventilation, we estimated the extent of inflammation and proteinous pulmonary edema by histological findings and BALF analysis. Compared with control group (Figure 2, top, left), HTV ventilation group developed increased inflammatory cell infiltration in lung parenchyma (Figure 2, top, right), whereas groups receiving pre- or post-treatment with EphA2 receptor Ab showed a significant decrease in the extent of inflammation (Figure 2, bottom, left and right). Similarly, groups receiving EphA2 receptor Ab demonstrated a significant reduction in BALF cell counts and lung injury scores compared with HTV ventilation group ($p < 0.01$) (Figure 3A and 3B). Although not statistically significant, total protein concentrations in BALF also trended lower in EphA2 receptor Ab treatment groups rather than in HTV ventilation group (Figure 3C).

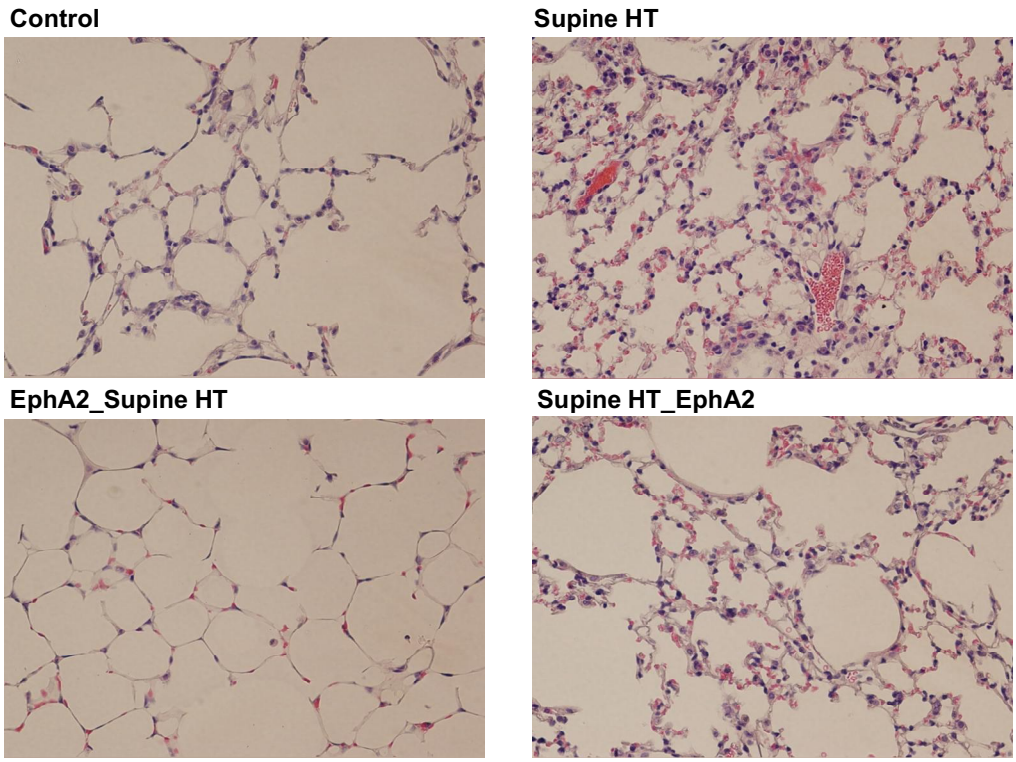


Figure 2. Blockade of EphA2 receptor ameliorated ventilator-induced lung injury, as shown by histopathologic features of mice lung tissues (hematoxylin and eosin stain, 400×magnification). Compared with control group (top, left), Supine HTV group showed a significant increase in inflammatory cell infiltration (top, right), whereas pre- or post-treatment with EphA2 Ab attenuated the degree of inflammation (bottom, left and right). *Control*, non-ventilated group; *Supine HT*, IgG injection 1 hour before mechanical ventilation + HTV ventilation; *EphA2_Supine HT*, EphA2 Ab injection 1 hour before mechanical ventilation + HTV ventilation; *Supine HT_EphA2*, HTV ventilation + EphA2 Ab injection 2 hours after mechanical ventilation.

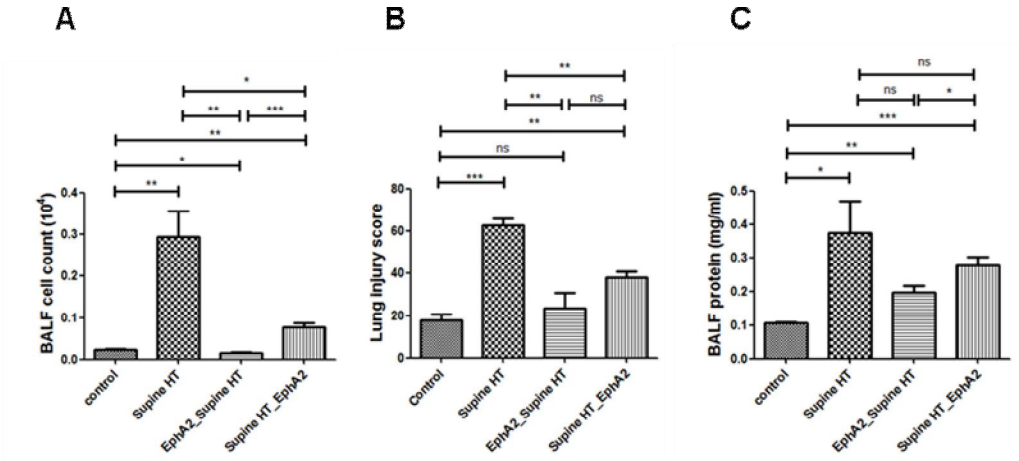


Figure 3. Pre- or post-treatment with EphA2 receptor Ab attenuated ventilator-induced lung injury, as demonstrated by decreased total cell counts (A), lung injury scores (B), and protein concentrations (C) in bronchoalveolar lavage fluid (n = 4/group). *Control*, non-ventilated group; *Supine HT*, IgG injection 1 hour before mechanical ventilation + HTV ventilation; *EphA2_Supine HT*, EphA2 Ab injection 1 hour before mechanical ventilation + HTV ventilation; *Supine HT_EphA2*, HTV ventilation + EphA2 Ab injection 2 hours after mechanical ventilation. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$ by two-way ANOVA and *post hoc* analysis with Bonferroni's correction. BALF, bronchoalveolar lavage fluid; ns, not significant

In addition, the degree of alveolar capillary barrier damage was assessed by electron microscopy. Compared with control group (Figure 4A and 4B), HTV ventilation group (Figure 4C and 4D) demonstrated extensive widening of basement membrane, separation of endothelium and epithelium from the basement membrane, swelling and apoptotic change of endothelial cells, and damaged epithelial cells. In contrast, groups receiving pre (Figure 4E and 4F) or post-treatment (Figure 4G and 4H) with EphA2 receptor Ab showed limited regions of blebs, minimal widening of basement membrane, and relatively little injury to the adjacent alveolar capillary barrier.

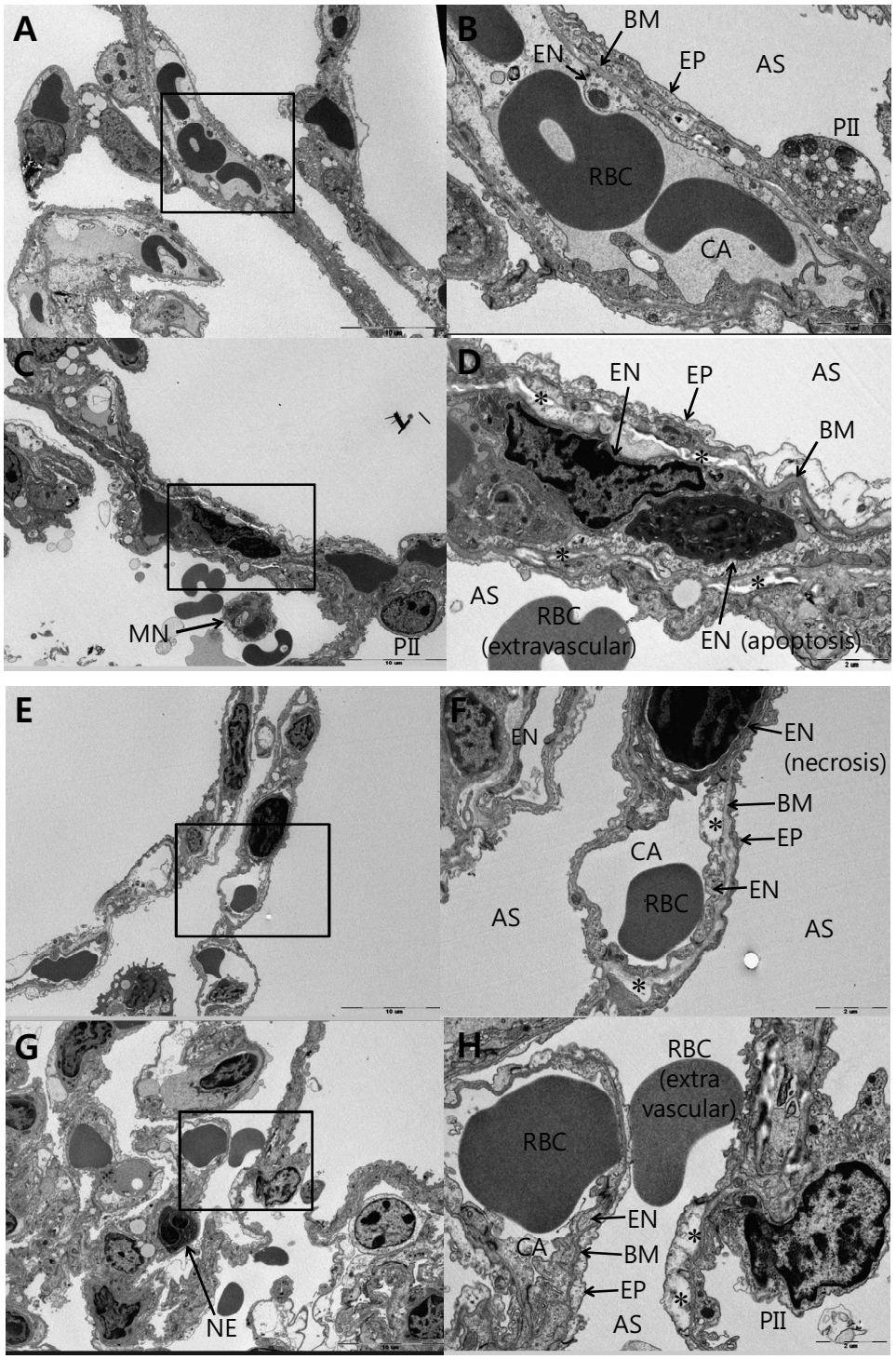
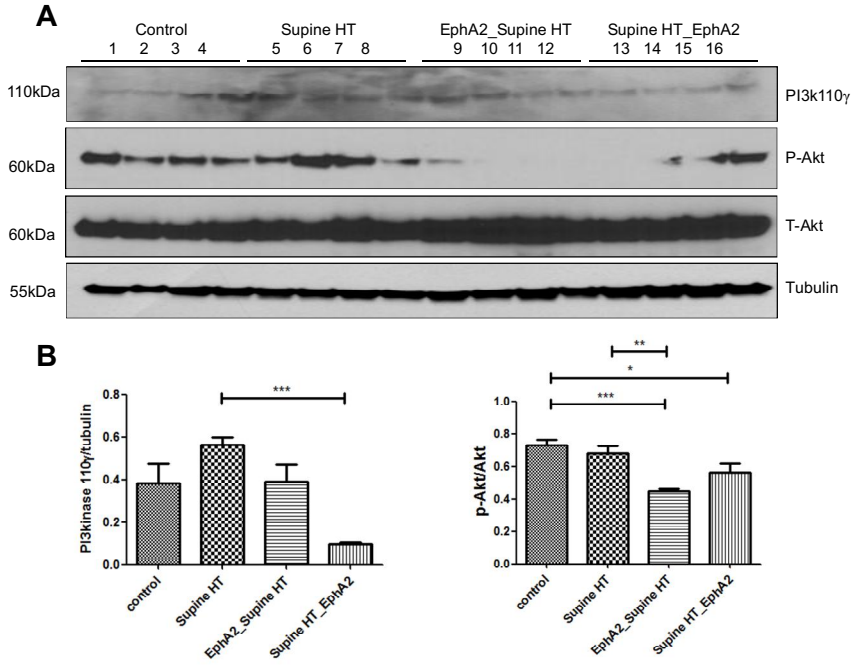


Figure 4. Pre- or post-treatment with EphA2 receptor Ab diminished ventilator-induced lung injury, as shown by transmission electron micrographic features of mice lung tissues. Compared with control group (A, B), Supine HTV group (C, D) showed swelling and apoptotic change in endothelial cells, damaged epithelial cells, extensive widening of basement membrane, and separation of endothelium and epithelium from the basement membrane (*), whereas groups receiving pre (E, F) or post-treatment (G, H) with EphA2 receptor Ab demonstrated minimal widening of basement membrane, limited regions of blebs (*), and relatively little injury to the adjacent alveolar capillary barrier. AS, alveolar space; BM, basement membrane; CA, capillary lumen; EN, endothelium; EP, epithelium; MN, mononuclear cell; NE, neutrophil; PII, type 2 pneumocyte; RBC, red blood cell. Scale bar = 10 m (A, C, E, G) and 2 m (B, D, F, H).

3. EphA2 receptor mediates ventilator-induced lung injury via PI3Kγ/Akt/NF-κB dependent manner

PI3Kγ/Akt/NF-κB signaling cascade plays a critical role in inflammatory response elicited by injurious mechanical ventilation.[20-23] It has not been identified thoroughly, however, whether EphA2 receptor mediates VILI via the same signaling pathway. Therefore, we investigated the effect of EphA2 antagonism on the activation of PI3Kγ, Akt, and NF-κB using EphA2 receptor Ab. As shown in Figure 5, groups receiving EphA2 receptor Ab showed a significant decrease in PI3Kγ activation and Akt phosphorylation (Figure 5A and 5B) compared with HTV ventilation group ($p < 0.01$). In addition, the p-IκBα/IκBα ratio was higher in EphA2 receptor Ab treatment groups compared with HTV ventilation group ($p < 0.05$) (Figure 5C and 5D), suggesting the downregulation of NF-κB expression by EphA2 receptor Ab and the potential downstream involvement of PI3Kγ/Akt/NF-κB dependent pathway in the EphA2 receptor-mediated signaling cascade for VILI.



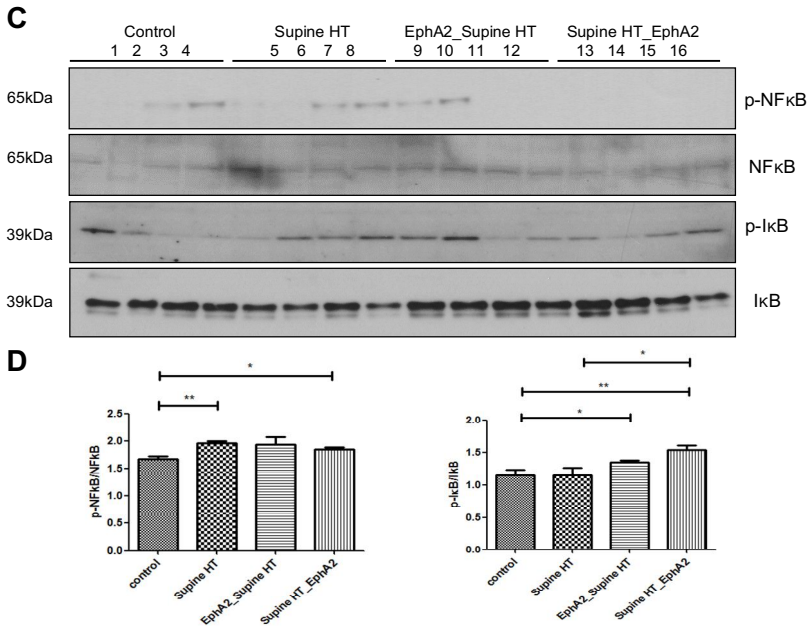


Figure 5. Pre- or post-treatment with EphA2 receptor Ab reduced the mechanical stretch-induced expression of PI3Kγ, Akt, and NF-κB in lung tissue, as shown by Western blots (A, C) and densitometry (B, D) of PI3Kγ, Akt, NF-κB, and IκB bands normalized to α-tubulin (n = 4/group). *Control*, non-ventilated group; *Supine HT*, IgG injection 1 hour before mechanical ventilation + HTV ventilation; *EphA2_Supine HT*, EphA2 Ab injection 1 hour before mechanical ventilation + HTV ventilation; *Supine HT_EphA2*, HTV ventilation + EphA2 Ab injection 2 hours after mechanical ventilation. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$ by two-way ANOVA and *post hoc* analysis with Bonferroni's correction. PI3Kγ, phosphoinositide-3 kinase γ; Akt, serine/threonine kinase-protein kinase B

4. EphA2 antagonism under injurious mechanical ventilation downregulates mTOR expression

To investigate whether mTOR pathway is involved in the EphA2 receptor-mediated signaling cascade, we evaluated the expression of P70S6 kinase, a major downstream target of mTOR,[29] by Western blot analysis. As shown in Figure 6, pre- or post-treatment with EphA2 receptor Ab reduced mechanical stretch-induced P70S6 kinase expression ($p < 0.01$), suggesting the potential downstream involvement of mTOR in EphA2 receptor-mediated signaling cascade for VILI.

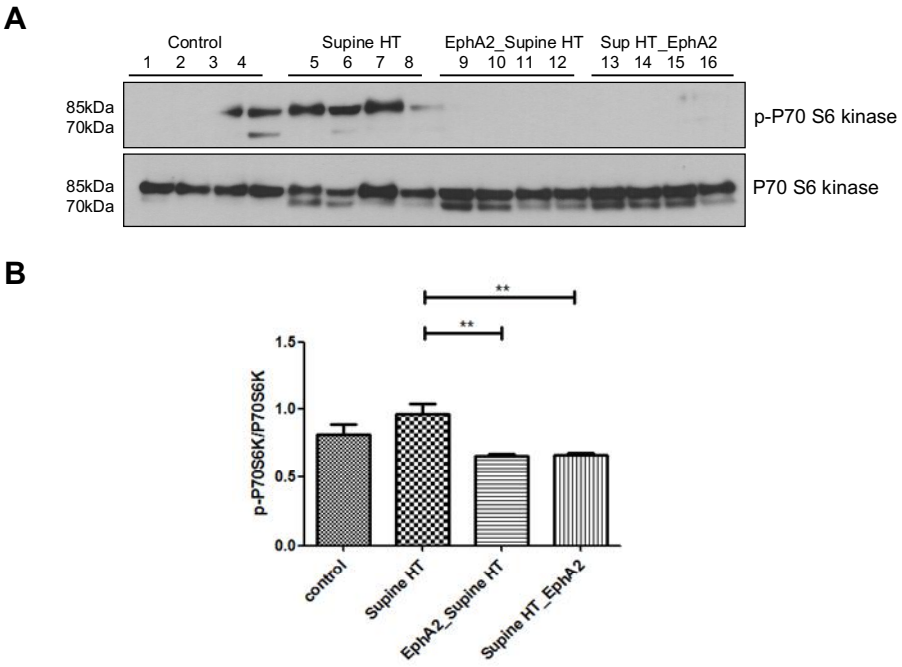


Figure 6. Pre- or post-treatment with EphA2 receptor Ab under injurious mechanical ventilation decreased mTOR expression in lung tissue, as shown by Western blots (A) and densitometry (B) of P70S6 kinase (a major downstream target of mTOR) bands normalized to α-tubulin (n = 4/group). *Control*, non-ventilated group; *Supine HT*, IgG injection 1 hour before mechanical ventilation + HTV ventilation; *EphA2_Supine HT*, EphA2 Ab injection 1 hour before mechanical ventilation + HTV ventilation; *Supine*

HT_EphA2, HTV ventilation + EphA2 Ab injection 2 hours after mechanical ventilation. *$p < 0.05$ and **$p < 0.01$ by two-way ANOVA and *post hoc* analysis with Bonferroni's correction. mTOR, mammalian target of rapamycin

5. Blockade of EphA2 receptor do not influence on the release of proinflammatory mediators

To clarify the effect of EphA2 receptor Ab on the release of proinflammatory cytokine/chemokine, we compared the levels of IL-1β, IL-6, and KC among groups. As expected, the proinflammatory cytokine/chemokine levels were increased by HTV mechanical ventilation. However, EphA2 receptor Ab did not significantly decrease the levels of those proinflammatory mediators (Figure 7A, 7B, and 7C).

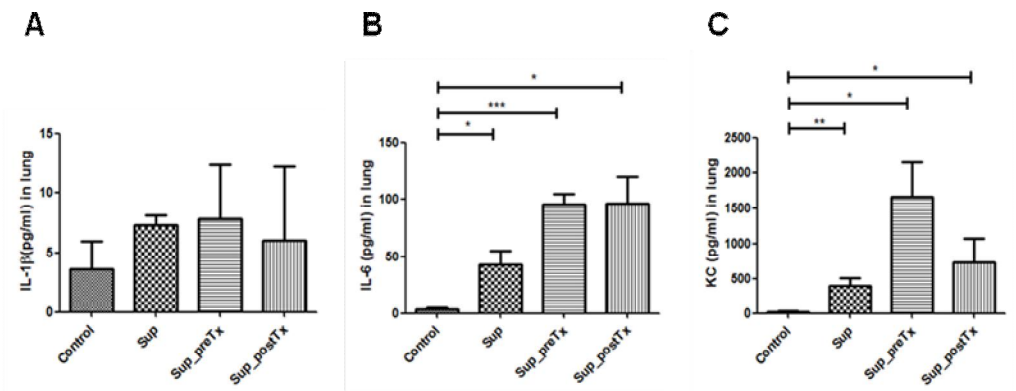


Figure 7. EphA2 receptor blocking did not influence on the release of proinflammatory mediators in lung tissue, as shown by ELISA-based array (n = 4/group). *Control*, non-ventilated group; *Supine*, IgG injection 1 hour before mechanical ventilation + HTV ventilation; *Sup_preTx*, EphA2 Ab injection 1 hour before mechanical ventilation + HTV ventilation; *Sup_postTx*, HTV ventilation + EphA2 Ab injection 2 hours after mechanical ventilation. *$p < 0.05$, **$p < 0.01$, and

***$p < 0.005$ by two-way ANOVA and *post hoc* analysis with Bonferroni's correction. IL, interleukin; KC, keratinocyte chemoattractant

6. The expression of EphA2 receptor and EphrinA1 in lung tissue is up-regulated in patients with respiratory infection

To explore the expression of EphA2/ephrinA1 in human respiratory disease, we measured EphA2/ephrinA1 levels in BALF of patients who had undergone bronchoscopic evaluation. Although not statistically significant, EphA2 levels showed a trend toward increase in patients diagnosed as having pneumonia with or without mechanical ventilatory support compared with control group (control, 0.05 ± 0.03 ng/ml; pneumonia without mechanical ventilatory support, 0.08 ± 0.05 ng/ml; pneumonia with mechanical ventilatory support, 0.12 ± 0.13 ng/ml; $p = 0.101$) (Figure 8A). Similarly, ephrinA1 levels were higher in patients with pneumonia with or without mechanical ventilatory support compared with control group (control, 0.03 ± 0.02 ng/ml; pneumonia without mechanical ventilatory support, 0.20 ± 0.15 ng/ml; pneumonia with mechanical ventilatory support, 0.63 ± 0.65 ng/ml; $p < 0.001$) (Figure 8B).

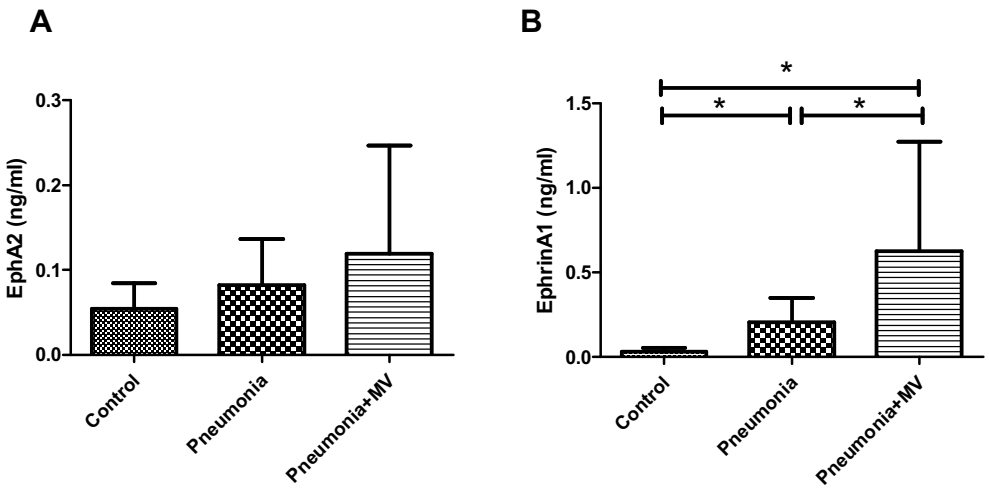


Figure 8. The levels of EphA2 receptor (A) and the ligand EphrinA1 (B) in lung tissue were higher in subjects diagnosed as having pneumonia with or without mechanical ventilatory support compared with control group. The mean ± SD values of EphA2 levels in control group (n = 15), pneumonia without mechanical ventilation group (n = 14), and pneumonia with mechanical ventilation group (n = 38) were 0.05 ± 0.03 ng/ml, 0.08 ± 0.05 ng/ml, and 0.12 ± 0.13 ng/ml, respectively ($p = 0.101$). The mean ± SD values of ephrinA1 levels in control group, pneumonia without mechanical ventilation group, and pneumonia with mechanical ventilation group were 0.03 ± 0.02 ng/ml, 0.20 ± 0.15 ng/ml, and 0.63 ± 0.65 ng/ml, respectively ($p < 0.001$). *$p < 0.05$ by two-way ANOVA and *post hoc* analysis with Bonferroni's correction. MV, mechanical ventilation

IV. DISCUSSION

In this study, we determined the role of EphA2/ephrinA1 and associated signaling mechanism in VILI model. Compared with control group, HTV mechanical ventilation group exhibited a significant increase in EphA2/EphrinA1 expression, which was partially blocked by pre- or post-treatment with EphA2 receptor Ab. As shown by BALF, histologic analysis, and electron micrographs, EphA2 antagonism ameliorated the extent of VILI. Moreover, EphA2 receptor mediated the complex inflammatory process in VILI, at least in part, through PI3Kγ/Akt/NF-κB signaling pathway, and possibly via mTOR pathway. To the best of our knowledge, this is the first study showing the role of EphA2/ephrinA1 and associated signaling mechanism in VILI model.

The effect of EphA2/ephrinA1 antagonism on lung injury has been controversial. Previous studies have demonstrated that EphA2 receptor is closely related with acute lung injury (ALI) or inflammation and EphA2 antagonism has been shown to ameliorate the extent of lung injury by various in vivo and in vitro models.[13,17-19] In contrast, others reported that EphA2-deficient mice exhibited an exaggerated airway inflammatory response by treatment with *Mycoplasma pulmonis* infection or ovalbumin sensitization.[30] In all these studies, however, the downstream signaling cascades of EphA2 receptor responsible for increased endothelial permeability had not been explained in detail. Furthermore, the role of EphA2/ephrinA1 and associated signaling pathways, especially in VILI model, have not been previously assessed. Since the lung cellular activation mechanism may be different depending on the type of insults[31] and the expression of Ephs/ephrins and their downstream signaling pathways are cell type- and microenvironment-dependent,[9,32] it is worth while establishing the exact role of EphA2/ehprinA1 and associated signaling mechanisms in the process of mechanical stretch-induced lung injury.

It is known that the interaction of EphA2/ephrinA1 is involved in the morphological changes of cytoskeletal architecture.[9,33] Moreover, EphA2 receptor has been shown to regulate endothelial cell migration and assembly through PI3K-mediated Rac1 GTPase,[34] suggesting that EphA2 is a key regulator of angiogenic remodeling in endothelium. Considering the vascular remodeling/angiogenic properties of EphA2/ephrinA1[35-37] and the propermeability nature of PI3Kγ/Akt-dependent pathway,[20-22] it is anticipated that EphA2/ephrinA1 should also mediate the complex process of mechanical stretch-induced biotrauma in a PI3Kγ/Akt-dependent manner. In fact, the involvement of EphA2 receptor-mediated PI3Kγ/Akt-dependent signaling cascade has been previously documented in cell migration/invasion[38-40] and wound healing.[41] Signal transduction pathways which connect EphA2 receptor activation to permeability change in epithelial/endothelial cells during mechanical stretch, however, have not yet been established in detail. Therefore, the present study is important in that it provided an evidence for the involvement of EphA2/ephrinA1 in the complex inflammatory process of VILI, partially through PI3Kγ/Akt/NF-κB-dependent pathway.

PI3Ks are isoforms of heterodimeric lipid-modifying enzymes for generating lipid second messengers involved in the activation of multiple intracellular signaling cascades.[42] Subclass IA PI3K consists of p110α, β, and δ catalytic subunits associated with the p85 family of adaptor proteins, whereas PI3Kγ belongs to subclass IB PI3K comprised of the single p110γ catalytic subunit and a 101 kD regulatory subunit (p101).[43] PI3Kγ signaling is controlled by β/γ subunits of heterotrimetic G proteins and its downstream pathways regulate various cell functions including cell growth, survival, migration, metabolic control, cytoskeletal remodeling, and angiogenesis.[43-46] In the process of VILI, PI3Kγ contributes to NF-κB activation, inflammatory cytokine gene activation, nitric oxide production, and edema formation.[20-22] Study results about the involvement of PI3Kγ in ALI, however, have been ambiguous. In an *ex vivo* model of VILI, PI3Kγ gene-deficient ($PI3K\gamma^{-/-}$) mice showed improved respiratory

mechanics and reduced edema/hyaline membrane formation.[20,21] In another study with the same model, mice pretreated with a non-selective PI3K inhibitor exhibited an attenuation in the release of inflammatory cytokines and a downregulation of NF-κB activation in inflammatory cells.[47] In contrast, the activation of PI3K-dependent pathways partially restored epithelial barrier function in an *in vitro* model of mechanical injury.[48]

Akt, also known as protein kinase B, is a major downstream target of PI3K and a multifunctional protein kinase for regulating endothelial cell survival, migration, nitric oxide synthase activity, and angiogenesis.[45] Akt can activate NF-κB pathway for organizing a complex network of angiogenesis[49] and controls cell growth and protein synthesis by mTOR phosphorylation.[50] As with the role of PI3K in VILI, however, conflicting data have been also reported about the effect of Akt activation on VILI. In an *in vivo* model of VILI, the activation of Akt by HTV mechanical ventilation resulted in lung inflammation, injury, and fibrosis.[20,21,23,51] On the contrary, the inhibition of Akt phosphorylation enhanced capillary leak and edema formation by mechanical ventilation in other *in vivo* model.[52] In the same manner, the inhibition of Akt activation augmented mechanical stretch-induced apoptosis in pulmonary microvascular endothelial cells in an *in vitro* model.[25] Others also reported that the anti-inflammatory effect of isoflurane during injurious mechanical ventilation was mediated via PI3K/Akt-dependent signaling pathway in an *in vivo* model of VILI.[53]

These seemingly apparent discrepancies in previous studies with regard to the effect of EphA2/ephrinA1-triggered and PI3Kγ/Akt-dependent signaling pathway in different lung injury models may be attributed to the context-dependent characteristics of EphA2/ephrinA1[9,32] and simultaneous activation of multiple PI3Kγ-mediated pathways with opposing effects.[21,22,54,55] Since there are many cross-talks between EphA2/PI3Kγ/Akt and other signaling pathways which can both enhance or suppress inflammation and lung injury, PI3Kγ and Akt activities may be influenced by these pathways.[22,52] For instance, activation of PI3K/Src pathway by ventilator-

induced lung overdistension appears to increase pulmonary vascular permeability and edema formation, whereas activation of the PI3K/Akt/GSK3β pathway tends to oppose this effect.[22] Thus, mechanical stretch-induced alveolar overdistension may activate EphA2/PI3Kγ/Akt-mediated signaling pathway with propermeability nature, which may be counteracted by other PI3K/Akt-dependent pathways and the resultant net balance among these pathways may determine the final inflammatory or endothelial permeability effect.

Serine/threonine kinase mTOR is one of the main downstream targets of PI3K/Akt, and PI3K/Akt/mTOR signaling pathway have a critical role in cellular proliferation, survival, and metabolism.[50] In addition, a growing body of evidence suggests the critical role of mTOR in the development of ALI.[56-58] While mechanical ventilation has been reported to induce mTOR expression,[59] only limited studies have focused on the function of mTOR in VILI model.[25] Therefore, its exact functions in the pathogenesis of VILI remain controversial. Since EphA2/ephrinA1 and mTOR seem to influence on the cellular actin-cytoskeletal structure in common,[9,33,60,61] it is expected that EphA2/ephrinA1 signaling cascade may be mediated, at least in part, through mTOR. Currently, however, there is no study concerning the crosstalk between these molecular targets in VILI model.

In the present study, we found that the blockade of EphA2 receptor suppressed the activation of mTOR, which is in line with the previous report showing that mTOR inhibition significantly reduced mechanical stretch-induced apoptosis in pulmonary microvascular endothelial cell.[25] Taken together, our data support the association between EphA2 receptor-mediated signaling process and mTOR pathway in the mechanically triggered cellular events. On the other hand, given that mTOR plays different roles in different cell types by a pathogen/stimuli-dependent manner,[62] it is not incomprehensible that the function of mTOR is either cytoprotective or detrimental depending on the types of insults in different ALI models. Furthermore, recent studies with various ALI models have mainly focused on epithelial cells or alveolar macrophages exploring the regulatory role of mTOR on inflammation or

programmed cell death in ALI, or have been even without investigation of cell-specific mechanism.[62] Therefore, more detailed studies with cell-specific methods are warranted to establish the relationship between EphA2 receptor and mTOR interaction in VILI or other models of ALI.

In the present study, blockade of EphA2/PI3Kγ/Akt-mediated signaling pathway did not influence on the proinflammatory cytokine/chemokine levels. Similar with our results, several investigators also demonstrated that suppressing PI3Kγ/Akt-mediated signaling cascade had no effect on the release of inflammatory cytokines.[20,21] In contrast, other researchers reported that PI3K inhibition prevented the release of inflammatory cytokine.[47] In our opinion, there are several possible mechanisms to explain this inconsistency. First, it is suggested that inflammatory cytokines have a minimal role in the immediate permeability increase due to HTV mechanical ventilation.[63,64] Second, silencing PI3Kγ-Akt mediated signaling pathway may attenuate the extent of VILI independently of inhibitory effects on cytokine/chemokine release but by enhancing pulmonary apoptosis,[21] reducing nitric oxide production,[20,65] and augmenting cAMP-dependent alveolar fluid clearance.[20] In addition, there are several evidences that PI3Kγ-Akt pathway has an anti-inflammatory property by modulating IL-10 or other immunoregulatory cytokines.[66,67] Therefore, it is possible that the blockade of EphA2/PI3Kγ/Akt-dependent signaling pathway in our VILI model may have attenuated permeability increase/edema formation in the lung without affecting the release of inflammatory cytokine/chemokine.

Third, besides Eph receptor tyrosine kinases, other components of innate immune system, such as damage-associated molecular patterns and their pattern recognition receptors, may also have been activated by injurious mechanical ventilation.[68] These receptors include Toll-like receptors,[69,70] nucleotide-binding oligomerization domain-like receptors,[71] and the receptor for advanced glycation end products.[72] Moreover, as previously mentioned, PI3Kγ/Akt/mTOR-dependent signaling pathway can be activated not only by EphA2/ephrinA1 but by diverse

stimuli from intra- or extracellular environment. Therefore, it is possible that those pattern recognition receptors or other receptor tyrosine kinases may also have recruited PI3Kγ/Akt/mTOR pathway or other inflammatory signaling cascades, resulting in the release of proinflammatory cytokine/chemokine regardless of EphA2 antagonism in the present study. Fourth, 24 ml/kg of the "high" tidal volume adopted in our VILI model may not have been sufficient to trigger significant cytokine release in the HTV mechanical ventilation group. Previous study revealed that tidal volumes up to 20 ml/kg are not enough to induce substantial overstretch in models using healthy young mice, and inflammatory cytokine levels did not increase up to 30 ml/kg of tidal volume compared with control group.[73] Consequently, it should be noted that the standard of clinically relevant high tidal volume remains elusive.

Unlike laboratory conditions where most confounding factors are under control, a considerable number of patients with lung injury may face delayed treatment in real clinical world, causing irreversible damage to the lung.[74] Considering this practical problem, therapeutic modality should ideally exhibit at least some effect even when it is applied late in the course of disease. In this regard, EphA2 receptor appears as a promising therapeutic target for VILI, because post-treatment with EphA2 receptor Ab in our study also partially suppressed the downstream signaling cascade and ameliorated the extent of VILI. In addition, the present human data from BALF demonstrated an increased expression of EphA2/ephrinA1 in the presence of pneumonia and mechanical ventilatory support. Though it is not clear whether this increase was due to the mechanical ventilation itself or merely reflected the disease severity, the up-regulation of EphA2/ephrinA1 expression in patients with respiratory infection may provide an additional evidence for the clinical utility of EphA2/ephrinA1 as a potential biomarker for respiratory infection or as a promising therapeutic target for modulating VILI. The efficacy and the clinical applicability of EphA2 antagonist, especially with respect to the treatment timing, need to be further validated.

There are several possible limitations in our study. First, since we did not use

knock-out mice in our experiments, the effect of EphA2 receptor blocking may not have been complete. Second, we cannot exclude the possibility that some of our data showing no statistically significant differences among groups were attributed to the relatively small numbers of mice in each group. Third, analogous to the previous studies, the techniques used in this study were neither truly quantitative nor able to localize cellular activation. Therefore, further researches with EphA2/ephrinA1 knock-out mice using flow cytometry-based or other cell-specific method and increasing the numbers per group might offer an additional basis for our results.

V. CONCLUSION

The present study demonstrates the critical role of EphA2/ephrinA1 in inflammatory process and related signaling mechanism in VILI. EphA2 antagonism attenuated the extent of lung injury without affecting the release of proinflammatory cytokines, which was mediated partly through PI3Kγ/Akt/NF-κB signaling pathway and possibly via mTOR pathway. Targeted approach for restoring increased epithelial/endothelial permeability while maintaining immune responses to mechanical insult may be safer and more effective for patients with VILI. In this respect, EphA2 receptor may be a potential therapeutic target for modulating VILI. Further investigations are required to evaluate the clinical safety and efficacy of EphA2 antagonism in patients with VILI.

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ABSTRACT (IN KOREAN)

인공호흡기 유도 폐손상 쥐모델에서 erythropoietin-producing hepatoma receptor tyrosine kinase A2 의 역할

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배경: Erythropoietin-producing hepatoma (Eph) receptor tyrosine kinases 와 이들의 리간드인 ephrins 는 다양한 생물학적인 기능을 가지며 발생과정과 출생 이후 전 시기에 걸쳐 세포 생태에 많은 영향을 미치게 된다. 이들 중 EphA2 수용체 및 이에 대한 리간드인 ephrinA1 은 상피 및 내피막의 투과성에 영향을 주어 폐의 염증반응 및 손상을 일으키는 데 중요한 역할을 하는 것으로 알려져 있다. 따라서, 본 연구에서는 인공호흡기 유도 폐손상 쥐모델을 사용하여 EphA2 수용체 차단의 효과 및 이에 관련된 신호전달경로를 밝히고자 하였다.

대상 및 방법: 야생형 수컷 C57BL/6J 유전형 쥐들을 무작위로 다음과 같이 4개의 실험군으로 나누었다 (n = 4/군); (1) 인공호흡기를 사용하지 않은 대조군, (2) 인공호흡기 적용 1시간 전 면역글로블린 G

(IgG) 를 투여한 뒤 높은 일회 환기량의 인공호흡기를 적용한 군, (3) EphA2 수용체 항체를 투여한 후 1시간 뒤 높은 일회 환기량의 인공호흡기를 적용한 군, (4) 높은 일회 환기량의 인공호흡기를 적용한 후 2시간 뒤 EphA2 수용체 항체를 투여한 군. 폐손상 정도는 기관지폐포 세척액 분석, 폐손상 점수 및 전자현미경사진으로 평가하였다. 폐조직 용해물에서 EphA2, ephrinA1, PI3Kγ, Akt, NF-κB, P70 S6 kinase 의 발현 정도는 특수 단백질 검출 검사법을 통해, 인터루킨1β, 인터루킨6, 케라틴생성세포 화학주성인자의 분비 정도는 효소 결합 면역분석 방법을 통해 확인하였다.

결과: EphA2/EphrinA1 의 발현 정도는 대조군에 비해 높은 일회 환기량의 인공호흡기를 적용받은 군에서 더 높았으며, EphA2 수용체 항체에 의해 감소하였다. EphA2 수용체 항체는 폐손상 정도를 경감시켰으며 PI3Kγ/Akt/NF-κB/mTOR 의 발현 정도를 낮추었다. EphA2 수용체 항체는 염증성 사이토카인과 케모카인 분비에는 영향을 미치지 않았다.

결론: 본 실험 결과는 EphA2/ephrinA1 이 PI3Kγ/Akt/NF-κB 및 mTOR 를 포함하는 신호전달경로를 통해 인공호흡기 유도 폐손상에 관여한다는 것을 보였으며, 인공호흡기 유도 폐손상을 억제시킬 수 있는 치료 대상으로서 EphA2/ehprinA1 의 활용 가능성을 제시하였다.

핵심되는 말: EphA2, ephrinA1, 인공호흡기 유도 폐손상, PI3Kγ, Akt, NF-κB, mTOR