NADPH oxidase 4 signaling in a ventilator-induced lung injury mice model

Sang Hoon Lee

Department of Medicine

The Graduate School, Yonsei University

NADPH oxidase 4 signaling in a ventilator-induced lung injury mice model

Sang Hoon Lee

Department of Medicine

The Graduate School, Yonsei University

NADPH oxidase 4 signaling in a ventilator-induced lung injury mice model

Directed by Professor Moo Suk Park

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

Sang Hoon Lee

December 2019

This certifies that the Doctoral Dissertation of Sang Hoon Lee is approved.

Thesis Supervisor: Moo Suk Park

Thesis Committee Member #1: Young Sam Kim

Thesis Committee Member #2: Jong Wook Shin

Thesis Committee Member #3: Ji-Hwan Ryu

Thesis Committee Member #4: Hyo Sup Shim

The Graduate School
Yonsei University

December 2019

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to several people who supported me during the time of writing this doctoral dissertation.

First of all, I am deeply indebted to my supervisor Prof. Moo Suk Park for his patient guidance throughout the research work. Without his advice and unconditional support, I could not have completed this dissertation.

I am also very grateful to the members of my thesis committee, Prof. Young Sam Kim, Prof. Jong Wook Shin, Prof. Ji-Hwan Ryu, and Prof. Hyo Sup Shim. Thank you for your time and valuable comments on the thesis. I also thank Mi Hwa Shin, who has helped me a lot during the experiment.

I owe a lot to my parents and my older brother, who helped me at every stage of my personal and academic life. Without their love and sacrifices, I could not have finished this dissertation. I would also like to thank my mother-in-law, my father-in-law, who encouraged and supported my studies.

I would like to dedicate this dissertation to my wife Hee Yeong Kim and my lovely daughters Chaewon Lee, and Heewon Lee to whom my expression of love and gratitude is never enough.

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

NADPH oxidase 4 signaling in a ventilator-induced lung injury mice model

Sang Hoon Lee

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Moo Suk Park)

Background: For patients with acute respiratory distress syndrome, a ventilator is essential to supplying oxygen to tissues, but it may also cause lung damage. In this study, we investigated the role of NOX4 in lung injury and the effect of NOX4 knockout (KO) mice and NOX4 inhibitors in a ventilator-induced lung injury (VILI) model.

Methods: Wild-type male C57BL/6J mice (20–28 g; Orient Bio, Sungnam, Korea) and NOX4 KO male mice (7–9 weeks, 20–28 g) were divided into five groups: (1) control group, wild-type mice + non-ventilator group; (2) high tidal ventilation (HTV) group, wild-type mice + HTV group; (3) NOX4 KO group, NOX4 knock-out mice + non-ventilator group; (4) NOX4 KO with HTV group; (5) NOX4 inhibitor during HTV, wild-type mice + HTV + post-treatment (anti-GKT 137831 inhibitor) group. In the ventilator lung injury model, the position was maintained at 24 mL/kg volume, 0 cm H_2O PEEP, 100/min respiratory rate, and 0.21 inspired oxygen fraction. In the NOX4 inhibitor group, mice were

treated for 2 hours at the time of ventilator use with an intraperitoneal injection of 50 μL anti-GKT 137831 inhibitor (BioVision cat # 9444-5) in 50 μL DMSO. After 5 h of HTV, mice in the ventilator group were euthanized and lung tissues were obtained for further analysis.

Results: Cell counts and concentrations of protein from the bronchoalveolar lavage fluid was significantly high in the HTV group. Cell counts were significantly lower ($p < 0.05$) in the NOX4 KO with HTV group compared to the HTV group, and the protein concentration tended to be less. In NOX4 inhibitor group, cell counts and protein concentrations were significantly lower than those in the HTV group ($p < 0.05$ and $p < 0.01$, respectively). VILI was the greatest in the HTV group; EphA2 levels were also the highest in this group. In the NOX4 KO mouse group and the NOX4 inhibitor group, EphA2 levels were significantly lower than those in the HTV group (both, $p < 0.01$). NOX4 mRNA levels were the most significantly elevated in the HTV group, followed by the NOX4 KO, and NOX4 inhibitor group.

Conclusion: In the VILI model, NOX4 expression is significantly associated with Eph-ephrin signaling. It may be possible to block ventilator-induced lung injury using NOX4 antibody.

Keywords: NADPH oxidase 4, ventilator-induced lung injury

NADPH oxidase 4 signaling in a ventilator-induced lung injury mice model

Sang Hoon Lee

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Moo Suk Park)

I. INTRODUCTION

Acute respiratory distress syndrome (ARDS) was first reported by Ashbaugh *et al.*[1] in 1967, and in 1994 at the American European Consensus Conference (AECC), ARDS was defined as a syndrome involving severe hypoxia ($PaO_2/FiO_2 \leq 200$ mmHg) with bilateral pulmonary infiltration in radiologic findings and non-cardiogenic pulmonary edema. Acute lung injury (ALI) was defined by less-severe hypoxemia ($PaO_2/FiO_2 \leq 300$ mmHg).[2] In 2012, the Berlin definition was established to treat patients according to severity, which facilitated determining a prognosis in ARDS.[3,4]

In 2003, Goss *et al.*[5] reported 17.6–64.0 ALIs per 100,000 people based on American hospital association data, and, in 2005, Rubenfeld *et al.* [6] reported 78.9 ALIs per 100,000 people by applying the AECC criteria.

Li *et al.*[7] reported results of a population-based study in Olmsted County, Minnesota. Based on 795 patients who were admitted to an intensive care unit (ICU) over 8 years, there were 81 cases of ARDS/ALI per 100,000 person-years

detected in 2001, but 38.3 cases per 100,000 person-years detected in 2008. This reduction in the incidence of ARDS/ALI was due to changes in ICU therapeutic practice. The use of low tidal volume ventilation in ARDS, established protocols for sepsis or pneumonia treatment, computerized data, and sufficient medical manpower might all have reduced the incidence of ARDS/ALI. However, there has been no change in the fundamental treatment of ARDS/ALI beyond an improvement in ICU quality management,[8-10] and, as populations age, ARDS/ALI will become a greater social and economic problem.[6] Therefore, it is important to determine the pathophysiology of ARDS/ALI through animal experiments and to identify the molecular mechanism that underlies ARDS/ALI to develop new therapeutic agents for these conditions.

Correcting hypoxemia through artificial respiration is one of the important therapies used to treat patients with ARDS/ALI, but the ventilator itself may cause lung injury and exacerbate existing ARDS/ALI.[11] Therefore, it is important to analyze the mechanism of pulmonary damage by ventilators to modify clinical management of hypoxemic patients.

In patients with ARDS/ALI, oxygen saturation in tissues is maintained by high levels of oxygen or high tidal volume with a ventilator. This could damages respiratory epithelial cells and endothelial cells and causes alveolar macrophages to secrete IL-1 and TNF-α, which, in turn, activates epithelial and endothelial cells, monocytes, and lymphocytes. As a result, various chemokines such as IL-8, CXCL1, and CXCL2/3 are secreted. These cells adhere to CXCR2 and recruit circulating neutrophils to inflammation sites, increasing vascular permeability of lungs (alveolar permeability) and exacerbating the inflammation. Therefore, in cases of ARDS/ALI, it is helpful to increase oxygen saturation by placing a patient in the prone position when a high concentration of oxygen or

high single respiration is needed. The survival rate in patients with severe ARDS has been shown to increase when they are in the prone position.[12,13]

In lungs, NADPH oxidase 4 (nicotinamide adenine dinucleotide phosphate-oxidase 4, NOX4) has been studied mainly because of its role in lung cancer. Zhang *et al.*[14] showed good agreement among the level of NOX4, cancer stage, and survival time in non-small-cell lung cancer (NSCLC). They also reported that overexpression of NOX4 in A549 cells promoted proliferation and invasion of cells, increased tumor size, shortened survival time, and stimulated lung metastasis when compared with the control group. Conversely, depletion of NOX4 has been shown to reduce the aggressiveness of NSCLC. In particular, the PI3K/Akt pathway regulates the expression of NOX4 via NF-κB, which leads to positive feedback in cell proliferation and invasion in NSCLC.[14] Additionally, Amara *et al.*[15] showed that NOX4 mRNA and protein expression increased in patients with idiopathic pulmonary fibrosis (IPF) and that TGF-β1 increased the expression of NOX4, α-SMA, and procollagen 1 (α1). However, NOX4 and PI3K pathways have rarely been studied in an ICU model. Erythropoietin-producing hepatocellular receptor A2 (EphA2) is one of the tyrosine kinases that is overexpressed on cell membranes and is expressed at a high level in cancer cells, including those in NSCLC.[16] Menges *et al.*[17] showed that EphA2 is linked with the PI3K-Akt pathway in cellular arrest.

Based on this understanding of this linkage, it is important to examine the correlation between signal pathways, including those with PI3K/Akt, fibrosis, and NOX4, in lung cancer or IPF and pulmonary damage by ventilators. In this study, we investigated the expression of NOX4 in a ventilator-induced lung injury (VILI) mouse model, as well as the potential therapeutic effect of a NOX4 inhibitor.

II. MATERIALS AND METHODS

1. Experimental animals and groups

All animal protocols were approved by the Institutional Animal Care Committee of the Medical College of Yonsei University (2015-0269). All animal experiments were conducted in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animals were supplied with food and water and were subjected to the same day and night light cycles. Wild-type male C57BL/6J mice (20–28 g; Orient Bio, Sungnam, Korea) and NOX4 knockout (KO) male mice (7–9 weeks 20~28 g, provided by Prof. Yoo) were divided into five groups as follows:

(1) Control group; wild-type mice + no ventilation

(2) High tidal ventilation (HTV) group; wild-type mice + HTV

(3) NCV NOX4 KO; NOX4 KO mice + no ventilation

(4) NOX4 KO with HTV group; NOX4 KO mice + HTV

(5) NOX4 inhibitor during HTV group; wild-type mice + HTV + anti-GKT 137831 inhibitor (Figure 1).

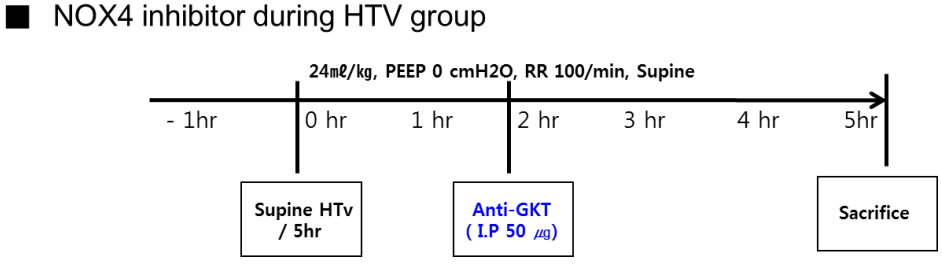


Figure 1. Time table for NOX4 inhibitor treatment in the HTV group
In the VILI model, mice were tracheostomized, and the ventilator was maintained for 5 h in the supine position with 24 mL/kg volume, 0 cmH_2O PEEP, 100/min respiration rate, and 0.21 inspired oxygen fraction.

For the NOX4 inhibitor during HTV group, 50 μL of DMSO with 50 μg of anti-GKT 137831 inhibitor (BioVision cat # 9444-5) was injected intraperitoneally at 120 min of ventilation with mice. Mice were euthanized after 5 h of HTV, and the lung tissues were obtained for further analysis.

2. Bronchoalveolar lavage (BAL)

For the BAL fluid (BALF) analysis, BAL was performed with a tracheal cannula and 1 cc of sterile saline. The BALF was centrifuged (4°C, 3000 rpm, 10 min), and the supernatant was stored at 80°C for further analysis. The cell pellet was reconstituted in 100 μL phosphate-buffered saline (PBS) and used for cell counts and cytospins. Total cell numbers in each sample were determined using a hemocytometer (Marienfield) according to the manufacturer′s protocol. A 90 μL aliquot of each sample was transferred to chamber slides, which were then inserted into a cytocentrifuge facing outward. The slides were centrifuged at 800 rpm for 5 min, removed from the cytocentrifuge (Shandon Cytospin 4 cytocentrifuge, Thermo Scientific, Waltham, MA), and dried prior to staining with Diff-Quik Stain Set (Dade Behring, Newark, DE) to assess inflammation. Protein concentrations in the BAL supernatant were determined using BCA assay (Thermo Fischer Scientific).

3. Histopathologic analysis

For the histopathology analysis, left lungs were inflated via low-melting point agarose (4%) in PBS through a tracheotomy incision at an H_2O pressure of 25 cm until the pleural margins became sharp. The inflation-fixed left lungs of

experimental mice were fixed in paraffin and cut to a thickness of 5 μm. After the slides were prepared, they were stained with hematoxylin and eosin, and lung injury scores were determined under light microscopy. These scores were calculated as described by Matute-Bello *et al*.[18] by using neutrophils in alveolar spaces, neutrophils in interstitial spaces, hyaline membranes, proteinaceous debris filling airspaces, and alveolar septal thickening as a scoring parameters. Lung sections were processed for immunohistochemistry using an anti-NOX4 (UOTRIB492, Abcam) antibody.

4. Western blotting and ELISA

Lung tissues were harvested and lysed in homogenization buffer (PRO-PREP Extraction solution, iNtRON Biotechnology). The samples were centrifuged at 13,000 × *g* for 30 min at 4°C. Concentrations of proteins in the supernatants were determined by BCA assay (Thermo Fischer Scientific). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked with 5% skim milk in TBS-T (TBS [170-6435, Bio-Rad Laboratories] and 1% Tween-20 [170-6531, Bio-Rad Laboratories]) for 1 h at room temperature. Then, the membranes were incubated overnight with primary antibody diluted in 5% skim milk and TBS-T at 4°C. After washing with TBS-T, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies and 5% skim milk in TBS-T for 1 h at room temperature and developed using a Super-Signal West Pico chemiluminescence detection kit (Pierce). The antibodies used in the present study included NOX4 (ab155071, Abcam), EphA2 (PA5-14574, Thermo Fisher Scientific), rabbit PI3 kinase 110γ

(Cell Signaling Technologies), rabbit α-tubulin (PA5-16891, Cell Signaling Technologies). Proteins resolved on western blots were quantified using ImageJ (Image Processing and Analysis in Java, NIH, USA) software.

Interleukin-1β (IL-1ß), interleukin 6 (IL-6), and interleukin 8 (IL-8) levels in lung lysates were measured using ELISA kits (Millipore) according to the manufacturer′s directions.

5. Real-time PCR

Total RNA was isolated from homogenized lungs using a GenElute Mammalian Total RNA Miniprep Kit with DNAse treatment. A High Capacity cDNA Reverse Transcription Kit was used to deliver RNA into cDNA. A real-time PCR analysis of ~25 ng cDNA was performed using TaqMan Universal PCR Master Mix for NOX4 (Mm 00479246_m1), GAPDH (Mm 99999915_g1), and pre-designed TaqMan Gene Expression Assays. The reaction was performed on a 7300 Real-Time PCR System (Applied Biosystems, Vienna). Data were analyzed using cyclophilin (Mm 00835365_g1) as a reference gene. No extraneous amplification was confirmed by inclusion of a no-template control.

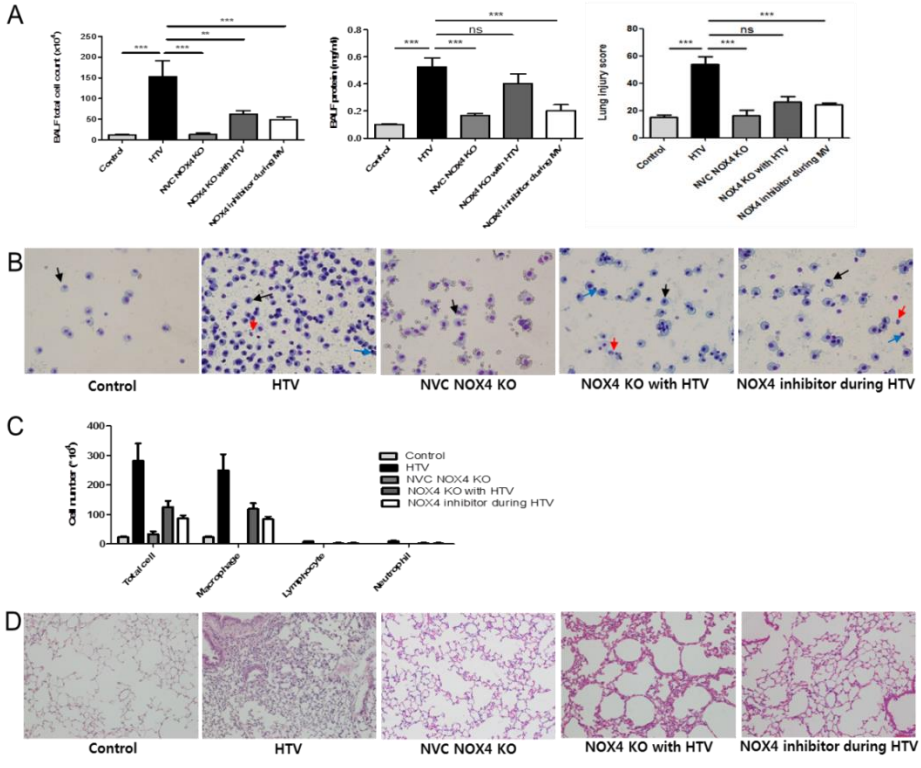
6. Statistical analysis

A statistical analysis was performed in Prism version 5.0 (GraphPad Software, Durham, NC), and the data were expressed as means ± standard deviations. Comparisons between groups were made using a two-way analysis of variance (ANOVA) and corrected using the Bonferroni correction method. In this study, $P < 0.05$ was considered significant.

III. RESULTS

1. Attenuation of VILI in NOX4 KO mice and mice receiving a NOX4 inhibitor

As shown in Figure 2, VILI, cell counts, and protein concentrations from bronchial cells were the lowest in the control group and significantly high in the HTV group ($p < 0.001$, Figure 2A). Cell counts were significantly lower in the NOX4 KO with HTV group than in the HTV group ($p < 0.01$), and protein concentrations tended to be less. In the NOX4 inhibitor during HTV group, cell counts and protein concentrations were significantly lower than those in the HTV group (both $p < 0.001$).



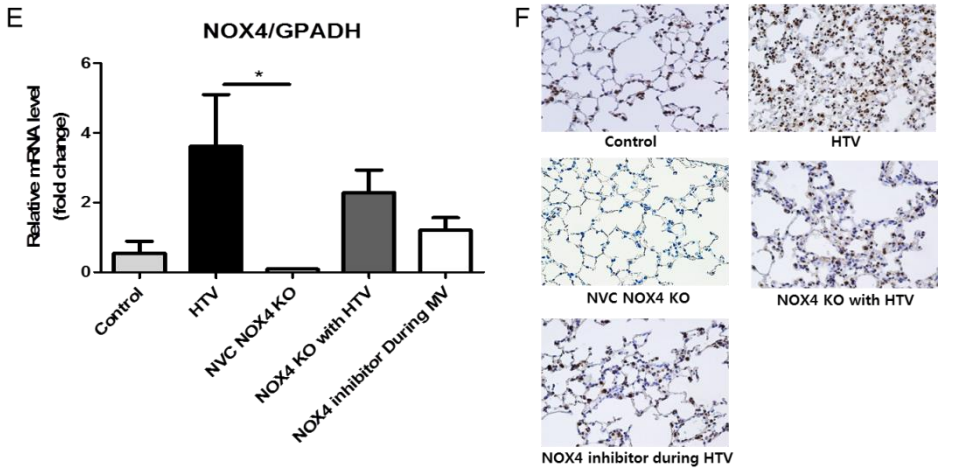


Figure 2. **NOX4 KO mice and NOX4 inhibitor attenuated VILI mice (n = 8, each group)**. (A) Total cell counts and protein concentrations. (B, C) Cell differentiation in bronchoalveolar lavage fluid (BALF) with a representative visual field. Cells were mainly macrophages (black arrow) with a few lymphocytes (blue arrow) and neutrophils (red arrow). (D) Histopathologic features of hematoxylin and eosin-stained mouse lung tissues showed a lesser extent of vascular engorgement, hyaline membrane formation, alveolar wall edema, and inflammatory cell infiltration in the NOX4 KO mouse and the treatment groups receiving NOX4 inhibitor than the HTV group (200× magnification). (E) Relative levels of NOX4 mRNA determined by real-time PCR (n=3, each group). (F) NOX4 immunohistochemistry (400× magnification). *p < 0.05, **p < 0.01, and ***p < 0.001, analyzed by two-way ANOVA with post hoc testing and Bonferroni correction.
BALF, bronchoalveolar lavage fluid; KO, knockout; HTV, high tidal ventilation

Figure 2B and C shows the differential cell counts from cytospins. Macrophages were predominant, and there were few lymphocytes in the BALF. In the histopathologic analysis (Figure 2D), the extent of leukocyte infiltration, capillary leakage, hyaline membrane formation, and alveolar wall edema was the greatest in the HTV group. Relative levels of NOX4 mRNA were higher in the HTV group, followed by NOX4 KO with HTV, the NOX4 inhibitor, control groups, and NOX4 KO (Figure 2E). Increased NOX4 immunostaining after HTV was reduced by NOX4 inhibition (Figure 2F).

2. VILI decreased with downregulation of NOX4, EphA2, and PI3K 110λ signaling

To further evaluate the effects of NOX4 inhibition on VILI, we examined NOX4, EphA2, and PI3K 110λ signaling by western blot analysis (Figure 3A). NOX4, EphA2, and PI3K 110λ expression levels were significantly higher in the HTV group compared to the control group ($p < 0.001$, $p < 0.001$, and $p < 0.05$, respectively; Figure 3B). Furthermore, in both the NOX4 KO group and NOX4 inhibitor group, the expression of these signaling molecules was significantly lower than that in the HTV group (Figure 3B). Treatment with the NOX4 inhibitor during HTV significantly attenuated VILI. These results indicated that NOX4, EphA2, and PI3K 110λ signaling pathways are involved in VILI and that a NOX4 inhibitor could be a therapeutic potential agent in the treatment of ALI/ARDS in VILI.

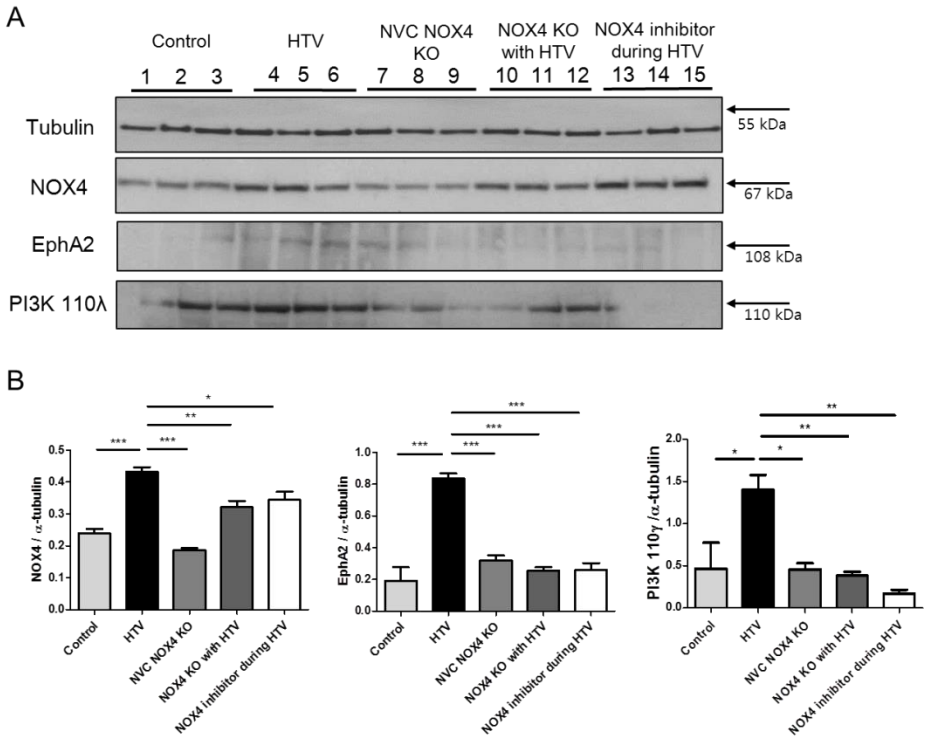


Figure 3. VILI was decreased in the NOX4 KO mouse group and NOX4 inhibitor group. (A) Western blot of NOX4, EphA2, and PI3K 110λ. (B) Densitometry of NOX4, EphA2, and PI3K 110λ. *p < 0.05, **p < 0.01, and ***p < 0.001, analyzed by two-way ANOVA with post-hoc testing and Bonferroni correction.
KO, knockout; HTV, high tidal ventilation

3. VILI upregulates EphA2 via NOX4 activation

Given the protective effect of NOX4 inhibitor on VILI, we postulated that NOX4 mediates VILI through a EphA2-dependent pathway. Therefore, we investigated the expression of NOX4 and EphA2 using EphA2 KO mice. First, we investigated the effects of EphA2 on VILI (Figure 4). Cell counts and protein concentrations in BALF were significantly higher than those in the HTV group and lower than those in the EphA2 KO group (both $p < 0.001$; Figure 4A). BALF cells are mainly macrophages with a few lymphocytes (Figure 4B).

Histopathologic analysis showed that the extent of lung injury was the most severe in the HTV group and less severe in the EphA2 KO group.

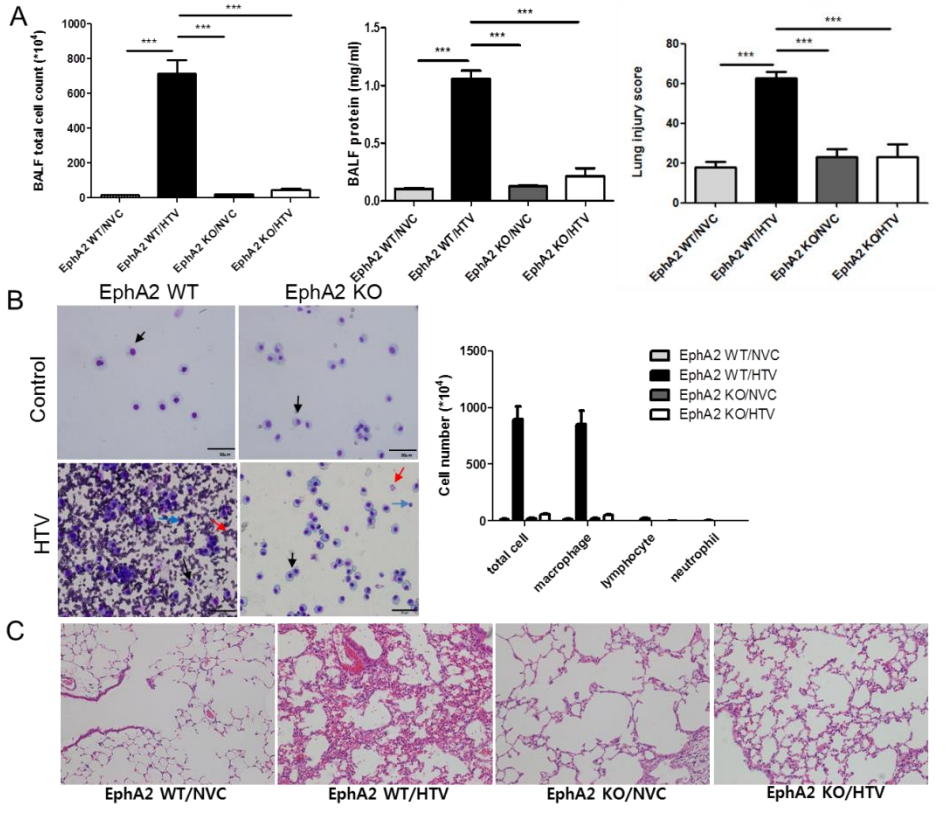


Figure 4. EphA2 affected VILI (n=3 in NVC group and n=5 in HTV group). (A) Total cell counts and protein concentrations. (B) Identification of cells in BALF with representative visual fields. Cells were mainly macrophages (black arrow) with a few lymphocytes (blue arrow), and neutrophils (red arrow). (C) Histopathologic features of hematoxylin and eosin-stained mouse lung tissues, which showed less vascular engorgement, hyaline membrane formation, alveolar wall edema, and inflammatory cell infiltration in the EphA2 KO mouse than in the HTV group (200× magnification). ***p < 0.001, analyzed by two-way ANOVA with post-hoc testing and Bonferroni correction. BALF, bronchoalveolar lavage fluid; WT, wild type; KO, knockout; NVC, normal ventilation control; HTV, high tidal ventilation

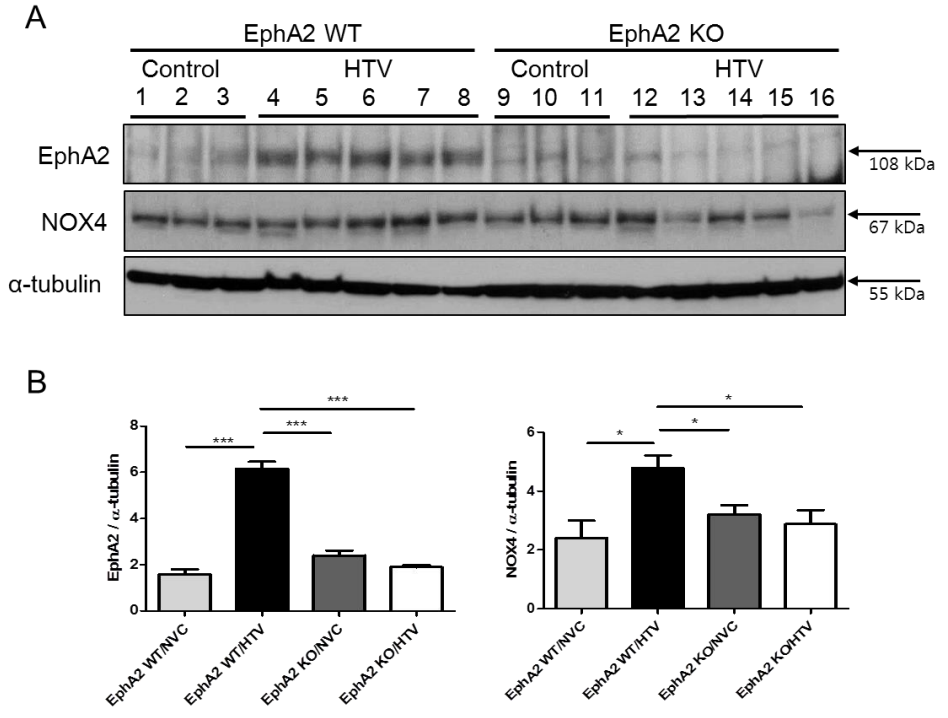


Figure 5. EphA2 levels increased in lung lysates after VILI, and NOX4 levels were similar to EphA2 levels in VILI regardless of EphA2. (A) Western blot of EphA2 and NOX4. (B) Densitometry of EphA2 and NOX4 in response to HTV and EphA2 KO. *p < 0.05, **p < 0.01, and ***p < 0.001, analyzed by two-way ANOVA with post-hoc testing and Bonferroni correction.
WT, wild type; KO, knockout; HTV, high tidal ventilation, NVC, normal ventilation control

Figure 5 shows the results of the western blot and densitometry analyses of EphA2 and NOX4. EphA2 expression was significantly higher in the HTV group and lower in the normal ventilation and EphA2 KO groups (both $p < 0.001$; Figure 5A and B). NOX4 expression was significantly higher in the HTV group, but it decreased in the EphA2 KO mouse and normal ventilation groups (both $p < 0.05$; Figure 5B). These results showed that NOX4 and EphA2

signaling was involved in VILI, and that EphA2 was activated through a NOX4-dependent pathway.

4. NOX4 inhibitor attenuated VILI by suppressing IL-6 and IL-8

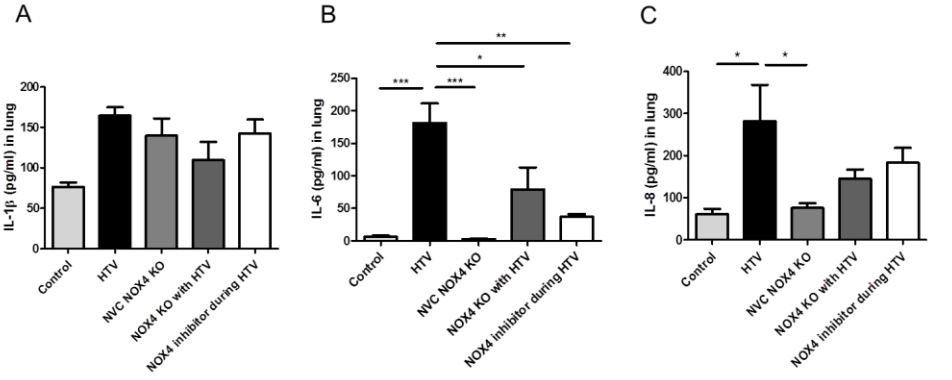


Figure 6. Inflammatory cytokine levels decreased in the NOX4 KO mouse and NOX4 inhibitor groups. (A) IL-1β, (B) IL-6, and (C) IL-8 levels in lung tissue lysates. *p < 0.05, **p < 0.01, and ***p < 0.001, analyzed by two-way ANOVA with post-hoc testing and Bonferroni correction.
KO, knockout; HTV, high tidal ventilation

Cytokine levels were measured by ELISA. The concentrations of cytokines (IL-6 and IL-8) were significantly higher in the HTV mouse group than in the control group (Figure 6). The expression of IL-6 was significantly decreased in both the NOX4 KO ($p<0.05$) and NOX4 inhibitor groups ($p<0.01$). The expression of IL-8 was significantly decreased in NOX4 KO mice ($p<0.05$).

IV. DISCUSSION

For patients with ARDS, a ventilator is essential for supplying oxygen, but it can also cause lung damage. In this study, we developed a VILI model. Then, we examined the role of NOX4 in VILI and the effects of NOX4 inhibition

through blockade of EphA2 using KO mice and NOX4 inhibitors in this VILI model.

NOX4 acts as an oxygen sensor for catalysis of molecular oxygen in various reactive oxygen species (ROS), and the resulting ROS undergo many biological reactions, including those involved in signal transduction, cell differentiation, and tumor cell growth. Eph-ephrin signaling, as part of receptor tyrosine kinase (RTK) pathways, have been implicated in many cellular processes, including vasculogenesis, angiogenesis, cell migration, axon guidance, embryonic development, fluid homeostasis, and injury repair.

Recently, Palumbo *et al*.[19] reported the role of NOX4 in the lungs and showed that ALI was more severe in aged mice than in young mice because of increased vascular permeability, albumin influx, and BAL neutrophils and proteins. In addition, they showed that ROS were elevated in aged or injured mice, suggesting that lung injury was associated with NOX4, a ROS-generating enzyme. They detected the senescence of endothelial cells bases on β-galactosidase activity and an increase in p16 level and studied regulation of the endothelial cell barrier in human lungs. Their results showed that membrane permeability caused by LPS was increased in senescent endothelial cells compared to that in young endothelial cells, and the expression of NOX4 was rapidly induced by LPS challenge via a proteasome/ubiquitin system. They found that pharmacological inhibition of NOX4 reduced the change in permeability due to LPS. Our study showed that NOX4 was involved in VILI, similar to its involvement in lung injury by LPS. Our results also demonstrated lung injury prevention by NOX4 inhibition.

Hong *et al*.[20] reported downregulation of PI3K 110γ, phospho-Akt, phospho-NF-κB p65, phospho-Src, and phospho-S6K via EphA2 antibody in an LPS-

induced lung injury model. The above two studies showed that NOX4 and EphA2 are involved in lung injury due to LPS and that NOX4 inhibition and EphA2 receptor inhibition are effective in limiting such lung injury. Therefore, with VILI, it can be inferred that the NOX4 and EphA2 pathways function similarly to that in LPS-induced lung injury and that there is a therapeutic effect from NOX4 or EphA2 inhibition.

A study of the EphA2/ephrinA1 signaling pathways in a VILI model was performed by Park *et al*.[21] Placing mice in a prone position reduced lung injury, and EphA2 antagonism downregulated the expression of PI3Kγ, Akt, NF-κB, and P70S6 kinase, resulting in lung protective effects. EphA2/ephrinA1 was shown to be a VILI therapeutic target in their study. Similarly, Leem *et al*.[22] reported that EphA2/ephrinA1 was elevated by bleomycin in a bleomycin-induced lung injury model, that elevation of IL-6 and TNF-a in PI3K-Akt led to lung injury, and that elevation of EphA2-ephrin A1 could be blocked by all-trans retinoic acid. As indicated by the studies described above, EphA2 levels are closely related to extent of lung injury in ICU patients, and, in our study, we found that EphA2 levels were higher in the severe lung injury group. Che *et al*.[23] showed that bleomycin-induced lung injury is caused by TGF-β/Smad3 signaling and oxidative stress, which can be regulated by a Chinese medicine called Shenks that increased expression of antioxidant-related genes Gclc and Ec-sod, both in vivo and in vitro, by increasing the transcription of oxidative-related genes, including Rac1 and Nox4. Zhang *et al*.[24] also showed overexpression of NOX4 in a bleomycin-induced lung injury model and reported that schizandrin B and glycyrrhizic acid were effective inhibitors of the TGF-β1/Smad3 signaling pathway in this model.

Lee *et al*.[25] measured plasma EphA2 receptor levels in a prospective study of

patients admitted to an ICU because of sepsis and determined disease severity based on acute physiology and chronic health evaluation (APACHE) II and sequential organ failure assessment (SOFA) scores to examine whether there was correlation between disease severity EphA2 levels. They confirmed a positive correlation between serum levels of the EphA3 receptor and severity of lung disease in ICU patients. In addition, when the area under the curve of EphA2 receptor levels was measured, it was 0.690 higher than the APACHE II scores. Additionally, they showed that EphA2 receptor levels were associated with sepsis severity and 28-day mortality. These results suggest that EphA2 levels are associated with VILI severity in ICU patients.

In summary, we found that a signaling pathway with NOX4, EphA2, and PI3K is associated with VILI and that there are several potential mechanisms by which NOX4 inhibition may affect VILI. In addition, we showed the potential for a NOX4 inhibitor to decrease VILI through EphA2 and PI3k 110λ signaling (Figure 7).

IV. CONCLUSION

In this study, we showed that NOX4 and Eph-ephrin signaling is involved in VILI and that a NOX4 inhibitor can play a therapeutic role in VILI. Further studies with human samples are needed to investigate the role of NOX4 signaling in VILI.

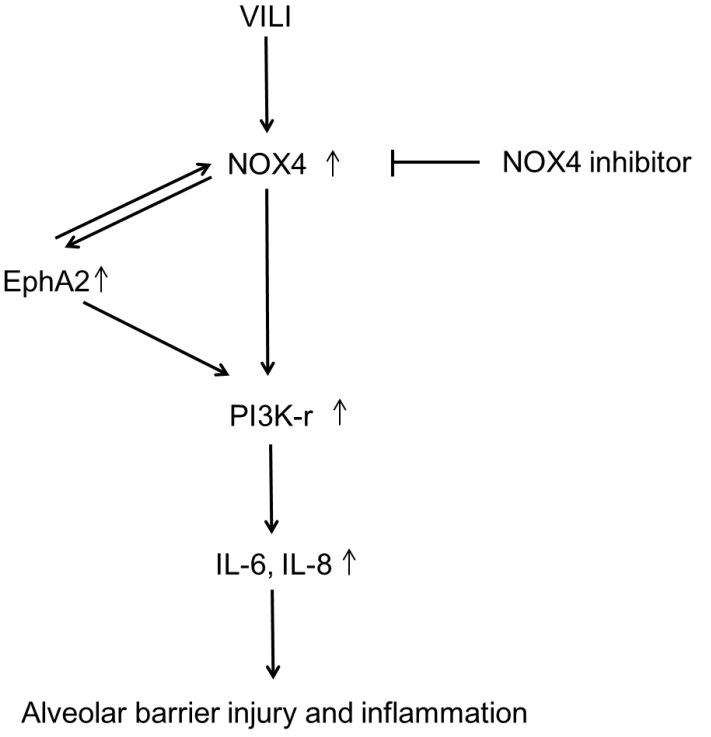


Figure 7. Potential mechanisms by which NOX4 inhibition attenuates VILI.
Our study suggests that NOX4 inhibition decreases VILI through EphA2, PI3K-r, and Nrf2 signaling.
VILI, ventilator-induced lung injury

REFERENCES

1. Ashbaugh DG, Bigelow DB, Petty TL, Levine BE. Acute respiratory distress in adults. Lancet 1967;2:319-23.
2. Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, et al. Report of the American-European consensus conference on ARDS: definitions, mechanisms, relevant outcomes and clinical trial coordination. The Consensus Committee. Intensive Care Med 1994;20:225-32.
3. Force ADT, Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, et al. Acute respiratory distress syndrome: the Berlin Definition. JAMA 2012;307:2526-33.
4. Fan E, Brodie D, Slutsky AS. Acute Respiratory Distress Syndrome: Advances in Diagnosis and Treatment. JAMA 2018;319:698-710.
5. Goss CH, Brower RG, Hudson LD, Rubenfeld GD, Network A. Incidence of acute lung injury in the United States. Crit Care Med 2003;31:1607-11.
6. Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, et al. Incidence and outcomes of acute lung injury. N Engl J Med 2005;353:1685-93.
7. Li G, Malinchoc M, Cartin-Ceba R, Venkata CV, Kor DJ, Peters SG, et al. Eight-year trend of acute respiratory distress syndrome: a population-based study in Olmsted County, Minnesota. Am J Respir Crit Care Med 2011;183:59-66.
8. Finfer S, Bellomo R, Boyce N, French J, Myburgh J, Norton R, et al. A comparison of albumin and saline for fluid resuscitation in the intensive care unit. N Engl J Med 2004;350:2247-56.
9. Spragg RG, Lewis JF, Walmrath HD, Johannigman J, Bellingan G, Laterre PF, et al. Effect of recombinant surfactant protein C-based surfactant on the acute

respiratory distress syndrome. N Engl J Med 2004;351:884-92.

10. Sprung CL, Annane D, Keh D, Moreno R, Singer M, Freivogel K, et al. Hydrocortisone therapy for patients with septic shock. N Engl J Med 2008;358:111-24.
11. Fang WF, Cho JH, He Q, Lin MC, Wu CC, Voelkel NF, et al. Lipid A fraction of LPS induces a discrete MAPK activation in acute lung injury. Am J Physiol Lung Cell Mol Physiol 2007;293:L336-44.
12. Fernandez R, Trenchs X, Klamburg J, Castedo J, Serrano JM, Besso G, et al. Prone positioning in acute respiratory distress syndrome: a multicenter randomized clinical trial. Intensive Care Med 2008;34:1487-91.
13. Taccone P, Pesenti A, Latini R, Polli F, Vagginelli F, Mietto C, et al. Prone positioning in patients with moderate and severe acute respiratory distress syndrome: a randomized controlled trial. JAMA 2009;302:1977-84.
14. Zhang CX, Lan T, Hou JC, Li J, Fang RD, Yang ZC, et al. NOX4 promotes non-small cell lung cancer cell proliferation and metastasis through positive feedback regulation of PI3K/Akt signaling. Oncotarget 2014;5:4392-405.
15. Amara N, Goven D, Prost F, Muloway R, Crestani B, Boczkowski J. NOX4/NADPH oxidase expression is increased in pulmonary fibroblasts from patients with idiopathic pulmonary fibrosis and mediates TGF beta 1-induced fibroblast differentiation into myofibroblasts. Thorax 2010;65:733-8.
16. Zhou Y, Sakurai H. Emerging and Diverse Functions of the EphA2 Noncanonical Pathway in Cancer Progression. Biol Pharm Bull 2017;40:1616-24.
17. Menges CW, McCance DJ. Constitutive activation of the Raf-MAPK pathway causes negative feedback inhibition of Ras-PI3K-AKT and cellular arrest through the EphA2 receptor. Oncogene 2008;27:2934-40.
18. Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, et al. An official American Thoracic Society workshop report: features

and measurements of experimental acute lung injury in animals. Am J Respir Cell Mol Biol 2011;44:725-38.

19. Palumbo S, Shin YJ, Ahmad K, Desai AA, Quijada H, Mohamed M, et al. Dysregulated Nox4 ubiquitination contributes to redox imbalance and age-related severity of acute lung injury. Am J Physiol Lung Cell Mol Physiol 2017;312:L297-L308.
20. Hong JY, Shin MH, Douglas IS, Chung KS, Kim EY, Jung JY, et al. Inhibition of EphA2/EphrinA1 signal attenuates lipopolysaccharide-induced lung injury. Clin Sci (Lond) 2016;130:1993-2003.
21. Park BH, Shin MH, Douglas IS, Chung KS, Song JH, Kim SY, et al. EphA2 Modulation Associates with Protective Effect of Prone Position in Ventilator-induced Lung Injury. Am J Respir Cell Mol Biol 2017; doi:10.1165/rcmb.2017-0143OC.
22. Leem AY, Shin MH, Douglas IS, Song JH, Chung KS, Kim EY, et al. All-trans retinoic acid attenuates bleomycin-induced pulmonary fibrosis via downregulating EphA2-EphrinA1 signaling. Biochem Biophys Res Commun 2017;491:721-6.
23. Chu H, Shi Y, Jiang S, Zhong Q, Zhao Y, Liu Q, et al. Treatment effects of the traditional Chinese medicine Shenks in bleomycin-induced lung fibrosis through regulation of TGF-beta/Smad3 signaling and oxidative stress. Sci Rep 2017;7:2252.
24. Zhang D, Liu B, Cao B, Wei F, Yu X, Li GF, et al. Synergistic protection of Schizandrin B and Glycyrrhizic acid against bleomycin-induced pulmonary fibrosis by inhibiting TGF-beta1/Smad2 pathways and overexpression of NOX4. Int Immunopharmacol 2017;48:67-75.
25. Lee SH, Shin JH, Song JH, Leem AY, Park MS, Kim YS, et al. Clinical implications of the plasma EphA2 receptor level in critically ill patients with septic shock. Sci Rep 2017;7:17612.

ABSTRACT (IN KOREAN)

인공호흡기 유발 폐손상 쥐모델에서 NADPH oxidase 4의 신호체계

<지도교수 박 무 석>

연세대학교 대학원 의학과

이 상 훈

배경: 급성호흡곤란증후군 환자에서 기계 환기는 조직에 산소를 제공하기 위해 필수적이라고 할 수 있다. 하지만 역설적으로 기계 환기는 폐손상을 가져올 수도 있다. 따라서 현 연구에서 우리는 인공호흡기 유발 폐손상에서 NOX4 의 역할을 규명하고, 이로 인한 폐손상에서 NOX4 유전자 제거 생쥐와 NOX4 억제제의 치료적 효과를 보고자 한다.

방법: 실험을 위한 C57BL/6J 마우스 (7-9 주, male, 20-28g; 오리엔트 바이오, 한국 성남)와 NOX4 KO 마우스 (7-9 주, male, 20-28g)는 다음과 같이 5 개의 그룹으로 나뉘어졌다; (1) 대조군, 마우스 + 비 기계환기; (2) HTV 그룹, 마우스 + 기계환기; (3) NOX4 KO 그룹, NOX4 KO 마우스 + 비기계환기; (4) NOX4 with HTV 그룹, NOX4 KO 마우스 + 기계환기 그룹; (5) NOX4 during HTV 그룹, 마우스 + 기계환기 + NOX4 억제제 처리 그룹. 인공 호흡기 폐 손상 모델에서, 기계환기는 24 mL/kg, 호기말양압 0 cm H2O, 100 회/분의 호흡 속도 및 0.21 흡기 산소 분율로 유지하였다. NOX4

억제제 처리 그룹에서는 인공호흡기 2 시간 째 50 μL 의 anti-GKT 137831 억제제 (BioVision cat # 9444-5)를 복강 내 주사로 주입하였다. 모든 기계환기를 사용한 그룹에서 5 시간의 기계환기 후 마우스를 안락사 시키고 추가 분석을 위해 폐 조직을 획득하였다.

결과: 기관지 폐포 세척액의 세포 수와 단백질 농도는 HTV 그룹에서 의미있게 높았다. HTV 그룹과 비교하여 NOX4 KO with HTV 에서 세포 수는 상당히 낮았고 ($p < 0.05$), 단백질 농도는 더 적은 경향이 있었다. NOX4 억제제 그룹에서, 세포 수 및 단백질 농도는 HTV 그룹보다 현저히 낮았다 (각각 $p < 0.05$ 및 $p < 0.01$). 기계환기에 의한 폐손상은 HTV 그룹에서 가장 컸으며, EphA2 level 도 HTV 그룹에서 가장 높았다. NOX4 KO 마우스 그룹 및 NOX4 억제제 그룹에서, EphA2 level 은 HTV 그룹보다 유의하게 낮았다 ($p < 0.01$). NOX4 mRNA 수준은 HTV 그룹에서 가장 유의하게 상승하였다.

결론: 현 연구에서 인공호흡기 유발 폐손상 모델에서 NOX4 발현은 Eph-ephrin 신호와 함께 유의하게 관련되어있음을 확인하였으며, 인공 호흡기 유발 폐 손상에서 NOX4 항체의 치료제로서의 가능성을 보여주었다.

핵심되는 말: NADPH oxidase 4, 인공호흡기 유발 폐손상