Oxidative stress by mitochondrial dysfunction contributes to the activation of necroptosis in acute respiratory distress syndrome

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ABSTRACT

Oxidative stress by mitochondrial dysfunction contributes to the activation of necroptosis in acute respiratory distress syndrome

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Background: Acute respiratory distress syndrome (ARDS) develops due to acute injury to the lungs caused by pulmonary or extrapulmonary abnormalities. Since the pathogenesis of ARDS is complex, the mechanisms of development of ARDS are not fully understood. Necroptosis is linked to cell death in ARDS. Oxidative stress has also been implicated in the pathogenesis of ARDS. Although the effect of necroptosis through oxidative stress is identified in the cellular system, the mechanisms for the activation of necroptosis in ARDS remain unclear.

Purpose: This study aims to confirm whether oxidative stress contributes to the receptor-interacting protein kinase 3 (RIPK3)-mediated necroptosis associated with ARDS.

Methods: The lung tissues from seven human subjects with ARDS were investigated for the involvement of RIPK3-mediated necroptosis and oxidative stress using immunoblot and immunostaining. We also investigated whether the oxidative stress due to mitochondrial dysfunction might exacerbate the RIPK3-mediated necroptosis in the lipopolysaccharide (LPS)-induced acute lung injury (ALI) mouse model.

Results We found that RIPK3- mediated necroptosis was significantly elevated in patients with ARDS compared to the non-ARDS subjects. Oxidative stress was increased in patients with ARDS due to the accumulation of 4-HNE, a marker for oxidative stress. Both RIPK3-mediated necroptosis and oxidative stress were elevated in the lung epithelial cells of the patients with ARDS. The oxidative stress due to mitochondrial dysfunction caused by oligomycin, an inhibitor of ATP synthase, significantly increased lung injury in the LPS-induced ALI mouse model compared to the ALI model without oligomycin treatment. Moreover, the oxidative stress due to mitochondrial dysfunction significantly increased the levels of RIPK3 and the release of damage-associated molecular patterns (DAMPs) by necroptosis in the lung during ALI compared to the group administered the dose without treatment of oligomycin.

Conclusion: The results of the human ARDS analysis showed that RIPK3-mediated necroptosis and oxidative stress were increased in ARDS, and the LPS lung injury mouse model with oxidative stress induced by mitochondrial dysfunction had increased RIPK3-mediated necroptosis, lung injury, and DAMPs release. Our results suggest that oxidative stress due to mitochondrial dysfunction might contribute to a critical mechanism for RIPK3-mediated necroptosis-induced lung injury during ARDS.

Key words: oxidative stress, necroptosis, acute respiratory distress syndrome, acute lung injury

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I. INTRODUCTION

Acute respiratory distress syndrome (ARDS) develops due to acute injury to the lungs caused by pulmonary or extrapulmonary abnormalities such as pneumonia, sepsis, trauma, multiple transfusions, aspiration of gastric contents, and pulmonary contusion.[1] ARDS has been described since 1967 and accounts for more than 10% of intensive care unit admissions; many studies have reported more than 30% mortality rate due to ARDS despite current treatment techniques.[2–4]

The aforementioned causes such as pneumonia, sepsis, trauma, multiple transfusions, aspiration of gastric contents, and pulmonary contusions induce diffuse alveolar damage, leading to excess fluid in both the interstitium and alveoli, a characteristic pathological finding of ARDS.[1] The inflammatory response and endothelial barrier disruption play important roles in developing ARDS.[5,6] Although the recent Berlin definition of ARDS does not distinguish between direct and indirect ARDS based on the cause,[7] direct ARDS caused due to pneumonia and aspiration is induced by severe injury to both the alveolar epithelium and the vascular endothelium, leading to the increased permeability

of the alveolar-capillary membrane.[8] However, indirect ARDS caused by lung injuries, such as non-pulmonary sepsis originating in the lungs and systemic endothelial damage, is induced by the intravascular inflammatory mediators.[9]

As a cause of direct ARDS, pneumonia activates several inflammatory pathways such as the toll-like receptor 4 (TLR4) signaling pathway.[10,11] However, all pneumonia patients do not progress to ARDS.[10,11] Since the pathogenesis of ARDS is complex, the mechanisms of the development of ARDS are not fully understood.

Necroptosis is linked to cell death during ARDS.[14] Necroptosis, a programmed form of necrosis, is regulated by the formation of a necrosome complex consisting of receptor-interacting proteins 1 and 3 (RIPK1 and RIPK3).[15,16] The elevation of RIPK3 levels promotes the activation of necroptosis.[17] Oxidative stress also has been implicated in the pathogenesis of ARDS.[12] Mitochondrial dysfunction plays a crucial role in developing oxidative stress in various types of cells in lung diseases.[12] In our previous study, we found that mitochondrial dysfunction promotes the activation of necroptosis in the human lung epithelial cells.[18] Although the regulation of necroptosis has been identified in the cellular system, the mechanisms for the activation of necroptosis in ARDS remain unclear.

Our study aims to confirm whether oxidative stress contributes to the RIPK3-mediated necroptosis associated with ARDS. To this end, we evaluated RIPK3-mediated necroptosis and oxidative stress in human ARDS lung tissue. We determined whether oxidative stress induced by mitochondrial dysfunction via oligomycin treatment increased the RIPK3-mediated necroptosis and necroptosis-induced damage-associated molecular pattern (DAMP) release in the lungs of mice with acute lung injury (ALI).

II. MATERIALS AND METHODS

1. Human study

Slides of lung sections from seven human ARDS patients were obtained according to the protocol approved by the Institutional Review Board of Severance Hospital, Yonsei University Health System, Republic of Korea (approval number: 4-2019-0831). Two ARDS lung tissues were obtained with the consent of patients with ARDS undergoing lung transplantation. Both patients were enrolled in a lung transplant patient cohort (approval number: 4-2013-0770). The non-ARDS lung tissues were obtained from the human-derived material bank (approval number: 4-2019-0447).

2. Animal study

The animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals (National Research Council, USA), approved by the Yonsei University Health System Institutional Animal Care and Use Committee (approval number: 2018-0158). Male 8-week-old C57BL/6 mice (ORIENT BIO, Gyeonggi, Republic of Korea) were intratracheally administered with lipopolysaccharide (LPS; 5 μg/g) derived from *Escherichia coli* 0111:B4 (InvivoGen, San Diego, CA, USA) for lung injury, whereas the control mice were treated with an equal volume of phosphate-buffered saline (PBS). In the groups with the promotion of necroptosis, 24 h and 1 h before treatment with LPS or PBS, oligomycin (0.5 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) was injected intraperitoneally to impair oxidative phosphorylation, thereby allowing necroptosis to proceed.[18,19] The mice were sacrificed using alfaxalone (76 mg/kg) and xylazine (10 mg/kg) one day after LPS or PBS

administration, the trachea was washed thrice with 0.9 mL of PBS through a tracheal catheter to obtain bronchoalveolar lavage fluid (BALF) and collect lung tissue.

3. Immunoblot analysis

Human lung tissues and whole right lung sections from the mice were used for immunoblot of RIPK3 protein. The lung tissues were homogenized in 1 mL of radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 0.1 % sodium dodecyl sulphate (SDS), 2 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 1 % sodium deoxycholate; 1 % Triton X-100; pH 7.5) and protease inhibitor cocktail. The homogenate was incubated for 20 min on ice and then centrifuged at 14,000 ×g for 15 min at 4 °C. The supernatant was collected and the same volume of 5 X SDS buffer was added to the supernatant. The mixture was then boiled for 5 min and stored at −80 °C. The proteins were separated by SDS-polyacrylamide gel electrophoresis on 4–5% Q-PAGE Tris-Glycine Novel Precast Gel (SMOBIO Technology, Hsinchu City, Taiwan). After electrophoresis, the proteins were transferred to the polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) and blocked in 5% non-fat milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) for 1 h at room temperature. The membranes were immunoblotted with primary antibodies against RIPK3 for the mouse (Cell Signaling Technology, Danvers, USA), RIPK3 for human (Abcam, Cambridge, UK), and β-actin (Sigma-Aldrich, St Louis, MO, USA) overnight at 4 °C and washed five times with TBS-T for 10 min each at room temperature. The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit, anti-rat, or anti-mouse secondary antibody (1:2000 dilution in TBS-T; Thermo Fisher Scientific,

Rockford, IL, USA) at 37 °C for 1 h and were washed five times with TBS-T for 10 min each at room temperature. The membranes were treated with chemiluminescence reagent (Thermo Fisher Scientific, Rockford, IL, USA) and exposed to photographic film.

4. Histopathological examination and lung injury scoring

The left lung of each mouse was fixed by perfusion with formaldehyde before routine processing and paraffin embedding. The slide sections were stained with hematoxylin and eosin (H&E) for histological examination. At least 20 high-power fields (400 X total magnification) were randomly selected due to the patchy nature of the injury and lung injury scores were calculated as described in the American Thoracic Society Workshop Report.[20] Human lung tissue slide sections were further stained for immunohistochemical analysis of anti-RIPK3 antibody (Abcam, Cambridge, UK) to confirm necroptosis. The immunohistochemistry was performed using the RIPK3 antibody with Dako Autostainer Link 48 platform (Dako, Glostrup, Denmark).

5. Immunofluorescence analysis

For immunofluorescence analysis, the lung tissues were sectioned from the paraffin-embedded tissue blocks at a thickness of 4 μm. The sections were permeabilized in 0.5 % Triton-X (Sigma-Aldrich, St Louis, MO, USA), blocked in CAS-Block™ Histochemical Reagent (Thermo Fisher Scientific, Waltham, MA, USA), and then stained with the following antibodies: polyclonal rabbit anti-4-hydroxynonenal (HNE) antibody (1:100) (Abcam, Cambridge, UK), monoclonal mouse anti-E-cadherin antibody (1:100) (Santa Cruz Biotechnology, Dallas, TX, USA). These sections were then incubated

with goat anti-rabbit IgG (H+L) Alexa Fluor 488 (1:100) (Thermo Fisher Scientific, Waltham, MA, USA), and goat anti-mouse IgG H&L Texas Red (1:100) (Abcam, Cambridge, UK) secondary antibody at 25 °C for 2 h. Fluoroshield™ with DAPI (Sigma-Aldrich, St. Louis, MO, USA) was used for nuclear staining. The stained brain sections were analyzed by THUNDER Imager Tissue (Leica Microsystems Ltd., Wetzlar, Germany). The stained brain 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 the measurements were performed with blinded conditions by two observers per experiment under identical conditions.

6. Quantification of protein levels and number of cells in BALF

The BALF was centrifuged (4 °C, 400 ×g, 10 min) and the protein content was measured in the supernatant using Pierce BCA Protein Assay Kit (Thermo Scientific Fisher, Waltham, MA, USA). After incubation for 30 min at 37 °C, the plate was cooled and the absorbance was read at 562 nm in a spectrophotometer. The cell pellet was reconstituted in 100 μL PBS and used to quantitatively and qualitatively analyze the cells. The total cell number in each sample was determined using a hemocytometer (Paul Marienfeld GmbH, Lauda-Königshofen, Germany) according to the manufacturer's protocol. An aliquot of each sample (90 μL) was transferred into the slide chambers and inserted into a cytospin with the slide facing outward. The slides were centrifuged at 600 rpm for 6 min and then removed and dried before staining. The slides were immersed in the three Diff-Quick fluids (Fixative, Solution I, and Solution II) and rinsed with purified water.

7. Statistical analysis

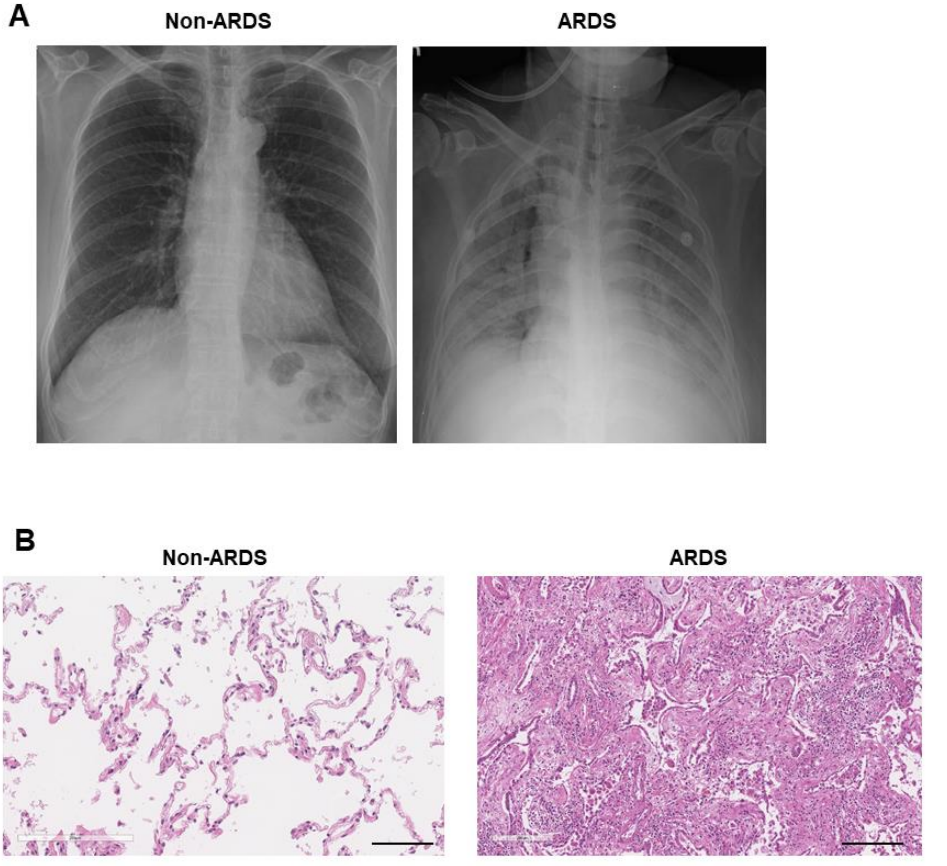
All data are represented as mean ± standard deviation (SD) or standard error of the mean (SEM). All statistical tests were analyzed using a two-tailed Student's t-test for comparison of two groups, and analysis of variance (ANOVA) (with post hoc comparisons using Dunnett's test) using a statistical software package (GraphPad Prism version 5.01, GraphPad Software Inc. (San Diego, CA, USA)) for comparison of multiple groups. p values (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$) were considered statistically significant.

III. RESULTS

1. The RIPK3-mediated necroptosis in the lung tissues of patients with ARDS

To investigate whether the activation of RIPK3-mediated necroptosis might promote the development of ARDS, we analyzed the activation of RIPK3-mediated necroptosis in the lung tissues of patients with ARDS who received lung transplantation. Retrospectively, we reviewed 241 consecutive lung transplantation patients between October 2012 and July 2019. Among the 241 patients, we specifically examined seven patients with ARDS. The chest X-ray revealed that the bilateral diffuse infiltration was different in patients with ARDS compared to non-ARDS (Figure 1A). The histopathological examination of ARDS patients showed high interstitial organizing fibrosis with fibrinous exudates, accumulation of intra-alveolar hemosiderin-laden macrophages, and type II pneumocyte hyperplasia, which is consistent with the proliferative (organizing) phase of diffuse alveolar damage in H&E staining (Figure 1B). We next investigated whether the RIPK3-mediated

necroptosis is elevated in the lung tissues of patients with ARDS. We measured the protein levels of RIPK3 in the lung tissues of patients with ARDS. The protein levels of RIPK3 are significantly higher in the lung tissues of patients with ARDS than in those of non-ARDS lung tissues (Figure 1C). These results suggest that RIPK3-mediated necroptosis is elevated in the lung tissues of patients with ARDS.



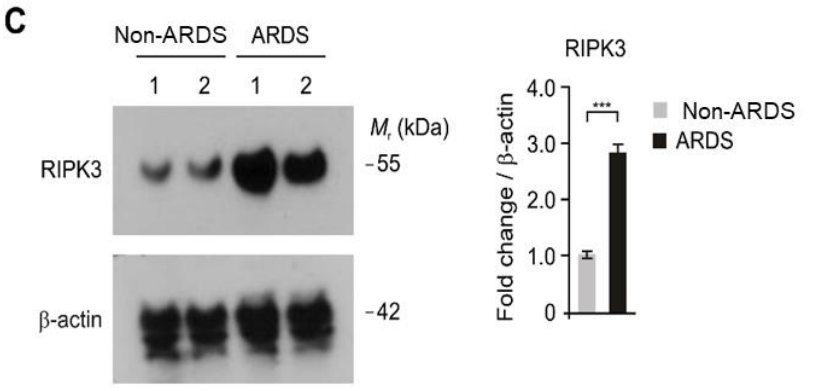
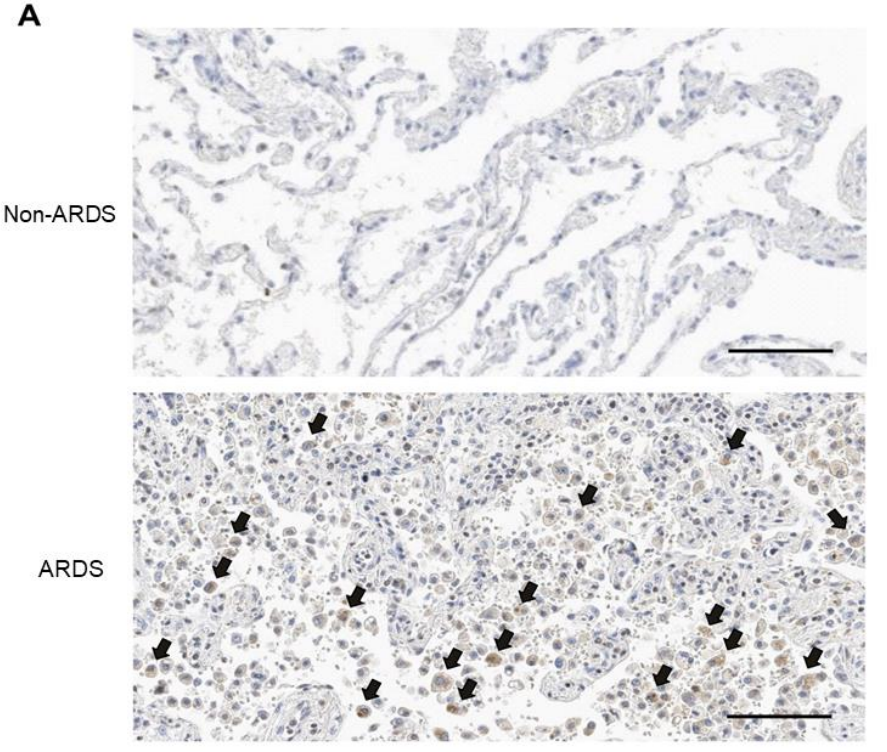


Figure 1. Expression of RIPK3-mediated necroptosis in lung tissues of patients with and without ARDS (A) The representative chest X-ray images of the patient with ARDS showed distinct infiltration on both sides of the lungs compared to that of non-ARDS. (B) Representative hematoxylin and eosin-stained image of lung tissues from the patient with ARDS (ARDS) showed high interstitial organizing fibrosis with fibrinous exudates, accumulation of intra-alveolar hemosiderin-laden macrophages, and type II pneumocyte hyperplasia compared to the non-ARDS subject. Scale bars, 200 μm. (C) Representative immunoblot images of RIPK3 (left) and quantification for RIPK3 protein levels (right) in lung tissues of patients with ARDS (ARDS) and non-ARDS subjects. RIPK3 expression was significantly increased in patients with ARDS and the protein levels of RIPK3 are significantly higher in the lung tissues of patients with ARDS than non-ARDS lung tissues. For immunoblots, β-actin was used as the loading control. Data are representative of three independent experiments. Data are mean ± standard deviation (SD). *** $p < 0.001$ using the two-tailed Student's *t*-test.

2. The RIPK3-mediated necroptosis in the lung epithelial cells of patients with ARDS

Next, we investigated whether RIPK3-mediated necroptosis contributes to cell death of lung epithelial cells in the lung tissue of patients with ARDS. We analyzed the expression of RIPK3 in the lung epithelial cells of lung tissues from patients with ARDS. Immunohistochemical staining revealed that the expression of RIPK3 was higher in lung epithelial cells of patients with ARDS (ARDS) than non-ARDS subjects (Figure 2A). Notably, the number of RIPK3-positive cells was significantly increased in patients with ARDS (ARDS) compared to non-ARDS subjects (Figure 2B). These results suggest that the RIPK3-mediated necroptosis was elevated in the lung epithelial cells of patients with ARDS.



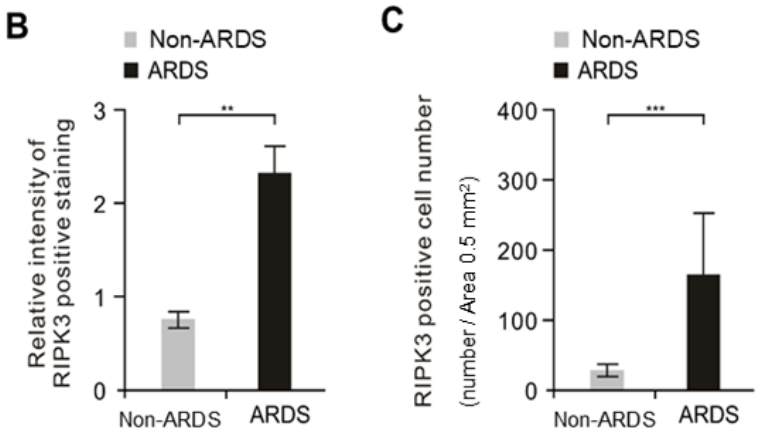


Figure 2. The RIPK3-mediated necroptosis in lung epithelial cells of patients with and without ARDS (A) Representative immunohistochemistry for RIPK3 in the lung tissues from the patient with ARDS and non-ARDS subjects showed that expression of RIPK3 was higher in the lung epithelial cells of the patient with ARDS than in the non-ARDS subject. The RIPK3-positive cells were indicated by black arrows. Scale bars, 200 μm. (B) The intensity of RIPK3 staining and (C) the quantification of the RIPK3-positive cells in the lung tissues from patients with ARDS and non-ARDS subjects. The intensity of RIPK3 staining and quantification of RIPK3-positive cells were significantly increased in the ARDS patients compared to non-ARDS subjects. (n = 10 images per individual subject). Data are mean ± standard deviation (SD). ** $p < 0.01$; *** $p < 0.001$ using the two-tailed Student's *t*-test.

3. The levels of oxidative stress in the lung epithelial cells of patients with ARDS

We investigated whether oxidative stress might be associated with the

activation of RIPK3-mediated necroptosis during ARDS. We analyzed the levels of oxidative stress using 4-HNE, a marker for oxidative stress, staining in epithelial cells of lung tissues from patients with ARDS (Figure 3). The levels of 4-HNE were elevated in E-cadherin-positive lung epithelial cells of lung tissues from patients with ARDS relative to non-ARDS subjects (Figure 3A and 3B). Consistently, the number of 4-HNE positive lung epithelial cells was significantly increased in patients with ARDS (ARDS) compared to non-ARDS subjects (Figure 3C). These results suggest that the levels of oxidative stress are elevated in the lung epithelial cells of patients with ARDS.

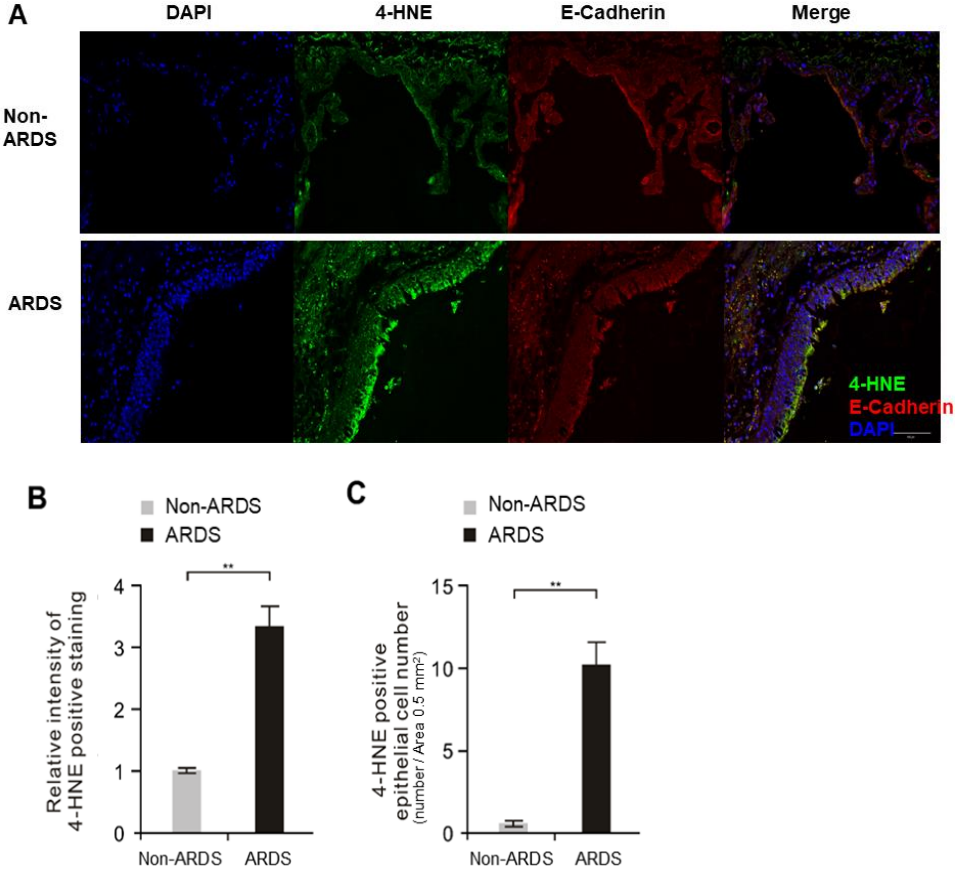
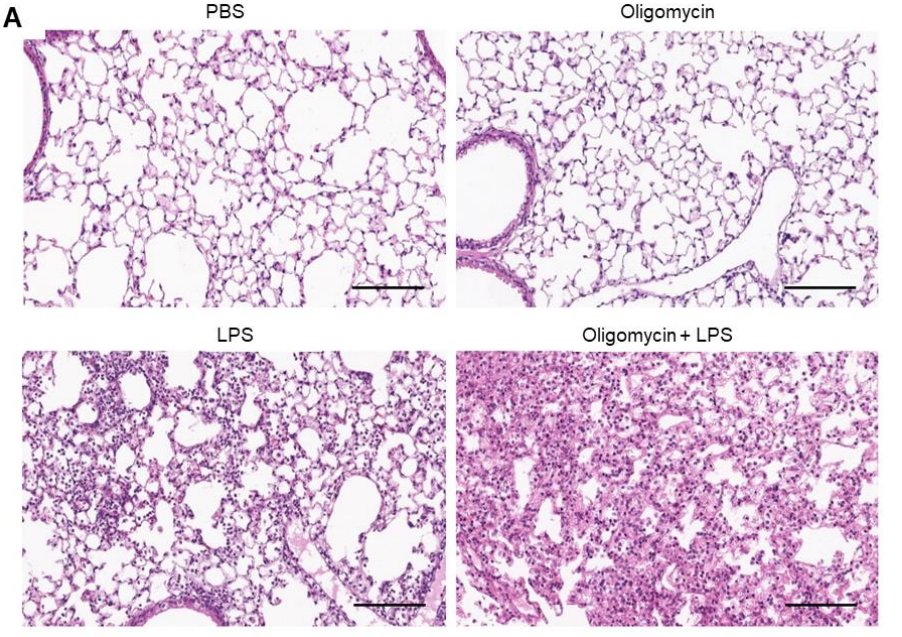


Figure 3. Oxidative stress in lung tissues from patients with and without ARDS (A) The expression of 4-HNE (green) in the epithelial cells of lung tissues from the patient with ARDS was elevated compared to the non-ARDS subject. The epithelial cells stained with E-Cadherin are shown in red. The DAPI-stained nuclei are shown in blue. Scale bars, 100 μm. (B) Quantification of intensity for 4-HNE-positive staining in the epithelial cells from immunofluorescence images in the lung tissues from patients with ARDS and non-ARDS subjects. The relative intensity for 4-HNE positive staining was significantly increased in ARDS patients compared to non-ARDS subjects. (n = 10 images per individual subject). Data are mean ± standard deviation (SD). **, $p < 0.01$ by Student's two-tailed *t*-test. (C) The number of 4-HNE positive epithelial cells from the immunofluorescence images in the lung tissues from patients with ARDS was significantly higher than non-ARDS subjects. (n = 10 images per individual subject). Data are mean ± standard deviation (SD). **, $p < 0.01$ by Student's two-tailed *t*-test.

4. Effect of oxidative stress in the LPS induced acute lung injury mouse model

We investigated whether oxidative stress due to mitochondrial dysfunction might exacerbate lung injury in a mouse model of ALI. We analyzed the effects of mitochondrial dysfunction via inhibition of mitochondrial oxidative phosphorylation by oligomycin, an oxidative phosphorylation uncoupler, on lung injury during ALI. We treated oligomycin in LPS-induced ALI mice. The levels of lung injury scores were measured by H&E staining (Figure 4A). The lung tissues treated with PBS or only oligomycin showed that the lung architecture was well-maintained and there was no increase in the

inflammatory cells. The LPS-treated group (LPS or LPS + oligomycin) showed a higher number of inflammatory cells than the non-LPS-treated group. Notably, the number of inflammatory cells was significantly elevated by oligomycin administration in the group treated with LPS compared to the group treated with only LPS (Figure 4A). Moreover, oligomycin administration resulted in significantly higher lung injury scores than the group treated with only LPS (Figure 4B). These results suggest that oxidative stress due to mitochondrial dysfunction exacerbated lung injury during ALI.



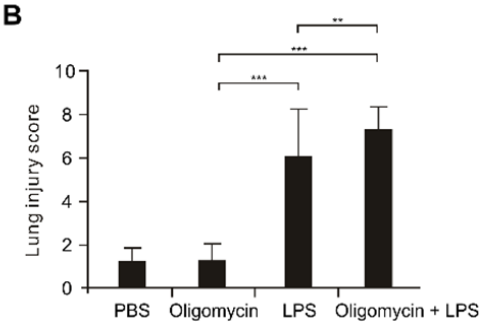


Figure 4. Lung injury of mouse in oxidative stress by mitochondrial dysfunction (A) Representative hematoxylin and eosin-stained images of the lung tissues of mice treated with oligomycin in the LPS-induced ALI model. The lung tissue in the mice treated with LPS and oligomycin showed significantly elevated inflammatory cells compared to the lung tissue in mice with only LPS. Scale bars, 200 μm. (B) Quantification of lung injury scores of mice with oligomycin treatment in the LPS-induced ALI model. The lung injury score of the group treated with LPS and oligomycin was significantly higher than that of the group treated with only LPS. (n = 20 images per individual subject). Data are mean ± standard deviation (SD). **, $p < 0.01$; ***, $p < 0.001$ by Student's two-tailed *t*-test or ANOVA.

5. The RIPK3-mediated necroptosis in the lung tissues of mice during ALI

We investigated whether oxidative stress induced by mitochondrial dysfunction might increase RIPK3-mediated necroptosis in the lung tissues of mice during ALI. We analyzed the protein levels of RIPK3 in the oligomycin-treated lung tissues. The levels of RIPK3 were induced in the lung tissues of mice with LPS relative to mice with PBS (Figure 5A and 5B). Notably, the levels of RIPK3 were significantly elevated by oligomycin treatment compared to LPS only (Figure 5A and B). These results suggest that oxidative stress induced by mitochondrial dysfunction elevated the RIPK3-mediated necroptosis in the lung tissue of mice during ALI.

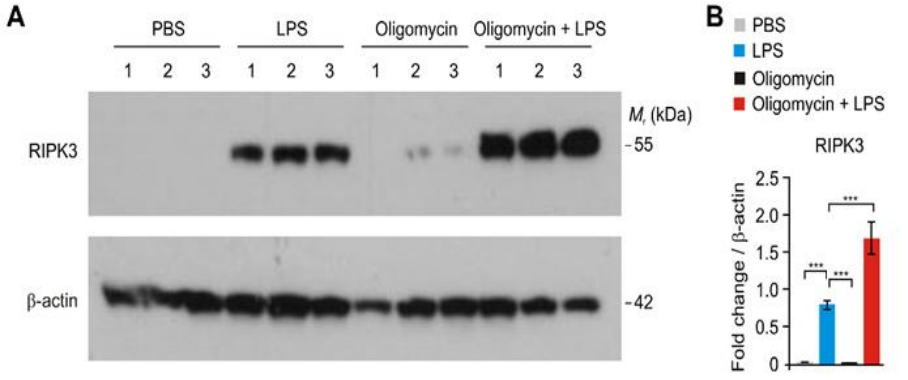


Figure 5. RIPK3-mediated necroptosis of mouse lung tissues in oxidative stress by mitochondrial dysfunction (A) Representative immunoblot images of RIPK3 protein and (B) Quantification of RIPK3 protein levels in the lung tissues upon oligomycin treatment in the LPS-induced ALI model. Immunoblot images of the lung tissue revealed that groups that were not treated with LPS (PBS, oligomycin) did not express RIPK3. However, in the lung tissue of mice treated with LPS, RIPK3 expression increased in both groups regardless of oligomycin administration. The levels of RIPK3 were significantly elevated by oligomycin treatment compared to the treatment with only LPS. For the immunoblots, β-actin was used as the loading control. Data are representative of three independent experiments. Data are mean ± standard deviation (SD). *** $p < 0.001$ using the two-tailed Student's *t*-test or ANOVA.

6. The release of DAMPs by necroptosis in the lung during ALI

We next investigated whether the activation of RIPK3-mediated necroptosis by oxidative stress due to mitochondrial dysfunction might lead to the release

of DAMPs in the BALF of the lung during ALI. We analyzed the protein levels as a parameter of DAMPs in the BALF of lungs from the mice treated with oligomycin during LPS-induced ALI. The protein levels of BALF were increased during LPS-induced ALI compared to the control (Figure 6A). Notably, oligomycin administration significantly increased the protein levels in BALF of the lung from the mice during LPS-induced ALI relative to only LPS treatment (Figure 6A). In contrast, oligomycin administration did not change the number of total cells in the BALF compared to the treatment with only LPS (Figure 6B). These results suggest that oxidative stress induced by mitochondrial dysfunction increases the release of DAMPs by RIPK3-mediated necroptosis in the lung during ALI.

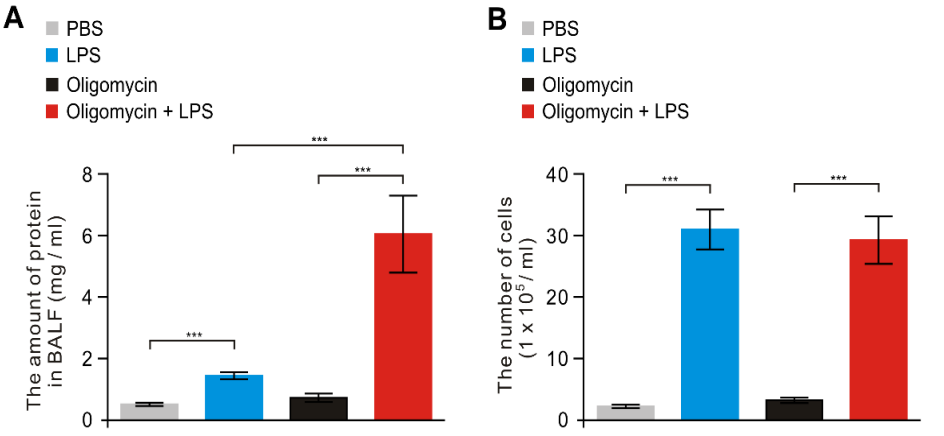


Figure 6. The release of DAMPs by necroptosis in the bronchoalveolar lavage fluid of mouse lung during ALI (A) Quantification of the amount of protein in the bronchoalveolar lavage fluid (BALF) from the lungs of mice treated with oligomycin in an LPS-induced ALI model. In the groups treated with LPS, oligomycin administration significantly increased the protein levels in BALF compared to the group treated with LPS only. Data are

representative of three independent experiments. Data are mean ± standard deviation (SD). *** $p < 0.001$ using the two-tailed Student's *t*-test or ANOVA. (B) Quantification of total cell counts in BALF from lungs of mice treated with oligomycin in the LPS-induced ALI model. In the groups treated with LPS, the oligomycin administration did not significantly change the number of total cells in the BALF compared to the administration of only LPS. Data are representative of three independent experiments. Data are mean ± standard deviation (SD). *** $p < 0.001$ using the two-tailed Student's *t*-test or ANOVA.

IV. DISCUSSION

In this study, we demonstrated that oxidative stress induced by mitochondrial dysfunction contributes to the RIPK3-mediated necroptosis in ARDS. Our results showed that the levels of oxidative stress and RIPK3-mediated necroptosis were elevated in patients with ARDS. We also showed that the oxidative stress due to mitochondrial dysfunction increased RIPK3-mediated necroptosis and the release of DAMPs in the lung tissues of the ALI mouse models.

The initial pathogenesis of ARDS called the exudative phase begins with innate immune cell-mediated damage of the alveolar endothelial and epithelial barriers, including apoptosis and necroptosis.[1] A previous study showed that apoptosis is linked to the cell death of the epithelial cells during ARDS.[21] Induction of apoptosis in the alveolar epithelial cells is related to the early stage of ARDS.[21] Additionally, previous studies suggest that RIPK3-mediated necroptosis is elevated in ARDS.[14,22,23] The levels of plasma RIPK3 were increased in patients with mechanical ventilator-induced lung injury.[14]

Furthermore, the RIPK3 plasma levels were increased in patients with ARDS in sepsis and trauma.[23] Consistent with the previous study, our results showed that the RIPK3-mediated necroptosis-mediated cell death is increased in the epithelial cells of the lung tissues of patients with ARDS. Furthermore, our results showed that the levels of oxidative stress are elevated in lung epithelial cells of patients with ARDS. Since the process of cell death is affected by cellular conditions such as mitochondrial function, the activation of apoptosis or necroptosis might be regulated by cellular stress during ARDS. Therefore, our results of the human ARDS lung tissue analysis suggest that oxidative stress could be an association factor for the progression of necroptotic cell death during ARDS.

Lung inflammation by infection with pneumonia is one of the most common causes of ARDS.[1,24,25] Lung inflammation can induce innate immunity, and thus, the necroptosis pathway through RIPK3 and MLKL.[1,24,25] Recent studies showed that necroptosis and apoptosis contribute to LPS-induced ALI in a mouse model.[26] This study suggests that the damage by necroptosis is more dominant in the alveolar epithelial cell than the damage by apoptosis in the LPS-induced ALI.[26] Similarly, we showed that RIPK3-mediated necroptosis was elevated in lung tissues during LPS-induced ALI. Moreover, our results suggest that oxidative stress induced by mitochondrial dysfunction exacerbates RIPK3-mediated necroptosis in lung tissues during LPS-induced ALI. The group with oxidative stress induced by mitochondrial dysfunction showed significantly increased lung injury and expression of RIPK3-mediated necroptosis compared to the other group without oxidative stress. Furthermore, our results suggest that oxidative stress induced by mitochondrial dysfunction is also critical for the activation of necroptosis in lung inflammation-induced

ARDS. Since our results showed that oxidative stress induced by mitochondrial dysfunction via oligomycin treatment significantly increased the levels of protein in the BALF of the lung and did not affect the number of cells in the BALF, our results could support that oxidative stress induced by mitochondrial dysfunction dominantly affects necroptotic cell death of lung epithelial cell relative to lung inflammation.

Our study had some limitations. First, the analysis was based on the human lung tissues but not blood or BALF, and while we could confirm an association between necroptosis and RIPK3, the exact nature of this relationship could not be determined. Since the non-ARDS human lung tissues used as a control for ARDS were not obtained from the normal subjects without disease but were obtained from the normal part of non-ARDS patients, there was a limit as a control. Third, the LPS-induced ALI model used as the ARDS model is a model using inflammatory reaction, so there is a limit to completely represent human ARDS caused by various causes. Fourth, all patients who underwent lung transplantation due to ARDS were not in the same phase of ARDS. However, the LPS-induced ALI mouse model showed an early phase of ARDS, and we could not establish the association between necroptosis and the other phases of ARDS, including the proliferative and fibrotic phases.

Although we tried to show the effects of oxidative stress induced by mitochondrial dysfunction on necroptosis in ARDS, further studies are required on the effects of oxidative stress induced by genetic deficiency or knockdown in the activation of necroptosis during ARDS or ALI. Further studies on the analysis of oxidative stress and necroptosis at different stages, including early or late stage ARDS, are also needed.

V. CONCLUSION

Our analysis of human ARDS found that RIPK3-mediated necroptosis and oxidative stress were increased, and the LPS lung injury mouse model with oxidative stress induced by mitochondrial dysfunction had increased RIPK3-mediated necroptosis, lung injury, and DAMPs release. Our results suggest that oxidative stress induced by mitochondrial dysfunction might be part of a critical mechanism leading to RIPK3-mediated necroptosis-induced lung injury during ARDS.

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ABSTRACT (IN KOREAN)

급성호흡곤란증후군에서 미토콘드리아 기능장애로 유도된 산화스트레스가 necroptosis의 활성화에 미치는 영향

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이 수 환

배경: 급성호흡곤란증후군은 폐 또는 폐 이외의 문제로 유발되는 폐의 급성 손상으로 발생한다. 급성호흡곤란증후군의 발병 기전은 복잡하며, 아직 모든 발병 기전이 밝혀지지는 않았다. 산화스트레스 (oxidative stress)와 세포사멸 종류 중 necroptosis는 급성호흡곤란 발생에 영향을 주는 것으로 알려져 있다. 이전에 보고된 세포실험 연구에서 산화스트레스가 necroptosis의 유도에 관여됨이 확인 되었다. 그러나 생체 내 (In Vivo)에서는 그 관련성이 아직 명확하게 밝혀져 있지 않다.
목적: 본 연구에서는 급성호흡곤란증후군 환자의 폐에서 산화스트레스와 RIPK3-mediated necroptosis가 발생함을 확인하고, lipopolysaccharide (LPS)로 유도된 급성 폐손상 마우스 모델에서 미토콘드리아 기능장애로 유발된 산화스트레스가 RIPK3-mediated necroptosis에 영향을 주는지 밝히고자 하였다.
방법: 7명의 급성호흡곤란증후군 환자의 폐 조직병리 슬라이드에서 면역 염색을 시행하였고, 2명의 급성호흡곤란증후군 환자의 폐 조직에서 단백질 면역 블랏을 시행하였다. 동물 실험에서 급성 폐손상 마우스 모델은 LPS를 기도내 주입하여 만들었다. 산화스트레스는 미토콘드리아 기능장애를 유발하는 oligomycin을 마우스 복강내 주입하여 발생시켰다. 급성 폐손상 마우스 모델에서 산화스트레스가 유도된 그룹과 유도되지 않은 그룹의 폐 손상 정도를 조직병리 염색을

이용하여 비교하였다. 그리고 RIPK3-mediated necroptosis 발생 정도를 단백질 면역 블랏과 면역화학 염색을 이용하여 비교하였다. 마우스의 기관지 세척으로 획득한 기관지 폐포세척액에서 necroptosis와 관련되어 분비되는 damage-associated molecular patterns (DAMPs)의 양을 비교하였다.

결과: 본 실험에서 사용된 급성호흡곤란증후군 환자의 폐 조직은 급성호흡곤란증후군이 없는 폐 조직에 비해 RIPK3-mediated necroptosis와 산화스트레스가 증가하였다. 그리고 RIPK3-mediated necroptosis 및 산화스트레스 모두 급성호흡곤란증후군 환자의 폐 상피세포에서 증가하였다. ATP synthase를 억제하는 oligomcyin을 투여하여 산화스트레스가 유도된 폐손상 마우스 모델에서 폐 손상 정도와 폐 조직의 RIPK3-mediated necroptosis의 발생이 oligomycin을 사용하지 않은 폐손상 마우스 모델에 비해 증가되었다. 그리고 oligomycin으로 산화스트레스가 유발된 폐손상 마우스 모델의 기관지 폐포 세척액에서 necroptosis로 인한 DAMPs 분비가 oligomycin을 사용하지 않은 폐손상 마우스 모델에 비해 증가하였다.

결론: 급성호흡곤란 증후군 환자의 폐 조직에서 RIPK3-meidated necroptosis와 산화스트레스가 증가하였다. 그리고 LPS로 유도된 급성 폐손상 마우스 모델에서 산화스트레스가 증가하면 폐손상 및 RIPK3-mediated necroptosis와 DAMPs 분비가 증가하였다. 이상의 결과들은 급성호흡곤란 증후군의 발생 과정에서 산화스트레스의 증가가 RIPK3-medicatied necroptosis의 활성화에 기여할 수 있음을 시사한다.

핵심되는 말: 산화스트레스, necroptosis, 급성호흡곤란증후군, 급성 폐손상