The role of EphA2/ephrinA1 pathway in hyperoxia-induced lung injury

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The role of EphA2/ephrinA1 pathway in hyperoxia-induced lung injury

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I want to dedicate this thesis to my lovely wife, Ji Yoon Han, and my son, Ha Seung Jung. When I was tired and struggling, their support and infinite love gave me courage during my research.

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ABSTRACT

The role of EphA2/ephrinA1 pathway in hyperoxia-induced lung injury

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Background: Oxygen therapy is an essential treatment for patients with acute respiratory distress syndrome. However, the inevitable development of hyperoxia-induced lung injury (HALI) may worsen patient prognosis. EphA2/ephrinA1 receptor-ligand signaling regulates the cytoskeleton and cell adhesion in various diseases and embryogenesis. We investigated whether EphA2/ephrinA1 signaling was associated with HALI in the present study.

Methods: Male wild-type C57BL/6J mice (20-24 g; Orient Bio) were exposed to >95% O_2 in a Plexiglass chamber. To evaluate the association between changes in EphA2/ephrinA1 signaling and exposure time on HALI, analysis was performed at 24 hr, 48 hr, and 72 hr compared with normoxia (21% O_2 room air). To analyze the effect of an EphA2 monoclonal antibody (mAb, 8 μg via tail vein, pretreatment), the mice were sacrificed at 72 hr after hyperoxia exposure. Total cell counts, protein concentration measurements, and cytokine Luminex® assays (Biotech®, Minneapolis, USA) were performed on bronchoalveolar lavage (BAL) fluid. Western blotting, immunohistochemical (IHC), and immunofluorescence (IF) staining were performed on mouse lung

tissue. To analyze the effect of the EphA2 mAb on survival, animal survival was observed by placing the mice in normoxic conditions after 72 hr of hyperoxia exposure.

Results: With increasing exposure to hyperoxia, the expression of phosphorylated EphA2 in mouse lung tissue significantly increased, and protein leakage and cytokine activation (IL-1β, TNF-α, IL-6, and MIP-2) in BAL fluid were increased ($p<0.05$). In addition, the expression of tight or adherens junctions proteins, such as ZO-2 and VE-cadherin, was disrupted due to hyperoxia. In the HALI mouse model, compared with phosphate-buffered saline (PBS), the EphA2 mAb attenuated cytokine activation (IL-10 and TNFα) in BAL fluid and lung injury scores ($p<0.05$). The EphA2 mAb attenuated oxygen stress and abrogated the degradation of tight junction or adherens junction proteins, which was accompanied by a decrease in the expression of the antiapoptotic protein Bcl-2 ($p<0.05$). Furthermore, the EphA2 mAb improved the survival of HALI mice ($p=0.045$).

Conclusion: EphA2/ephrinA1 signaling is significantly associated with HALI. EphA2 mAb treatment prevented alveolar-endothelial barrier damage and improved survival.

Key words: ephA2, ephrinA1, hyperoxia-induced lung injury

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I. INTRODUCTION

Various diseases that cause acute respiratory failure lead to a decrease in the oxygen absorption capacity of the human lung. In particular, acute respiratory distress syndrome (ARDS) is a devastating and fatal clinical syndrome with 30-50% mortality.[1,2] Most patients with severe ARDS inevitably require high concentration oxygen therapy with an invasive mechanical ventilator. However, this high concentration oxygen therapy may exert dual effects. High concentration oxygen therapy is essential for treating acute respiratory failure through adequate tissue delivery of oxygen to meet tissue oxygen requirements. However, excessive oxygen delivery can produce hyperoxia and generate toxic reactive oxygen species.[3,4] Hyperoxia can cause oxygen-induced pulmonary injury[5-8], reperfusion-associated injury[9], the loss of lung function[10], and an increase in mortality.[11]

It is still unclear how supplementary oxygen should be appropriately applied.[12-14] Excessive and prolonged exposure to oxygen (after a minimum of 48 hr of $FiO_2 \geq 0.5$) that exceeds the capacities of physiological antioxidants such as superoxide dismutase and catalase can cause radiological and cellular

changes in the lung similar to those of ARDS.[7,15] Common findings in both severe oxygen toxicity and ARDS include pulmonary edema, hyaline membrane formation, thickening of pulmonary arterioles, and worsening of the PaO_2/FiO_2 ratio.[16,17] Therefore, understanding the mechanisms by which hyperoxia contributes to lung pathogenesis is crucial to limiting the potentially harmful effects of oxygen toxicity in the clinic.

The erythropoietin-producing hepatoma (Eph) receptor and ephrin ligand family affect the pathogenesis of many diseases.[18-20] The Eph/ephrin receptor-ligand family are a group of cell surface proteins that may play essential roles in injury[21] and inflammation.[22] A total of 10 EphA receptors and 6 EphB receptors have been discovered. Sequence differences within the extracellular ligand-binding domain distinguish EphA and EphB receptors. Eph ligands are known as ephrins and include 6 glycosylphosphatidylinositol-linked ephrinA ligands and 3 transmembrane ephrin-B ligands.[23,24] Each Eph receptor preferentially binds specific Ephrin ligands. According to several studies, the EphA2 receptor and ephrinA1 ligand affect inflammation via vascular endothelial injury.[20,25] EphA2 receptors can promote monocyte and macrophage spreading through integrin-mediated cell adhesion.[26] The expression of EphA2 was increased in bleomycin-induced lung injury, and EphrinA1 was associated with NF-κB activation in endothelial cells.[27] We previously proved that the inhibition of EphA2/EphrinA1 signaling attenuated lipopolysaccharide-induced lung injury[28] and ventilator-induced lung injury.[29]

Therefore, the pathogenesis of hyperoxia-induced lung injury (HALI) might be associated with the mechanism of action of Eph/ephrin signaling. We hypothesized that EphA2/ephrinA1 signaling played an essential role in the pathogenesis of HALI and that blocking EphA2/ephrinA1 would protect against HALI.

II. MATERIALS AND METHODS

1. HALI mouse model

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Yonsei University College of Medicine (Seoul, Korea) and were conducted according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Male wild-type C57BL/6J mice (20-24 g; Orient Bio, Sungnam, Korea) were provided food and water and subjected to a similar day and night light cycle.

Eight- to ten-week-old male mice were exposed to room air (>21% O_2) or 100% O_2 in a sealed Plexiglass hyperoxia chamber (airflow: 2 L/min) for 72 hr as described previously.[30] We regularly checked that the oxygen concentration in the chamber was maintained above 95% with an oximeter. First, we examined changes over time (at 0 hr, 24 hr, 48 hr, and 72 hr) to evaluate whether the HALI mouse model was appropriate and whether HALI was associated with changes in EphA2/ephrinA1 signaling. At each time point, the mice were humanely killed by a lethal dose of ketamine and xylazine. Second, to confirm the effect of the EphA2 monoclonal antibody (mAb), changes in each group were evaluated at 72 hr of exposure to hyperoxia. Twelve mice were randomly divided into three groups: the control group, which was exposed to room air and administered phosphate-buffered saline (PBS); the HALI with PBS group; and the HALI with EphA2 mAb group. We administered the EphA2 mAb (R&D Systems, Inc., Minneapolis, MN 55413, USA) via the tail vein to evaluate the effect of the EphA2 mAb on HALI. Third, prolonged exposure to hyperoxia for more than 72 hr will kill most mice. However, survival analysis can be assessed if the mice are placed in room air after 72 hr of exposure to hyperoxia, depending on the extent of HALI. Without any treatment, approximately 60-80% mortality can occur. Therefore, we evaluated the preventive effect of

the EphA2 mAb on HALI using this method (Figure 1).

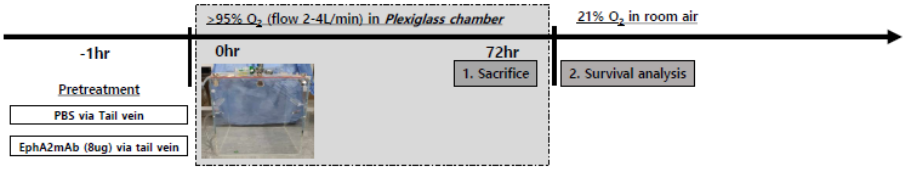


Figure 1. Experimental design of the hyperoxia-induced lung injury (HALI) mouse model
By maintaining 100% 2-4 L/min O2, a hyperoxic environment of >95% was created in the Plexiglass chamber, as measured by oximetry. PBS and the EphA2 mAb (pretreatment) were administered via the tail vein. First, the effect of the EphA2 mAb on HALI at 72 hr was analyzed. Second, after 72 hr of hyperoxia, survival was analyzed after exposure to atmospheric oxygen.

2. Isolation of bronchoalveolar lavage (BAL) cells, lung tissues, and cell counts

BAL was performed through a tracheal cannula using two 1-ml aliquots of sterile saline. The BAL fluid was centrifuged (4°C, 400 × g, 10 minutes), and the supernatant was stored at -80°C for further analysis. The cell pellet was reconstituted in 100 µl of PBS and used to quantitatively and qualitatively analyze the cells. According to the manufacturer's protocol, total cell numbers in each sample were determined using a hemocytometer (Marienfield). A 90-µl aliquot of each sample was transferred to slide chambers and inserted into a cytospin with the slide facing outward. The slides were centrifuged at 600 rpm for 6 minutes, removed from the centrifuge and dried before being stained. The slides were immersed in three Diff Quick solutions (Fixative, Solution I, and Solution II) and rinsed with purified water. The protein content of the BAL supernatant was measured using a Pierce® BCA protein assay (Thermo Fisher Scientific, Rockford, IL, USA). After being incubated for 30 minutes at 37°C, the plate was cooled, and the absorbance was measured at 562 nm with a

spectrophotometer.

3. Histopathological grading of lung injury

The right lung was isolated and stored at -80°C before protein extraction after the pulmonary vasculature was flushed with saline under low pressure. The left lung was inflated via tracheostomy with 10% formaldehyde in PBS at a pressure of 25 cm H_2O until the pleural margins became sharp. The lung was then excised and fixed overnight in 10% formaldehyde in PBS, embedded in paraffin and sectioned at a thickness of 5 μm. Left lung sections were stained with hematoxylin and eosin (H&E) and subjectively evaluated by light microscopy. The histopathology was reviewed in a blinded manner by two qualified investigators. Five easily identifiable pathological processes were scored using the weighted scale presented in the official ATS workshop report.[31]

4. Cytokine analysis of BAL fluid

According to the manufacturer's protocol, the levels of several cytokines in BAL fluid were measured using a Magnetic Luminex® Screening Assay Kit. All samples and standards were analyzed in duplicate using a Luminex MAGPIX Instrument (Biotech®, Minneapolis, USA).

5. Protein extraction and western blotting

Lung tissues were homogenized in 1 ml of RIPA buffer (50 mM Tris-HCl, pH 7.5; 0.1% sodium dodecyl sulfate [SDS]; 2 mM ethylenediaminetetraacetic acid; 150 mM NaCl; 1% sodium deoxycholate; 1% Triton X-100) containing a protease inhibitor cocktail. The homogenate was incubated for 20 minutes on ice and then centrifuged at 14,000 × *g* for 15 minutes at 4°C. The supernatant was collected, and an equal volume of 5× SDS buffer was added to the supernatant. The mixture was then boiled for 5 minutes and stored at -80°C.

Proteins were separated by 1.4-15% Q-PAGE™ Tris-glycine novel precast gels (SMOBIO Technology, Inc., Hsinchu City, Taiwan). After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA, USA) and blocked in 5% nonfat milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) at room temperature for 1 hr.

The membranes were immunoblotted with primary antibodies against EphA2, EphrinA1, Bcl-2, ZO-2 (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), EphA2, EphrinA1 (Thermo Fisher Scientific Rockford, IL61101, USA), phospho-EphA2, E-cadherin, phospho-AKT, AKT (Cell Signaling Technology, Inc., Denver, USA), EphB4, ephrinB2, VE-cadherin, NOX4, keap-1, VEGF (Abcam plc, Cambridge, CB2 0AX, UK), and β-actin (Sigma-Aldrich, St Louis, MO, USA) overnight at 4°C and washed five times with TBS-T for 10 minutes each at room temperature. The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit, anti-rat, or anti-mouse secondary antibodies (1:2000 dilution in TBS-T; Thermo Scientific, Rockford, IL, USA) at 37°C for 1 hr and washed five times with TBS-T for 10 minutes each at room temperature. The membranes were treated with electrochemiluminescence reagent (Thermo Scientific) and exposed to photographic film.

6. Immunohistochemistry (IHC) and immunofluorescence (IF) staining

Individual mouse lung tissues were fixed overnight in formalin and embedded in paraffin. All samples of mouse lung regions were serially sectioned. The slides were then stained using antibodies against VEGF, VE-cadherin, Occludin-5 (Abcam, plc, Cambridge, CB2 0AX, UK, 1:50) using an immunostainer (DAKO Autostainer Link 48). The slides were also counterstained with hematoxylin using an H&E stainer (Leica Autostainer XL,

CV5030)

For immunofluorescence analysis, lung tissues were sectioned from paraffin embedded tissue blocks at a thickness of 4 μm. Sections were permeabilized in 0.5 % Triton-X (T8787, Sigma-Aldrich, St Louis, MO, USA), blocked in CAS-Block™ Histochemical Reagent (008120, Thermo Fisher Scientific, Waltham, MA, USA), and then stained with the following antibodies: polyclonal rabbit anti-4-HNE antibody (1:100) (ab46545, Abcam, Cambridge, UK), monoclonal mouse anti-E-cadherin antibody (1:100) (sc-8426, Santa Cruz Biotechnology, Dallas, TX, USA). Sections were then incubated with goat anti-rabbit IgG (H+L) Alexa Fluor 488 (1:100) (A11008, Thermo Fisher Scientific, Waltham, MA, USA), and goat anti-mouse IgG H&L Texas Red (1:100) (ab6787, Abcam, Cambridge, UK) secondary antibody at 25 °C for 2 h. Fluoroshield™ with DAPI (F6057, Sigma-Aldrich, St. Louis, MO, USA) was used for nuclear staining. Stained lung sections were analyzed by THUNDER Imager Tissue (Leica Microsystems Ltd., Wetzlar, Germany). Stained lung sections were quantified by LAS X image-processing software (Leica Microsystems Ltd., Wetzlar, Germany) and ImageJ software v1.52a (Bethesda, MD, USA). To ensure objectivity, all measurements were performed with blinded conditions by two observers per experiment under identical conditions.

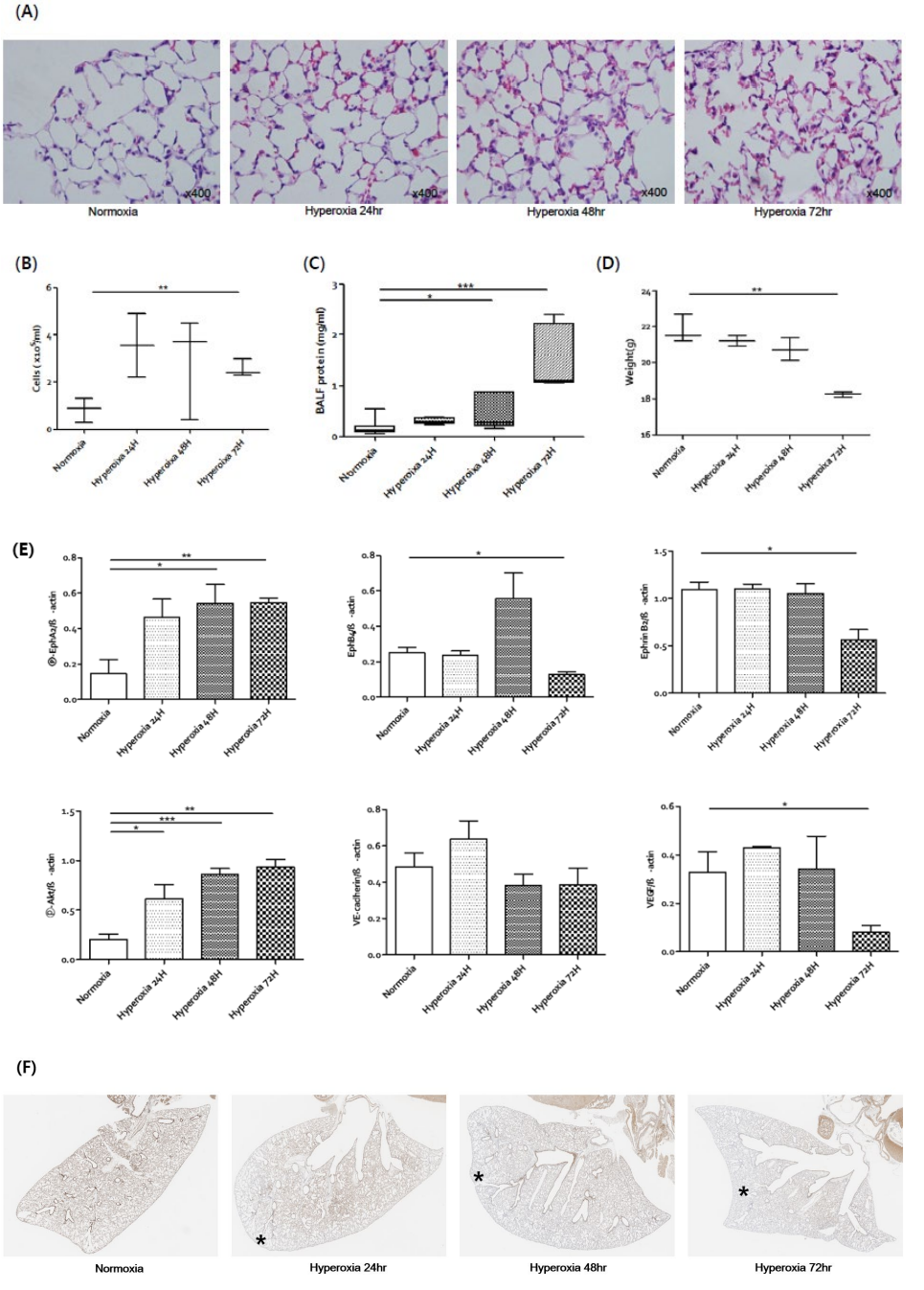
7. Statistical analysis

The data are presented as the mean ±SD or SEM unless otherwise indicated. Comparisons between groups were performed using unpaired Student's *t*-tests or ANOVA with Bonferroni multiple comparisons tests. Statistical analysis was performed using Prism version 5.0 (GraphPad Software). Kaplan-Meier survival plots and log-rank tests were used to compare the survival results. Statistical significance was set at $p<0.05$ or $p< 0.01$.

III. RESULTS

1. Association between HALI and EphA2/ephrinA1 signaling

To investigate how EphA2/ephrinA1 expression is affected by exposure to hyperoxia, we analyzed the expression of these proteins over time in mouse lung tissue by western blotting. According to a previous study, the inflammatory response and oxidative stress in the alveolar-endothelial barrier increase with prolonged exposure to hyperoxia.[3,32] In our study, compared with normoxia, after 24 hr, 48 hr, and 72 hr of hyperoxia, histological lung damage became increasingly severe, and protein leakage was increased. Interleukin (IL)-1β, TNF-α, IL-6, and MIP-2 levels in BAL fluid increased as the hyperoxia time increased, as analyzed by a Luminex® cytokine assay. The results confirmed that the alveolar-endothelial barrier was impaired, as evidenced by a decrease in the expression of tight junction or adherens junction proteins such as VE-cadherin, VEGF, and ZO-2, similar to the findings of a previous study[33] Over time, prolonged hyperoxia induced antiapoptotic signals, such as phosphorylated Akt or Bcl-2.[34] Transient Akt activation and increased Akt phosphorylation in primary human lung microvascular endothelial cells under hyperoxia are associated with early damage to the endothelium.[35] Interestingly, as hyperoxia exposure time increased, VE-cadherin expression decreased, mainly in the lung peripheral area, as shown by IHC staining. Furthermore, phosphorylated EphA2 protein expression increased, although the mechanism of Eph/ephrin signaling in HALI is not yet well understood. These results suggest that EphA2/ephrinA1 but not EphB4/ephrinB2 is involved in the pathophysiology of HALI. (Figure 2)



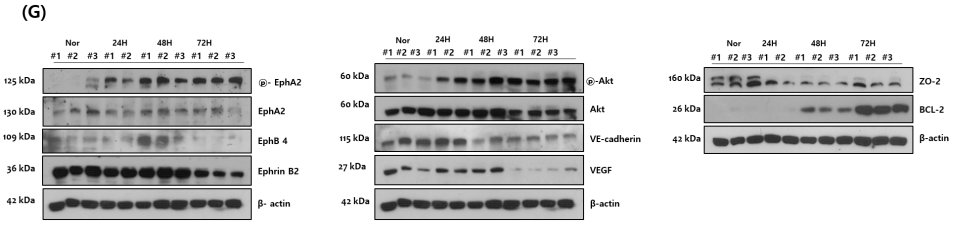


Figure 2. EphA2/ephrinA1 signaling in the HALI mouse model (n=3, each group)
(A) Histopathological features of hematoxylin and eosin (H&E)-stained mouse lung tissues showed that the extent of vascular engorgement, hyaline membrane formation, alveolar cell edema, and inflammatory cell infiltration increased over time after exposure to hyperoxia (24 hr, 48 hr, and 72 hr) (×400 magnification). (B) In bronchoalveolar lavage (BAL) fluid, total cell counts increased initially but decreased over time. (C) Protein concentrations in BAL fluid continued to increase over time. (D) The weights of the mice were also reduced by exposure to hyperoxia. (E) The time course of the changes in cytokines in BAL fluid of mice exposed to hyperoxia was analyzed using the Luminex® cytokine assay. IL-1β, TNF-α, IL-6, and MIP-2 increased with exposure to hyperoxia. (F) As the hyperoxia exposure time increased, VE-cadherin expression decreased, mainly at the edges of the mouse lungs (asterisk), as determined by immunohistochemical (IHC) staining. (G) Western blot analysis showed that as the hyperoxia exposure time increased, the expression of phosphorylated EphA2 increased, and the expression of VEGF, VE-cadherin, and ZO-2 decreased. (*p<0.05, **p<0.01, ***p<0.001).

2. Protective effect of the EphA2mAb on HALI mouse model

We divided the mice into three groups (normoxia, hyperoxia plus intravenous PBS, and hyperoxia plus intravenous EphA2 mAb) to evaluate the preventive effect of the EphA2 mAb on HALI. The EphA2 mAb was administered by intravenous injection through the tail vein because initial endothelial cell damage mainly occurred during HALI[34], and the role of Eph/ephrin signaling in endothelial cells has been elucidated in recent studies.[36] We decided to administer a dose of 8 μg because 8 μg of the EphA2 mAb induced more protein leakage in BAL fluid than 4 μg of the EphA2 mAb (Figure 3).

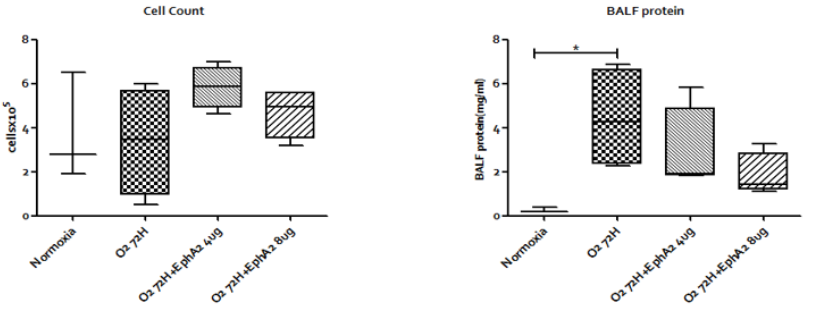
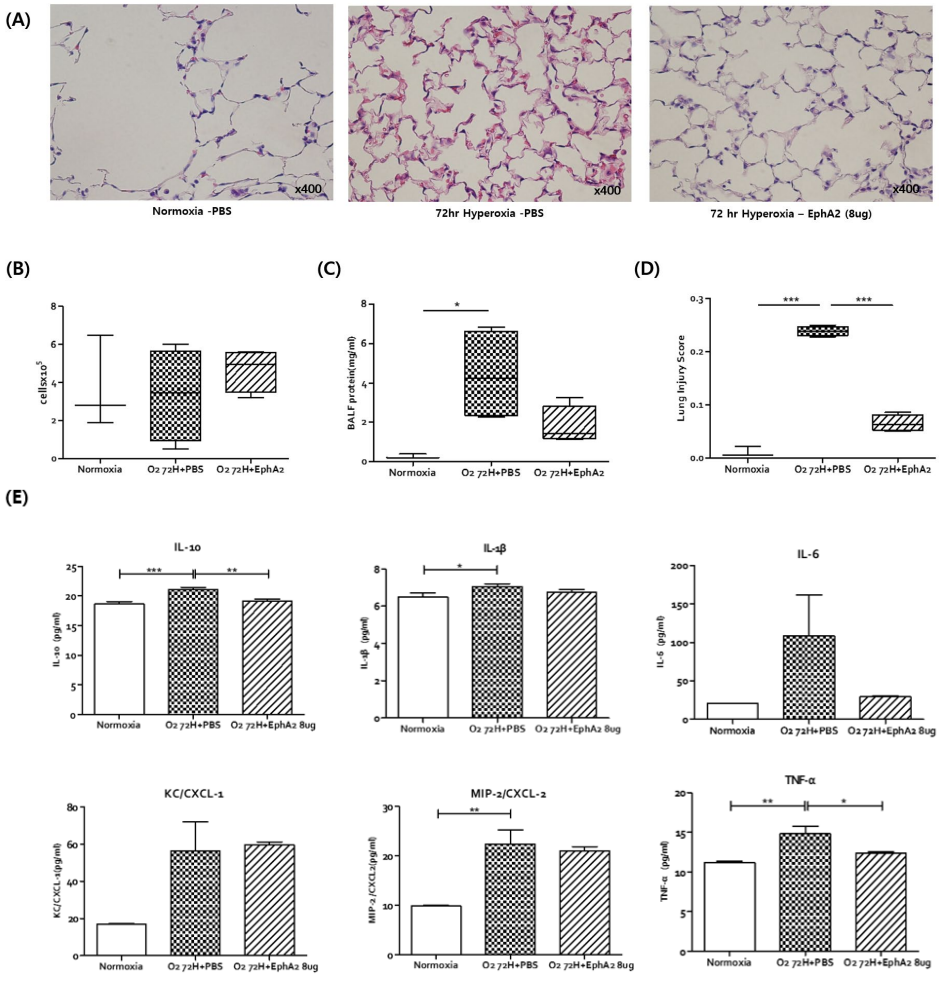


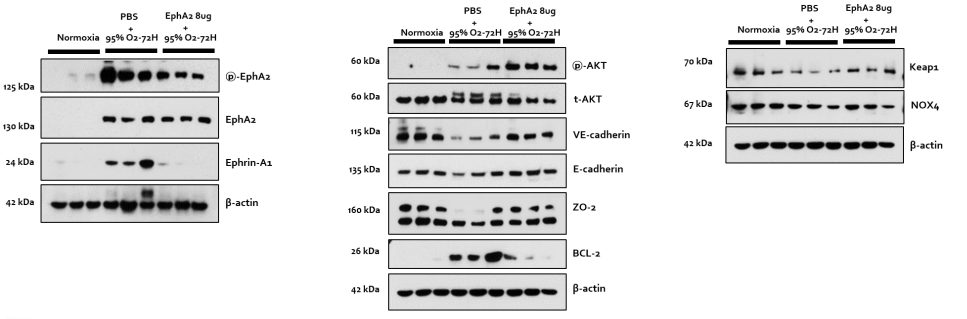
Figure 3. Eight micrograms of the EphA2 mAb showed a better effect on reducing protein leakage in BAL fluid than four micrograms of the EphA2 mAb (*p<0.05, **p<0.01, ***p<0.001).

Figure 4 shows that the EphA2 mAb had a protective effect against HALI in the mouse model. The lung injury score was significantly improved by the EphA2 mAb compared with that in the hyperoxia control group (p=0.012). EphA2 mAb treatment decreased protein leakage in BAL fluid. However, there were no differences in total cell counts among the three groups. Cytokine analysis of BAL fluid showed that the EphA2 mAb induced decreases in IL-10 and TNF-α. A decrease in IL-6 was observed but was not statistically significant. It was difficult to observe the secondary inflammatory cascade after prolonged hyperoxia for 72 hr. Western blot analysis confirmed the attenuation of EphA2 phosphorylation and the suppression of EphrinA1 in response to the EphA2 mAb. The EphA2 mAb restored tight junction or adherens junction proteins, such as VE-cadherin, E-cadherin, and ZO-2. Interestingly VE-cadherin expression in the distal lung, which was lost during hyperoxia, was maintained by EphA2 mAb treatment, as shown by IHC staining. EphA2 mAb treatment also protected VEGF and Claudin-5 expression in the lung during HALI. Although it is difficult to determine the specific pathway, these results confirmed that Akt phosphorylation was increased and Bcl-2 expression was

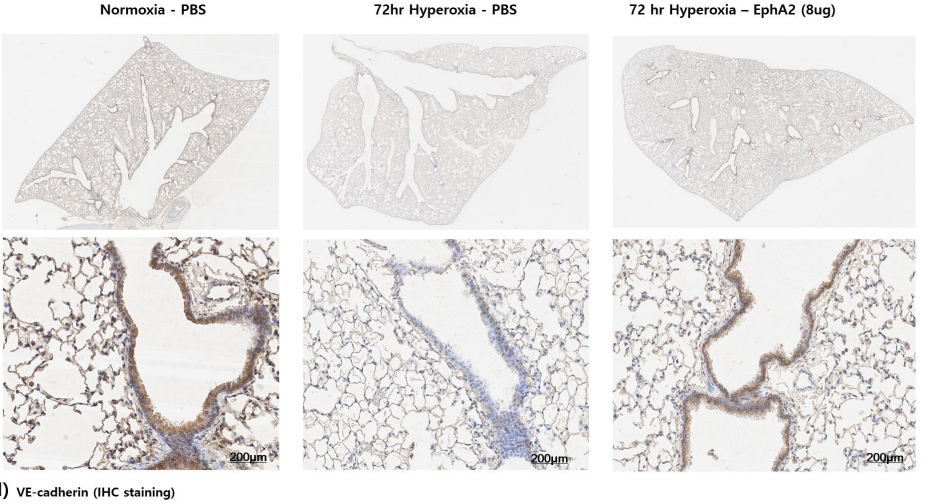
reduced in the EphA2 mAb treatment group. We also investigated the effect of EphA2mAb oxidative stress under HALI using IF with 4-Hydroxynonenal (HNE) and E-cadherin. 4-HNE is a crucial product of lipid peroxidation. However, it is quickly metabolized because of its high toxicity. It can identify the levels of oxidative stress. 4-HNE expression on epithelial cell was increased in hyperoxia control group. But EphA2mAb treatment decreased oxidative stress on epithelial cell.



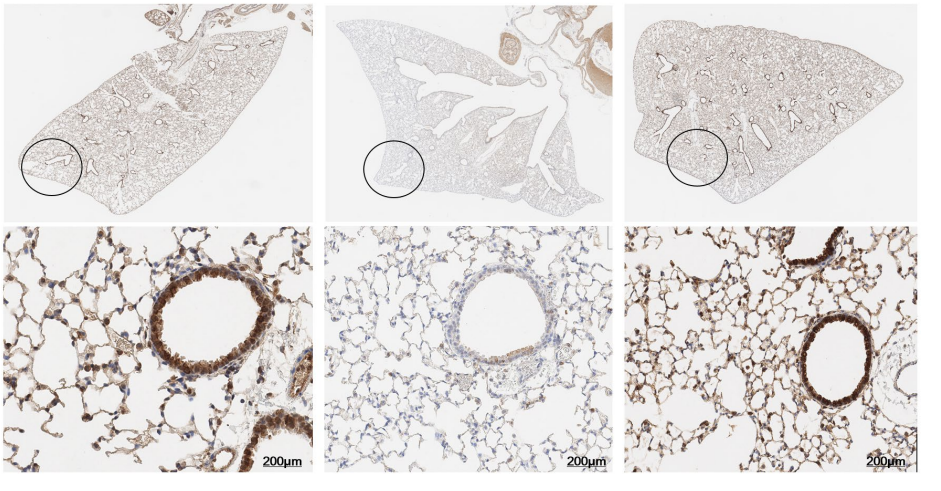
(F)



(G) VEGF (IHC staining)



(H) VE-cadherin (IHC staining)



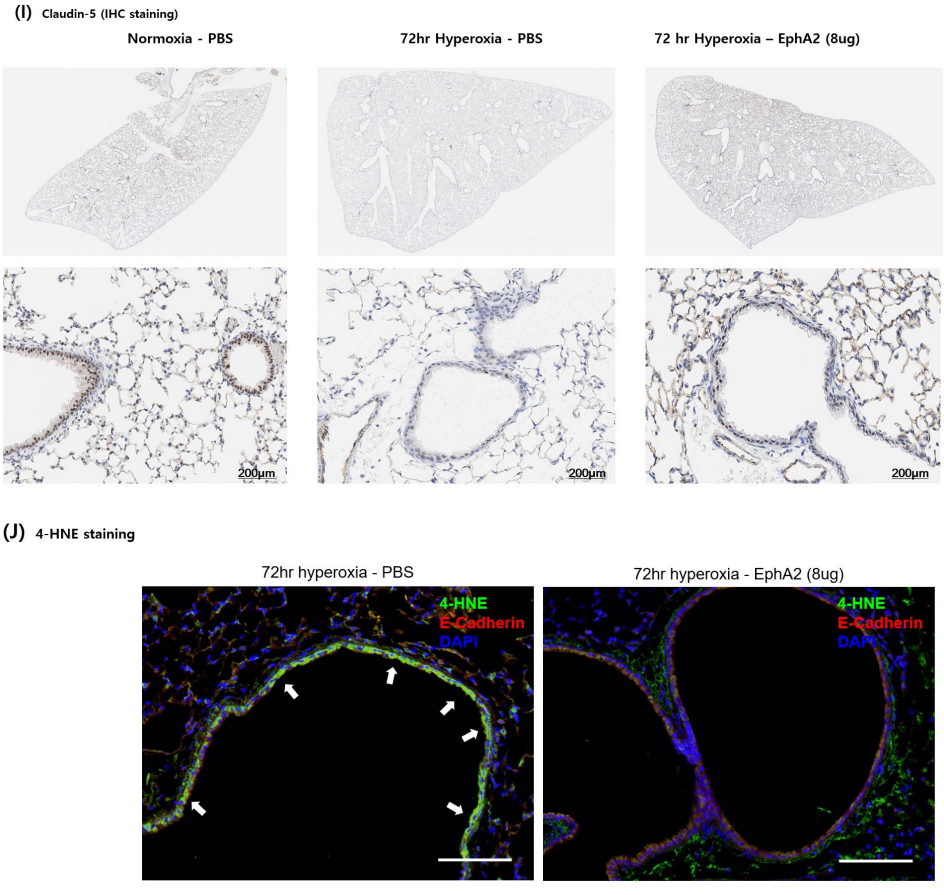


Figure 4. The EphA2 mAb attenuated HALI in model mice (n=4, each group)
(A) H&E-stained mouse lungs showed that the extent of vascular engorgement, hyaline membrane, and alveolar cell edema was decreased in the EphA2 mAb treatment group. (B) Total cell counts in BAL fluid increased, but there was no difference. (C) The EphA2 mAb treatment group showed a decrease in the protein concentrations in BAL fluid. (D) The lung injury score decreased in the EphA2 mAb treatment group. (E) Cytokine analysis of BAL fluid showed that IL-10 and TNF-α decreased in the EphA2 mAb treatment group. (E) Western blot analysis showed that phosphorylated EphA2 and EphrinA1 were suppressed by the EphA2 mAb. Treatment with the EphA2 mAb restored the expression of VE-cadherin and E-cadherin. The EphA2 mAb further activated phosphorylated Akt and decreased the expression of Bcl-2. (G, H, I) The EphA2 mAb restored VEGF, VE-cadherin, and Claudin-5 expression at the edges of the mouse lungs, as shown by IHC staining. (J) The EphA2 mAb suppressed oxidative stress in lung during HALI. Representative immunostaining for 4-HNE(green) in epithelial cells (E-Cadherin, Red) of lung during HALI. (*p<0.05, **p<0.01, ***p<0.001)

3. Survival benefit of the EphA2mAb on HALI mice

Survival analysis was performed to determine the degree of hyperoxia-induced lung damage after exposure to relative hypoxia at atmospheric oxygen (21%) after 72 hr of prolonged hyperoxia (>95%) insult in a Plexiglass chamber. We used this method to evaluate the protective effect of the EphA2 mAb on the HALI mouse model. We divided the mice into two groups: hyperoxia with intravenous PBS and hyperoxia with intravenous EphA2 mAb. EphA2 mAb pretreatment induced a survival benefit against 72 hr of hyperoxia insult (p=0.045), as shown in Figure 4. The reason for this improvement in survival is thought to be due to protection of the alveolar-endothelial barrier. (Figure 5)

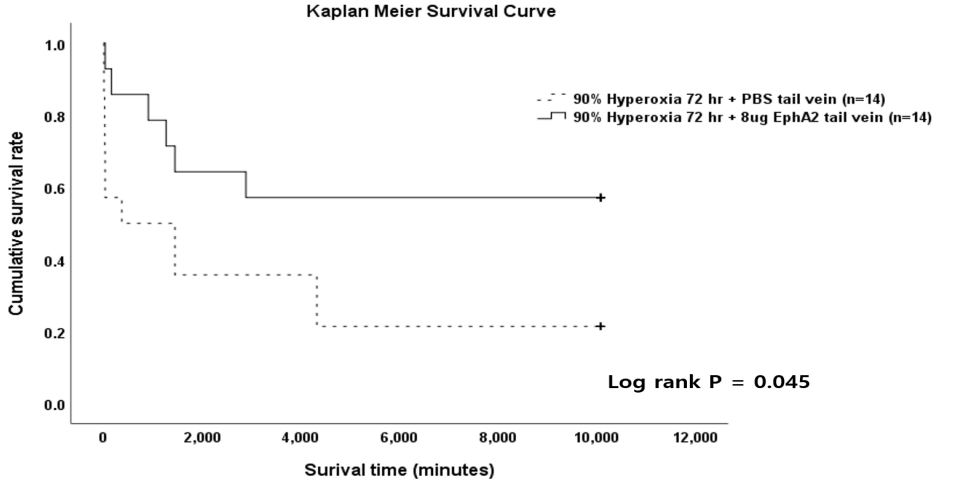


Figure 5. The EphA2 mAb increased survival in the HALI mouse model (n=14, each group)
The EphA2 mAb improved the survival rate at 72 hr of HALI insult compared with that of PBS (p=0.045).

IV. DISCUSSION

We found that EphA2/ephrinA1 was associated with the pathophysiology of HALI and that modulating the EphA2/ephrinA1 pathway using the EphA2 mAb reduced HALI and improved survival in vivo.

Human alveolar pO2 is approximately 100 mmHg or 14% and is precisely controlled in a narrow range to maintain homeostasis. Hyperoxia is a condition used by humans to treat various diseases, such as pneumonia, ARDS, pulmonary edema, and sepsis. Reactive oxygen species produced by high concentration oxygen therapy that exceeds the antioxidant buffering system, which is inevitably used for cellular oxygenation in the body, can eventually induce HALI. Hyperoxia is closely associated with bronchopulmonary dysplasia and proliferative retinopathy in neonates.[37] However, there is still controversy about adult lung damage caused by hyperoxia clinically[38,39], although many experimental studies have demonstrated that hyperoxia causes lung damage.[3,40] Because there is still no treatment to reduce hyperoxic lung damage, clinicians should treat patients with caution while weighing the risks and benefits associated with hyperoxia. A previous study showed that the pulmonary endothelium is a primary target for HALI during the first hours, sequentially activating platelet aggregation and inflammatory cascades and disrupting the alveolar-endothelial barrier.[41] Interestingly, EphA2/ephrinA1 signaling has many similarities with HALI pathophysiology. For example, hyperoxia disrupts alveolar-capillary integrity and activates inflammatory cells. EphA2/ephrinA1 signaling modulates the cytoskeleton and intercellular adhesion and repulsion in specific tissues and inflammatory cells.

Eph proteins affect the cytoskeleton with short-distance cell-cell communication as one of the superfamilies of transmembrane Tyr kinase receptors.[42] These Eph receptors have been classified as EphA and EphB, which preferentially bind to

membrane-anchored or transmembrane ephrin ligands such as EphrinA or EphrinB. Eph/ephrin protein expression is different in various tissues and cells and in normal and pathological conditions. Recently, Eph/ephrin signaling has been shown to play an essential role in various diseases, including cancer, wounds, ischemia-reperfusion injury, nerve injury, and inflammation, although these roles were initially discovered during embryonic development in the patterning of the nervous system.[18] Notably, a recent review of Eph/ephrin signaling indicated that Eph/ephrin signaling was involved in adult endothelial biology.[36]

Three significant families of receptor tyrosine kinases and ligands, VEGF[43], angiopoietins[44], and Eph/ephrin[45], are known to be regulators of vascular permeability in the context of angiogenesis. However, the mechanism by which Eph/ephrin regulates vascular permeability compared with VEGF or angiopoietins is not clear. Some studies have shown that the proangiogenic effects of EphA2/EphrinA1 are related to Rho family GTPase and the quinine exchange factor Vav.[45,46] EphA2 directly alters the phosphorylation of claudin-5 in the epithelial cell barrier[47]. Rearrangement of tight junctions and adherens junctions occurs if EphrinA1 signaling increases, which induces vascular permeability. Furthermore, increased EphrinA1 expression causes a decrease in tight junction proteins such as claudin-5, ZO-1, and VE-cadherin and shifts in these proteins from the membrane to the cytoskeleton are accompanied by functional declines in hLMVECs in vitro [27]. It has been reported that VEGF increases paracellular permeability by activating EphA2 through PI3K/Akt and Erk 1/2[48], but the study used a brain endothelial cell line, the effects have not been confirmed in the lung endothelium. It is unknown whether EphA2/ephrinA1 precisely regulates tight junction or adherens junction proteins.

Our group previously clarified the protective effect of ephA2/ephrinA1 in a

lipopolysaccharide-induced lung injury model[28] and ventilator-induced lung injury model[29]. One study showed that edematous alveolar septae were the main sites of increased EphA2 expression in rat lung injury due to viral infection and hypoxemia.[49] However, to date, no studies have shown the effect of EphA2/ephrinA1 on HALI. Among the Eph and ephrin proteins that are currently known, EphA2/ephrinA1 and EphB4/ephrinB2 are expressed in human lung microvascular endothelial cells, and their roles have been identified. Both pairs of proteins are increased due to inflammation or injury.[36] EphA2/ephrinA1 signaling plays a vital role in lung inflammation or damage[27-29,50,51], but EphB4/ephrinB2 has been shown to be associated with tissues or cancers other than the lungs.[52,53] In our study, we could not find any association between EphB4/ephrinB2 protein expression and HALI. The level of phosphorylated EphA2 protein increased over time under hyperoxia and correlated with HALI pathophysiology.

In this study, hyperoxia induced various cytokines or chemokines, such as IL-10, IL-1β, IL-6, KC/CXCL-1, MIP-2/CXCL-2, and TNF-α, 72 hr of hyperoxia exposure. Many studies have revealed that HALI is associated with various cytokines and chemokines, such as IL-33[54], IL-10[55], IL-6[56], and IL-1[57,58]. These studies showed that IL-33-, IL-6-, or IL-10-knockout mice are susceptible to HALI, and exogenous ILs can attenuate HALI. In HALI, cytokines and chemokines do not directly regulate the alveolar-capillary barrier. These cytokines or chemokines are highly likely to play roles in late inflammatory cascades. Therefore, therapeutics that directly control alveolar-endothelial barrier function are needed. Our study proved that some cytokines and chemokines were decreased after the administration of the EphA2 mAb. This result is thought to be a secondary effect induced by protecting the alveolar-endothelial barrier.

We showed that pretreatment with the EphA2 mAb alleviates lung injury in

HALI by preserving tight junction or adherens junction proteins. Consolidating epithelium-endothelial junction proteins is thought to reduce hyperoxic lung damage and lung vascular permeability. Some studies have reported an association between phosphorylated Akt and an increase in VE-cadherin in patients with chronic subdural hematoma[59], and activation of Akt in endothelial cells protects against hyperoxic stress.[35] Although the specific mechanism is not yet known, the administration of the EphA2 mAb led to an increase in phosphorylated Akt, which may be related to an increase in VE-cadherin expression or reduced oxidative stress (4-HNE) in epithelial cells. However, further research is needed. Additionally, the protective effect of the EphA2 mAb induced a decrease in the expression of antiapoptotic proteins, such as Bcl-2. Previous studies have shown conflicting results regarding the expression of antiapoptotic Bcl-2 in HALI.[60,61] Although the reason was not clear, the decrease in Bcl-2 expression is thought to result from the reduction in HALI because of the protective effect of the EphA2 mAb.

Our study has many limitations. First, we did not elucidate the exact mechanism of EphA2/ephrinA1 in HALI because we could not use EphA2/ephrinA1-knockout mice. Second, we have not provided any scientific evidence about why alveolar endothelial cells are targeted in HALI, even based on previous studies. Future studies should elucidate this mechanism. Finally, we could not examine the effect of repeated EphA2 mAb administration on HALI. Our Plexiglass chamber was sealed during hyperoxia exposure, making it difficult to administer additional doses of the EphA2 mAb. However, since clinicians already predict when hyperoxia will be administered clinically, pretreatment with the EphA2 mAb is thought to be appropriate.

V. CONCLUSION

In conclusion, we found that blocking EphA2/ephrinA1 signaling may be associated with preserving tight junction and adherens junction proteins in a mouse model of HALI. EphA2 mAb treatment can protect against HALI and improve survival by protecting alveolar-endothelial barrier function.

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ABSTRACT (IN KOREAN)

고산소유발 폐 손상에서 EphA2/ephrinA1 신호전달의 역할

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정 경 수

배경: 산소요법은 급성호흡곤란 증후군 환자에게 필수적인 치료법이다. 그러나 불가피하게 투여되는 고농도산소는 폐손상을 일으켜 환자의 예후를 악화시킬 수 있다. EphA2/ephrinA1 수용체-리간드 신호는 배아 발생뿐만 아니라 다양한 질병에서 세포의 골격과 부착을 조절하는 것으로 알려져 있다. 우리는 EphA2/ephrinA1 신호가 고농도산소에 의한 폐 손상과 관련성과 치료효과에 대해 연구하였다.

방법: 실험을 위한 수컷 C57BL/6J 마우스 (20-24g; 오리엔트바이오, 한국 성남)를 플렉시글라스챔버 (Plexiglas chamber)를 통해 95 % 이상 고농도 산소에 노출하였다. 고농도 산소 노출 시간에 따른 폐 손상에서 EphA2/ephrinA1 신호 변화의 연관성을 평가하기 위해 대기 중 산소 (21 % O2)와 비교하여 24 시간, 48 시간, 72 시간에 분석을 수행하였다. 다음으로 EphA2 단일 클론 항체 (마우스 꼬리 정맥, 8μg, 전처리)의 효과를 분석하기 위해 고산소농도 노출 후 72시간에 마우스를 해부하

였다. 실험 방법으로 기관지 폐포 세척액에서 총 세포 수, 단백질농도 및 사이토카인 분석을 시행하였다. 그리고 마우스 폐 조직에서 western blot 및 면역 조직 화학 염색을 시행하였다. EphA2 단일 클론 항체의 효과에 따른 생존 분석은 72 시간의과 산소 노출 후, 대기 중 산소 상태에 옮겨 생존을 관찰하였다.

결과: 고산소농도 노출 시간이 증가함에 따라 마우스 폐 조직에서 인산화 된 EphA2의 발현이 증가하였다. 기관지 폐포 세척액에서 단백질 누출이 유의하게 증가하였다. 또한 ZO-2, E-cadherin 및 VE-cadherin과 같은 밀착 또는 접착 접합의 발현이 고농도산소 노출 시간이 길어질수록 감소하였다. 고산소에 의한 폐 손상 마우스모델에서 인산염 완충 식염수와 비교하여 EphA2 단일 클론 항체는 기관지 폐포 세척액에서 단백질 누출을 감소시키는 경향을 보였고, 사이토 카인 활성화 (IL-10, TNFα) 및 폐 손상 점수를 감소시켰다. ($p<0.05$) EphA2 단일 클론 항체는 단단한 접합 또는 부착 접합 단백질의 분해를 저해하고 산화스트레스를 감소시킴으로써, Bcl-2 같은 항-아폽토시스 단백질 발현의 감소를 유도하였다. ($p<0.05$) EphA2 단일 클론 항체는 고산소에 의한 폐 손상 마우스 생존 실험에서 유의하게 생존을 향상시켰다. (p=0.045)

결론: 현 연구를 통해서 EphA2/ephrinA1신호는 고산소에 의한 폐 손상과 밀접한 관련이 있다. EphA2 단일 클론 항체의 투여는 폐포-내피 장벽을 유지하고 생존율을 개선했다.

핵심되는 말: ephA2, ephrinA1, 고산소유발 폐 손상