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The involvement of NLRX1 in pulmonary hyperoxic acute injury

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The involvement of NLRX1 in pulmonary hyperoxic acute injury

Directed by Professor Myung Hyun Sohn

The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Master of Medical Science

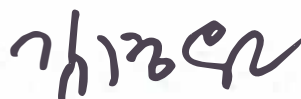
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December 2022

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2022년 12월 25일

김혜린 드림

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ABSTRACT

The involvement of NLRX1 in pulmonary hyperoxic acute injury

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(Directed by Professor Myung Hyun Sohn)

Hyperoxia is frequently used for treating acute respiratory failure, but it can cause acute lung injury. Nucleotide-binding domain and leucine-rich-repeat-containing family member X1 (NLRX1) is localized in mitochondria and related to reactive oxygen species production, inflammation, and apoptosis, which are the features of hyperoxic acute lung injury (HALI). However, the contribution of NLRX1 in HALI has not been addressed, so we examined to demonstrate the role of NLRX1 in hyperoxia. A murine model of HALI was generated in wild-type mice (WT) and NLRX1^{-/-} mice by exposing them to over 95% oxygen for 72 h. As a result, NLRX1 expression was elevated in mice exposed to hyperoxia. In acute lung injury, levels of inflammatory cells, protein leakage, cell cytotoxicity, and pro-inflammatory cytokines were diminished in NLRX1^{-/-} mice compared to WT mice. In survival test, NLRX1^{-/-} mice showed alleviated mortality under hyperoxic conditions, and apoptotic cell death and caspase expression and

activity were reduced in NLRX1^{-/-} mice. Furthermore, levels of MAPK signaling proteins ERK 1/2, JNK, and p38 were decreased in NLRX1-deficient mice than in WT mice exposed to hyperoxia. This study reveals that the genetic deficiency of NLRX1 dampens hyperoxia-induced apoptosis, suggesting NLRX1 acts as a pivotal regulator of HALI.

Key words: acute lung injury, apoptosis, hyperoxia, MAPK signaling pathway, NLRX1

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I. INTRODUCTION

Oxygen is essential for human survival, and it is sometimes used as adjuvant therapy for patients with respiratory failure and to increase oxygen delivery to peripheral tissues in patients with severe lung or heart disease¹. However, it has been demonstrated that prolonged exposure to a high concentration of oxygen increased DNA fragmentation and levels of reactive oxygen species (ROS), which induce apoptosis in pulmonary tissues. Excessive accumulation of these free radicals leads to acute and chronic lung injury².

Hyperoxia is defined as a supraphysiological concentration and pressure of oxygen in cells, tissues, or organs. When high concentrations of oxygen pass through the lungs, oxygen gradually replaces nitrogen as the primary gas in alveoli and puts the lungs in an absorption atelectasis state, which is a loss of lung volume due to the resorption of oxygen that does not occur with nitrogen. The

rise in partial pressure of oxygen in blood results in increased binding energy between oxygen and hemoglobin, preventing carbon dioxide from binding to hemoglobin. Finally, elevated blood carbon dioxide levels lower blood pH and decrease overall lung function³. Hyperoxia increases oxidant production via the mitochondrial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway, and prolonged exposure causes mitochondria dysfunction^{4,5}. Hyperoxic acute lung injury (HALI) is characterized by damage and death of endothelial and epithelial cells, which results in leakage of alveolar capillary proteins^{1,6}. Continuous exposure to hyperoxia also contributes to the pathogenesis of various lung diseases, including chronic obstructive pulmonary disease (COPD), asthma, idiopathic pulmonary fibrosis (IPF), and acute respiratory distress syndrome⁷.

The protein nucleotide-binding oligomerization domain and leucine-rich-repeat-containing family member X1 (NLRX1) is a mitochondrial targeting sequence, which is ubiquitously expressed and localized in mitochondria^{8,9}. Mitochondria are a major source of ROS, which can induce oxidative stress, mediate damage, and initiate inflammatory responses in the lung^{10,11}. However, the lungs are equipped with an antioxidant defense system to minimize infection and preserve the lung functions¹². Recent research evidence demonstrate that NLRX1 is involved in a variety of biological functions, including modulation of mitochondrial function, ROS generation, autophagy and apoptosis^{8,13-16}. These studies determined that NLRX1 acts as a mitochondrial controller of apoptotic cell death during ischemia-reperfusion injury and acute cellular injuries^{13,17}. In addition, other studies showed that the NLRX1 is involved in numerous diseases, including COPD, cancer, deafness and tumorigenesis^{16,18,19}. However, studies on NLRX1 signaling during hyperoxia have not yet defined its role.

Mitogen-activated protein kinase (MAPK; ERK 1/2, JNK, and p38) signaling pathways are known to mediate key cellular processes, including the regulation of cell survival and death. The MAPK pathways may act as either activators or inhibitors, depending on the cell type and the stimuli^{20,21}. Studies on various

pulmonary diseases, including respiratory failure, fibrosis, neutrophilic inflammatory disease, and hyperoxia reported that MAPK pathways were activated in response to increased apoptosis^{6,22-26}. In addition, it has been suggested that NLRX1 modulates MAPK pathways in immune responses to a variety of stimuli, including virus or fungal infection and tumorigenesis²⁷⁻³⁰.

In the present study, we aimed to identify the role and mechanisms of NLRX1 in hyperoxia induced acute lung injury. We hypothesized that deletion of NLRX1 would have effects on mouse survival and lung damage in hyperoxic condition. To test the hypothesis, we identified the basic factors of acute lung injury, from inflammation to mortality and apoptosis, and finally determined signaling pathway which are involved in NLRX1 deficiency under hyperoxic conditions.

II. MATERIALS AND METHODS

1. Mice

Mice (strain: C57BL/6; 6-8 weeks old; male) were purchased from Orient Bio Inc. (Seongnam, South Korea). NLRX1 knock-out (NLRX1^{-/-}) mice were kindly provided by Dr. J. P. Ting (University of North Carolina). All animals used were sex-and age-matched, of the same genetic background, and housed under a 12 h dark-light cycle under specific pathogen-free (SPF) conditions. Animals had free access to water and food during the research. Animal experiments were approved by the Institutional Animal Care, Use Committee (IACUC) of the affiliated university (protocol No. 2021-0178; Seoul, Korea), and the study was conducted in compliance with the ARRIVE guidelines.

2. Oxygen Exposure

WT (NLRX1^{+/+}) and NLRX1 knock-out (NLRX1^{-/-}) mice were exposed to >95% oxygen (Hyperoxia, HO) using cages enclosed in an air tight Plexiglas chamber (57x42x37 cm, JEUNG DO BIO & PLANT Co., Ltd., Seoul, Korea). The pressure inside the chamber was normalized to atmospheric pressure. Oxygen levels were constantly monitored for the duration of the experiment (72h) using an oxygen analyzer (MaxO₂⁺A, MAXTEC, Salt Lake City, UT, USA.). As controls, sex- and age-matched WT and NLRX1^{-/-} mice were housed in similar conditions under normoxia (room air, RA). All methods were performed in accordance with the relevant guidelines and regulations.

3. Bronchoalveolar Lavage (BAL) fluid

During sacrificing, mice underwent blunt dissection of the trachea following anesthesia. A small-caliber tube was inserted into the airway; 0.9ml of phosphate-buffered saline (PBS) was injected into the lungs, was collected, and this

collection process was repeated once more so that a total of 1.8ml BAL was collected from each mouse. The collected BAL fluid was centrifuged at 3,000rpm for 5 min at 4°C, and then separated into cell pellet and supernatant. The cell pellet was dissolved with PBS, mixed in a 1:1 ratio with Trypan Blue Solution, and the cells were counted using a hemocytometer. BAL cells were centrifuged onto slides using a Cytospin centrifuge (Thermo Fisher scientifics, Waltham, MA). Cell differentiation was assessed using a Diff-Quik stain kit (Merck, Darmstadt, Germany). The supernatant was stored at -80°C and used for various protein analyses and assessment of LDH activity.

4. Histology and Immunohistochemistry (IHC)

The left lobes of sacrificed mice lung were fixed in 4% formalin for 3 days and embedded in paraffin. Lung tissues were cut into 5- μ m sections and stained using hematoxylin and eosin (H&E) to analyze airway inflammation. To assess immunohistochemistry, sections were deparaffinized twice by washing with xylene for 10 min and rehydrated in 100% (5 min, twice), 95% (5 min), and 70% (5 min) alcohol. The tissues were then heated with retrieval buffer (DAKO, A/S, Glostrup, Denmark) for 20 min in a steamer and allowed cool to room temperature (18-22°C) for 20 min. After washing the sections, they were placed in peroxidase blocking solution (DAKO) for 5 min, and then in protein block solution (DAKO) for 1 hr. The NLRX1 antibody (Proteintech, Rosemont, IL) or normal rabbit IgG (Santa cruz Biotechnology, Inc, Dallas, TX) were prepared as 1:500 dilutions and loaded into sections, followed by incubation at 4°C overnight. The color was developed with DAB solution (DAKO), and the reaction was stopped using deionized water; finally, the immunostained tissue sections were mounted on slides with an aqueous-base mounting medium.

5. Bicinchoninic acid (BCA) assay

Protein leakage in BAL was evaluated using a PierceTM BCA Protein Assay Kit (Thermo Fisher scientifics, Waltham, MA). The BAL fluid supernatant and standard were dispensed into a 96-well plate for 25 μ l aliquots, and 200 μ l working reagent was dispensed in each well. The plate was placed in an incubator at 37°C and allowed to react for 30 min. The protein concentration in BAL fluid was measured at absorbance at 562 nm using a microplate reader (Molecular Devices, CA, USA).

6. Lactate dehydrogenase (LDH) assay

LDH concentration in BAL fluid was measured using a cytotoxicity detection kit (Roche Applied Science, Mannheim, Germany). We dispensed 100 μ l of BAL fluid sample and 100 μ l of the reagent mixed with the catalyst and dye solution in a 96-well plate, and this was allowed react at room temperature for 30 min. After the reaction was completed, the reaction was stopped by adding 50 μ l of stop solution, and LDH concentration was measured at an absorbance of 492/690 nm using a microplate reader.

7. Real-time PCR

After mice were sacrificed, lung tissues were homogenized with T10 Basic Ultra-Turrax[®] homogenizer (IKA Labortechnik, Staufen, Germany) and lysed in Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The relative mRNA expression levels were measured by reverse transcription and real-time PCR; 1 μ g of total RNA was synthesized to cDNA by reverse-transcribed with ReverTra Ace q PCR RT Master Mix Kit (Toyobo Co., Ltd., Osaka, Japan). Real-time PCR was performed in 20 μ l reactions containing 10 μ l Power SYBR Green[®] PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 1 μ l cDNA template, 1 μ l forward primer, 1 μ l reverse primer, and

deionized water to the desired volume. Quantitative PCR was performed with the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Waltham, MA) according to the manufacturer's protocol. Primer pairs for real-time PCR were manufactured by MBItech (Hanam, South Korea). The levels of mRNA were normalized to IPO8. Fold changes were calculated using the $2^{-\Delta\Delta CT}$ method.

8. ELISA

The levels of interleukin-6 (IL-6) and C-C motif chemokine ligand 2 (CCL2) were quantified in BAL fluid by ELISA (R&D systems, Minneapolis, MN, USA) as per manufacturer's instructions; 96-well plates were each coated with anti-mouse cytokine antibodies overnight at room temperature (18-22°C). The wells were blocked with PBS containing 1% BSA for 1 h. Dilution standards and samples were then incubated for 2 h. Bound cytokines were detected using incubation with anti-mouse-cytokine antibodies for 2 h. Samples were then washed with streptavidin-horseradish peroxidase for 20 min and TMB substrate solution (KPL) for a further 20 min. The reaction was stopped using 2 N sulfuric acid, and the colorimetric reactions were read using a microplate reader at 450 nm.

9. Western blot

Lung tissues were gently homogenized and lysed in RIPA buffer (Thermo fisher scientific, Waltham, MA, USA). The protein concentrations were measured using the Bradford assay. Equal amounts of protein samples were loaded on gel and separated by 8%~12% SDS-PAGE electrophoresis and were then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Membranes were blocked in Tris buffered saline containing 0.1% Tween 20 (TBST) with 5% skim milk and incubated overnight at 4°C with primary antibodies. After washing with TBST, the membranes were incubated at room

temperature with secondary antibodies for 1 h. The protein signal was analyzed using Image J software. Protein samples were normalized to GAPDH. The primary antibodies were used as follows: NLRX1 (Proteintech), and BAX, Cyto C, P-ERK 1/2, T-ERK 1/2, P-JNK, T-JNK, P-p38, T-p38, and GAPDH (all from Cell Signaling Technology).

10. TUNEL assay

To analyze apoptosis, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was carried out using an in-situ Cell Death Detection Kit (AP) (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. TUNEL assay detected the apoptotic cells in lung tissue samples. Pictures were taken a light microscope at 400× magnification in five random fields for each section and the rate of TUNEL-positive cells was calculated.

11. Caspase activity

Total protein was extracted from the lungs using RIPA lysis buffer solution and homogenizer according to the manufacturer's protocol. In a 96-well plate, 25 µl of Caspase-Glo® 3/7 Reagent (Promega, Madison, WI) and 25 µg of 1 mg/ml protein are added in a 1:1 ratio and incubation was performed for 30 min and luminescence was recorded at 30 minute intervals from 30 minutes to 3 hours. Caspase-Glo® 8 Assay and Caspase-Glo® 9 Assay protocols were conducted using the same methods as Caspase-Glo® 3/7 Assay.

12. Statistics

For the animal studies, values were expressed as means ± SEM. Most results were evaluated using Student's *t*-test. Cell count results were compared using two-way ANOVA, and survival analysis was corrected for multiple comparisons

by the log-rank test. All evaluations were performed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). In all analyses, $p < 0.05$ was considered statistically significant.

III. RESULTS

1. NLRX1 expression increases in mouse lung under hyperoxia conditions

To demonstrate whether NLRX1 is modulated by hyperoxia ($\geq 95\%$ oxygen), we established an experimental murine model of hyperoxia and analyzed the expression levels of NLRX1. Wild-type mice were exposed to either hyperoxic air or room air (RA) as a control for 72 h. NLRX1 mRNA and protein expression in hyperoxia-treated mice were higher than those in the control mice (Fig. 1A-1C). In addition, NLRX1 immunohistochemistry staining in lung tissues demonstrated that NLRX1 level is elevated after hyperoxia exposure (Fig. 1D and 1E).

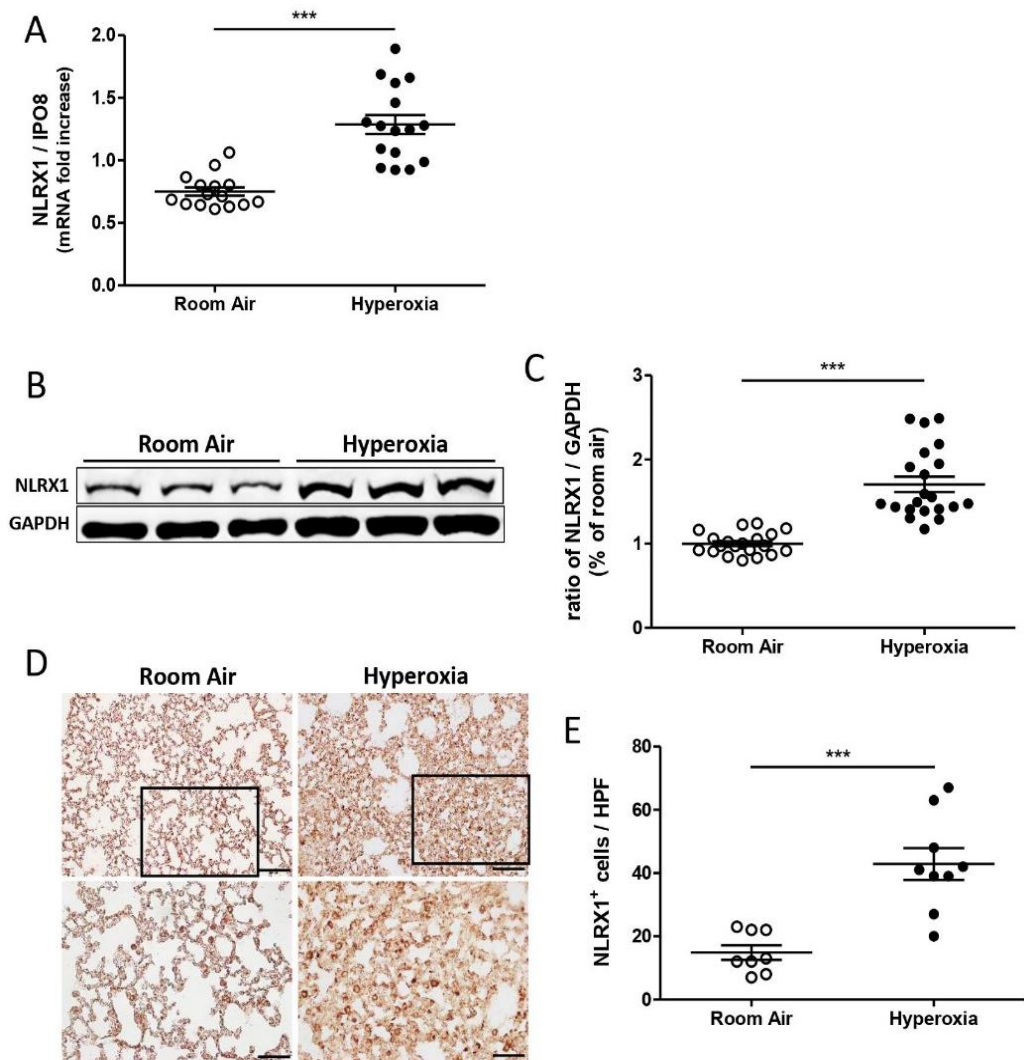


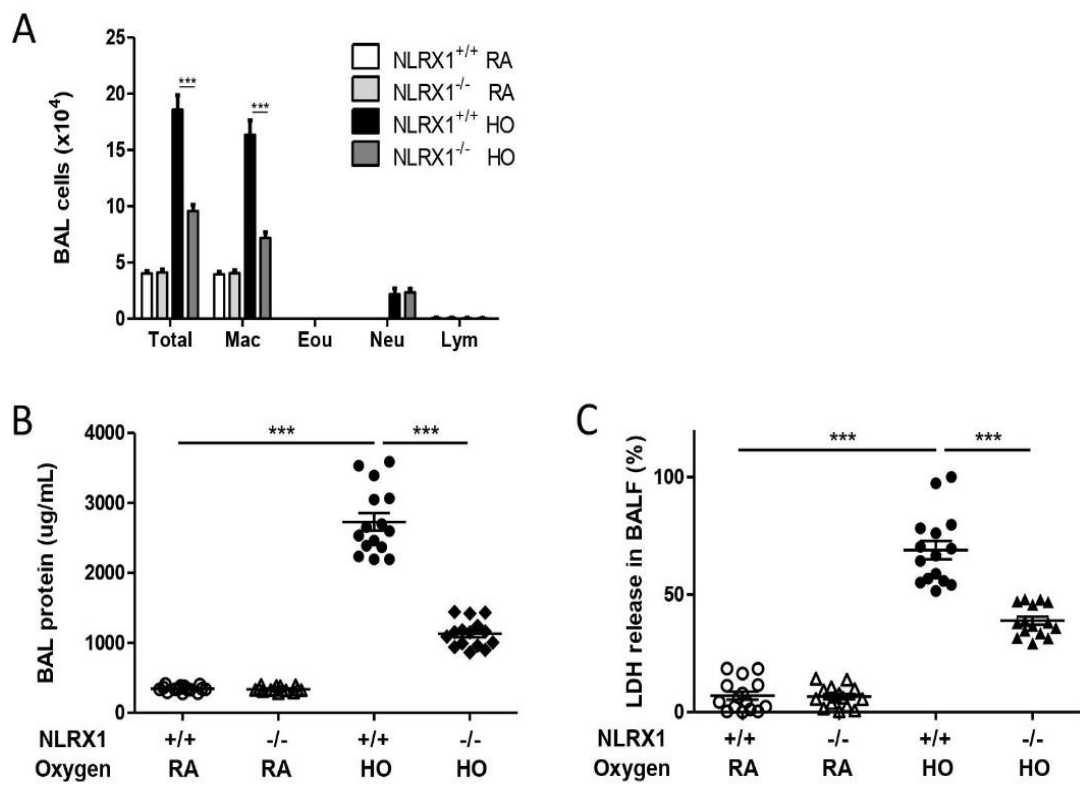
Fig. 1. NLRX1 expression levels are increased in a murine model of hyperoxia. Wild-type mice were exposed to room air or hyperoxia ($\geq 95\%$ oxygen) respectively for 72 hours. After 72 h, mice were sacrificed and collected lung tissues were analyzed. (A) The levels of NLRX1 mRNA were measured by real-time PCR. (B) NLRX1 protein expression was evaluated via western blot using lung lysates, and (C) the signal intensity of NLRX1 was quantified. (D) Immunohistochemical staining of NLRX1 was assessed in mouse lung tissue, and (E) NLRX1 positive cells were counted. Scale bars, 100 μm and 50 μm . Results are presented as the mean \pm SEM and are representative of at least three independent experiments ($n = 8-21$ mice per group). *** $p < 0.001$ (Student's t test). HPF; High power field

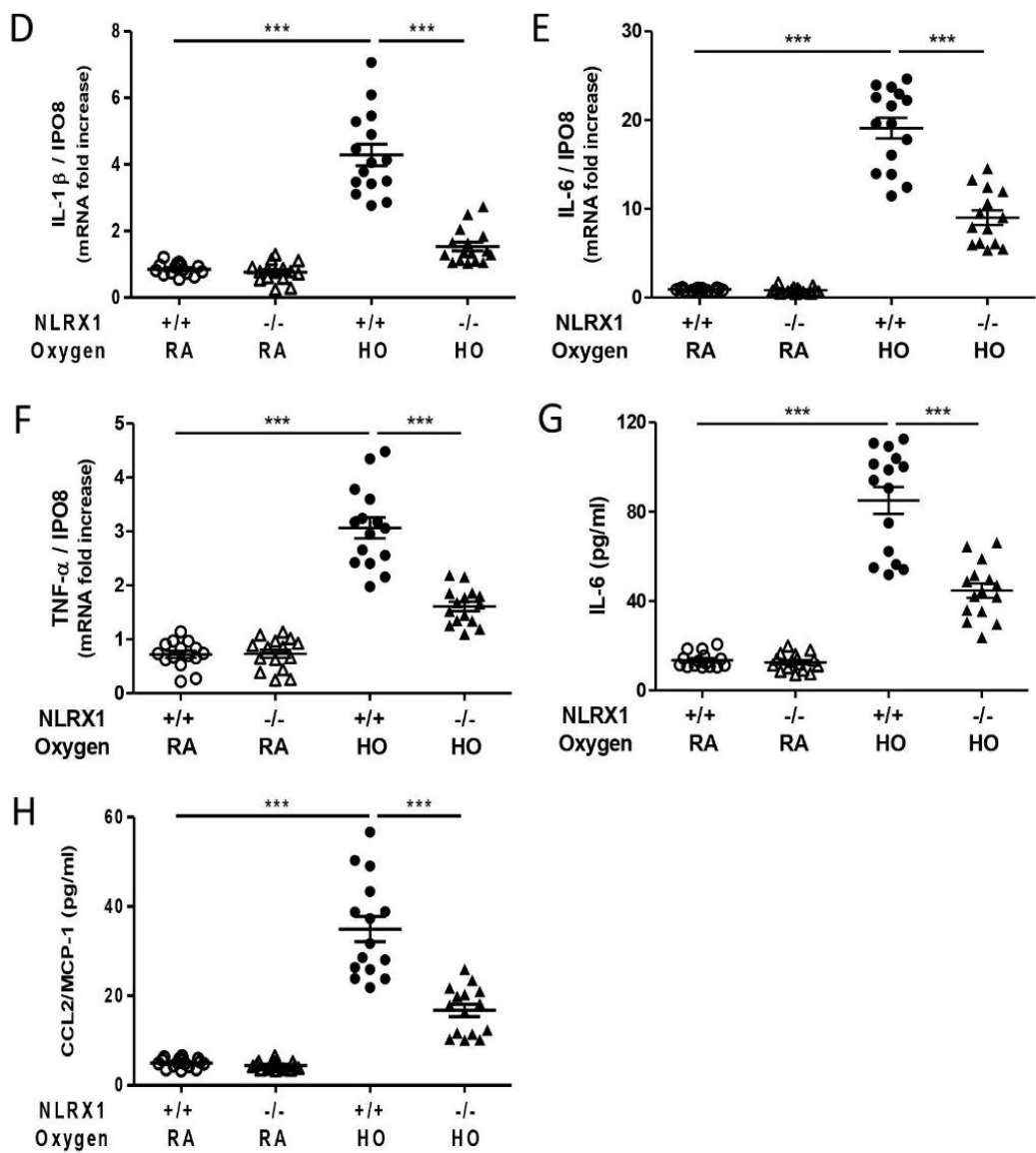
2. NLRX1 deficiency attenuates the hyperoxia-induced lung injury

Since hyperoxia increased the NLRX1 levels, we compared the severity of acute lung injury using WT mice and NLRX1^{-/-} mice. The number of inflammatory cells from bronchoalveolar lavage (BAL) fluid, total protein concentrations, and cell cytotoxicity were augmented by hyperoxia in WT mice. NLRX1^{-/-} mice showed reduced inflammatory responses compared to WT mice in response to hyperoxia exposure (Fig. 2A-2C).

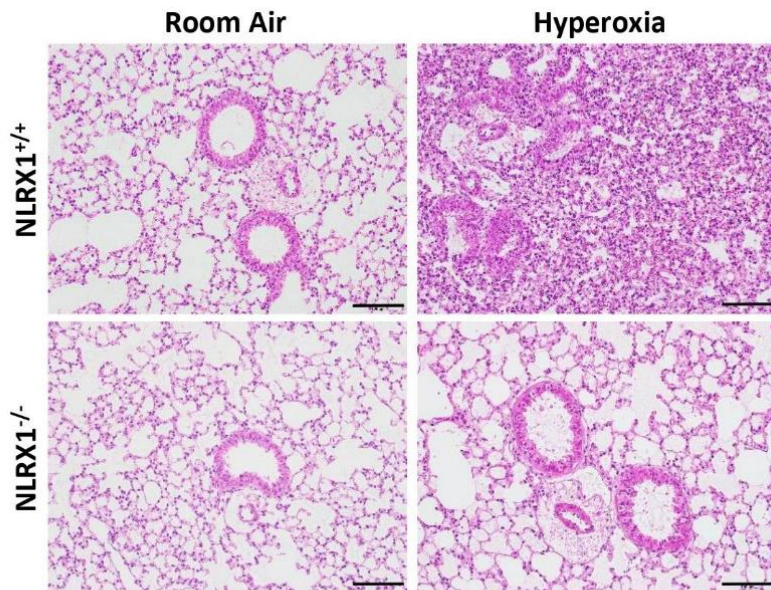
To determine whether NLRX1 contributes to hyperoxia-induced proinflammatory cytokine expressions, we examined the mRNA and protein levels of proinflammatory cytokines, such as IL-1 β , IL-6, TNF- α , and CCL2, using real-time PCR and ELISA. Increased mRNA and protein expression in lung tissues and BAL fluid were detected in hyperoxia treated mice, but NLRX1^{-/-} mice showed alleviated inflammatory responses compared to WT mice (Fig. 2D-2H). Hematoxylin and eosin staining of lung tissues revealed dampened inflammation in the lungs of NLRX1^{-/-} mice compared to that in the lungs of WT mice (Fig. 2I).

To evaluate the incidence of mortality, the end point of acute lung injury and survival was measured in WT and NLRX1^{-/-} mice under prolonged hyperoxia exposure. NLRX1^{-/-} mice had significantly longer survival than WT mice (median survival; LD₅₀ was 106h and 92h in NLRX1^{-/-} and WT mice, respectively) (Fig. 2J). Thus, these data confirmed that hyperoxia-induced acute lung injuries were significantly diminished in NLRX1^{-/-} mice.





I



J

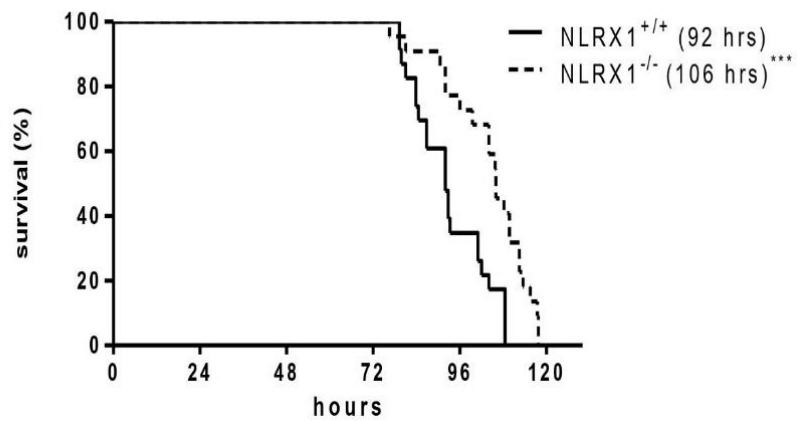
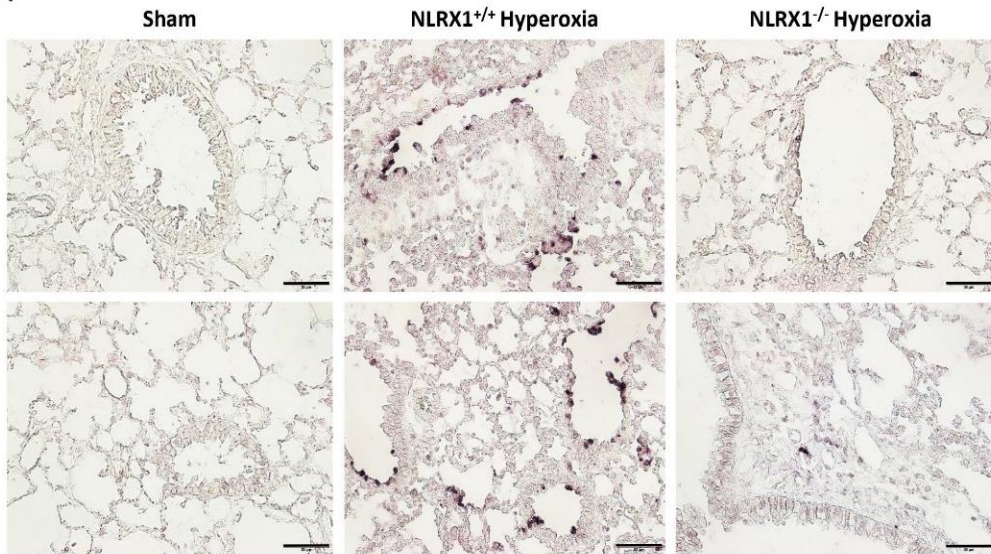


Fig. 2. NLRX1 deficiency dampens the inflammation and mortality induced by hyperoxia. Wild-type mice (NLRX1^{+/+}) and NLRX1 knock-out mice (NLRX1^{-/-}) were exposed to both room air (RA) and hyperoxia (HO, ≥95% oxygen) for 72 h. (A) Inflammatory cells from bronchoalveolar lavage (BAL) fluid were counted. (B) Total protein concentration of BAL fluid was quantified by BCA assay. (C) Cell cytotoxicity in BAL fluid was assessed using LDH assay. (D-F) mRNA levels of inflammatory cytokines IL-1 β , IL-6, and TNF- α were examined using real-time PCR in lung tissues. (G, H) CCL2 and IL-6 levels were analyzed by ELISA in BAL fluid. Results are presented the mean \pm SEM and are representative of at least three independent experiments (n = 15-18 mice per group). (I) Lung tissues were histologically analyzed for lung injury and inflammation by optical microscope after staining with hematoxylin and eosin (H&E). Scale bars, 100 μ m. (J) Mortality rates in WT and NLRX1^{-/-} mice were measured by survival test (NLRX1^{+/+}; n = 23, NLRX1^{-/-}; n = 22) and corrected for multiple comparisons by the log-rank test. The median survival (LD₅₀) is in parentheses. Data represent at least three separate experiments. ****p* < 0.001 (two-way ANOVA and Student's *t* test).

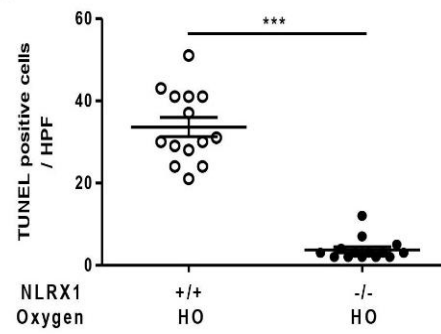
3. NLRX1 knockout reduces hyperoxia-induced apoptosis

Following our observation of the difference in mortality rates based on NLRX1 knockout and evidence from previous studies that showed a direct association between hyperoxia and apoptosis, we investigated whether NLRX1 modulates hyperoxic apoptosis using TUNEL assay and found that the number of apoptotic cells was dramatically reduced in lung tissues of NLRX1^{-/-} mice than in those of WT mice (Fig. 3A and 3B). To ascertain the impact of NLRX1^{-/-} on apoptotic signaling, proapoptotic members of the B cell lymphoma (Bcl)-2 family, *Bax* and *Bak*, were examined by real-time PCR (Fig. 3C and 3D). Activation of *Bax* and release of cytochrome *c*, which are essential for initiating the apoptotic cascade, were also confirmed by western blot (Fig. 3E-3G). These results suggest that signaling related to apoptosis declined due to the loss of NLRX1 (Fig. 3C-3G).

A



B



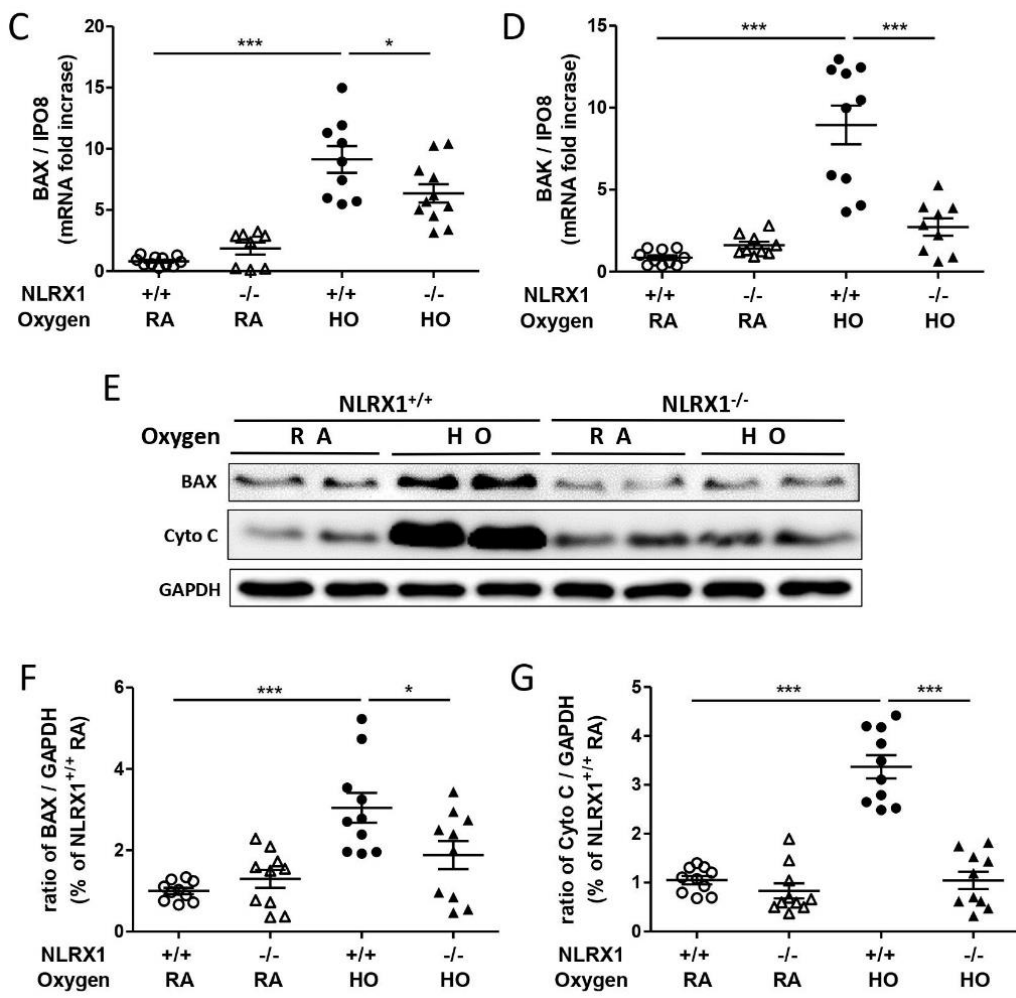


Fig. 3. Absence of NLRX1 decreases the number of apoptotic cells and signaling molecules under hyperoxic conditions. (A) NLRX1^{+/+} room air (sham), NLRX1^{+/+} hyperoxia, and NLRX1^{-/-} hyperoxia mice lung sections were stained using TUNEL assay and (B) TUNEL-positive cells were counted. Scale bars, 50 μ m. (C, D) mRNA levels of *Bax* and *Bak* were measured by real-time PCR. (E) Protein levels of BAX and Cytochrome C (Cyto C) were analyzed using western blot of lung lysates, and (F-G) the ratio of molecules was calculated. Results are presented as the mean \pm SEM and are representative of at least three independent experiments (n = 8-14 mice per group). * p < 0.05, *** p < 0.001 (Student's t test).

To further elucidate the role of NLRX1 in apoptosis, the caspase cascade was assessed using real-time PCR and caspase activity assay. We selected the representative and pivotal components of the apoptosis pathway, initiator caspase-8 and -9, effector caspase-3 and -7. In our hyperoxic model, caspase expression and activity were significantly augmented in WT mice. However, hyperoxia-treated NLRX1^{-/-} mice showed reduced expression and activity of these caspase proteins tested as compared to hyperoxia-treated WT mice (Fig. 4A-4F). Thus, NLRX1 deficiency decreased apoptosis as well as inflammation and mortality.

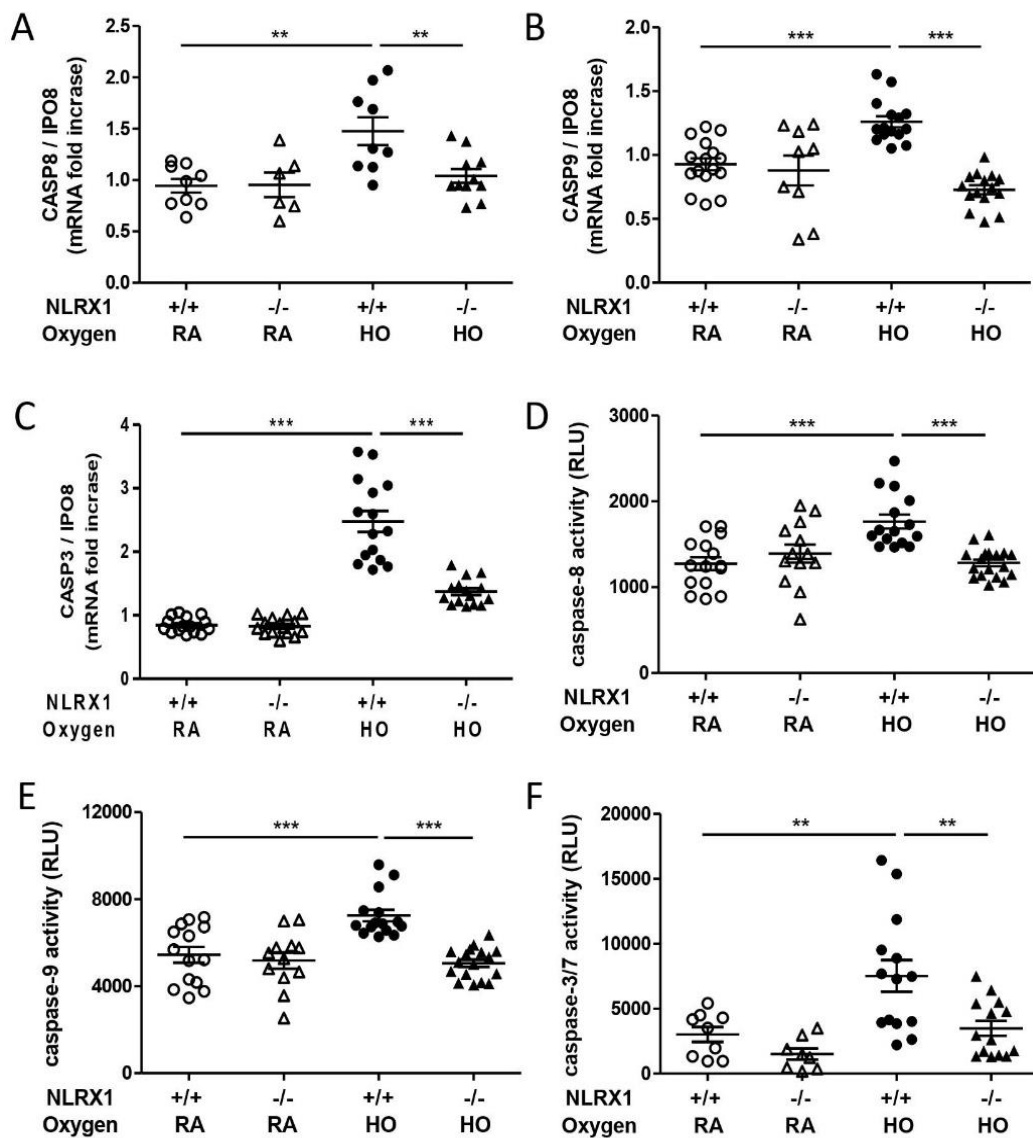
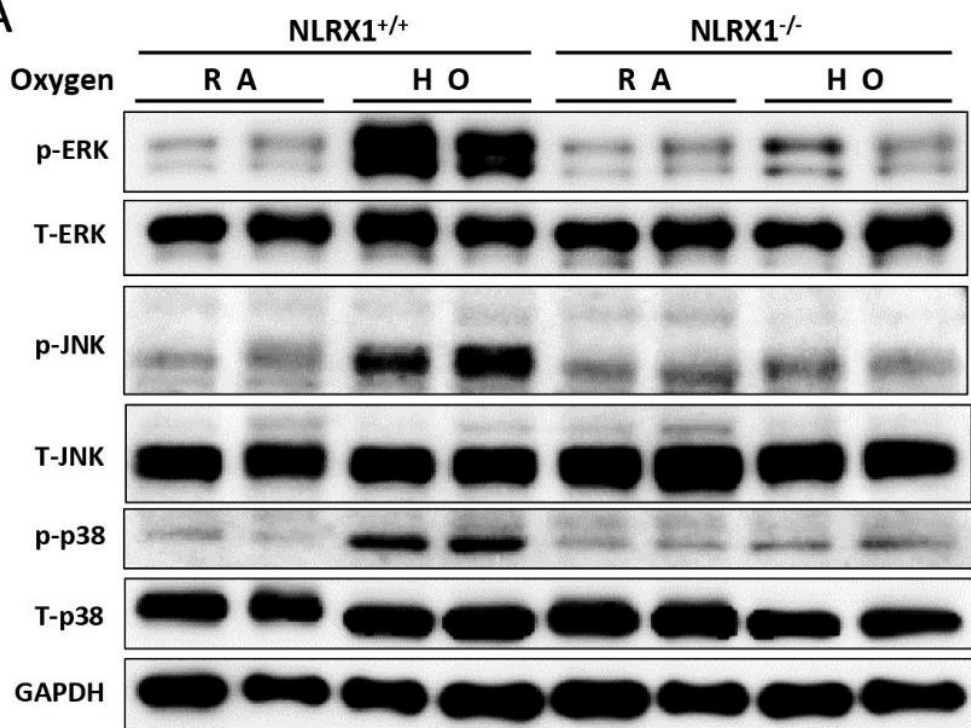


Fig. 4. NLRX1 deficiency reduces the expression of caspase and caspase activity in hyperoxic acute lung injury. (A-C) The mRNA levels of Caspase (CASP) -8, -9, and -3 were evaluated by real-time PCR. (D-F) Caspase -8, -9, and -3/7 activities were analyzed by luminescence using protein from lung lysates. Results are presented as the mean \pm SEM and are representative of at least three independent experiments (n = 6-18 mice per group). ** p < 0.01, *** p < 0.001 (Student's t test).

4. NLRX1 deficiency suppresses the MAPK signaling pathways under hyperoxic conditions

Phosphorylation of MAPK signaling pathways is known to be modulated by hyperoxia. NLRX1 is also known to regulate the MAPK signaling pathway. Thus, we investigated whether MAPK pathways were involved in NLRX1 function in hyperoxic lung injury. We evaluated the phosphorylation levels of ERK 1/2, JNK, and p38 in lung lysates through western blotting. As expected, hyperoxia treatment increased the phosphorylation levels of the MAPK pathways in wild-type mice. However, NLRX1 deficiency reduced the increase in MAPK pathway phosphorylation in response to hyperoxia (Fig. 5A-5D). These data support that the apoptotic role of NLRX1 mediates MAPK signaling during hyperoxia.

A



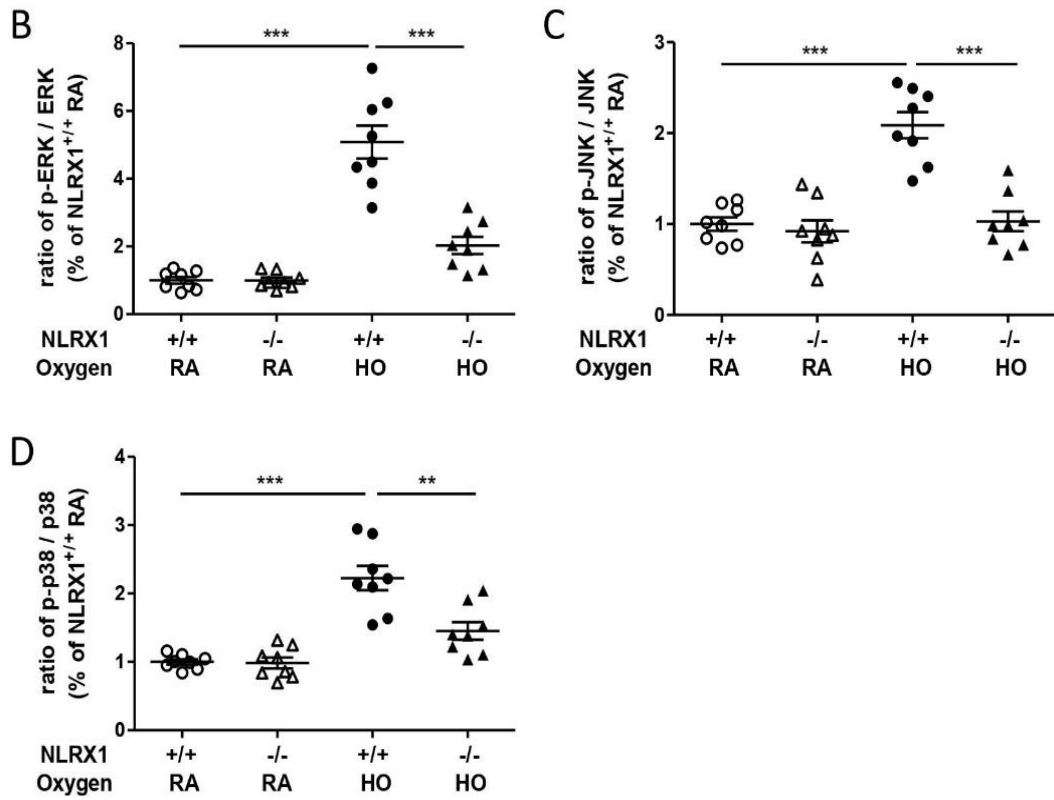


Fig. 5. NLRX1 regulates MAPK signaling pathways in hyperoxia. (A) Phosphorylation of MAPK pathways (ERK 1/2, JNK, and p38) was measured by western blot analysis using lung lysates, and (B-D) the ratios of molecule signal intensity were quantified. Results are presented as the mean \pm SEM and are representative of four separate experiments (n = 8 mice per group). ** $p < 0.01$, *** $p < 0.001$ (Student's t test).

IV. DISCUSSION

In this study, we found that NLRX1 knockout alleviates HALI, particularly via inhibiting apoptosis induction. Recently, an accumulation of data on NLRX1 suggests that it has a protective role in various diseases such as cancer, COPD, inflammatory bowel disease, and ischemia-reperfusion injury²⁹. A recent review has confirmed that NLRX1 inhibits the MAVS/RLH pathway, modulates the innate immune responses and the CD4⁺ T cells through immunometabolism²⁹. On the contrary, other studies, including our experimental hyperoxic model, found that the absence of NLRX1 also had protective effects. In one study, deficient of NLRX1 was advantageous in host defense against vesicular stomatitis virus through increased IFN-I production and reduced autophagy¹⁴. NLRX1 silencing has been shown to decrease intracellular ROS generation and apoptosis involving JNK signaling in HEI-OC1 cells induced by cisplatin⁸. NLRX1 deficiency has been shown to elevate fatty acid metabolism to prevent diet-induced hepatic steatosis³¹. In cochlear hair cells, NLRX1 was essential for hair cells maturity and hearing but was also associated with age-related increase in apoptosis through the JNK pathway¹⁹.

In previous studies, NLRX1 was associated with various pulmonary diseases, such as influenza A virus infections, invasive pulmonary aspergillosis, and COPD and aging^{29,32,33}. However, there have been no investigations of NLRX1 and HALI in a murine model. NLRX1 is known to modulate ROS production, inflammatory leukocyte infiltration response, cell death, and MAPK pathways, which are implicated in the pathogenesis of HALI^{30,34}. Further, we confirmed that increased NLRX1 expression was significantly associated with HALI.

Hyperoxia is known to impair the alveoli and lung structures, interfere with lung development, and cause fibrosis. These properties increase the permeability of the lungs and exacerbate alveolar protein leakage³⁵. This was evident in our data where total protein concentrations analyzed using BCA assay were elevated in

BAL fluid from mice exposed to hyperoxia. In addition, hyperoxia directly increased cell cytotoxicity, thereby increasing lactate dehydrogenase (LDH) release during cell death. These damages cause inflammatory responses, leading to increasing inflammatory cell (macrophage, neutrophil, lymphocyte) infiltration and pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α and CCL2) production. Despite the prolonged exposure to a high concentration of oxygen, NLRX1-deficient mice showed alleviated inflammatory responses and pathological changes. Thus, NLRX1 may regulate hyperoxia-induced lung injury.

Cell death, specifically apoptosis and necrosis, has been demonstrated to be involved in hyperoxia-mediated acute lung injury (ALI) pathogenesis³⁶. In recent studies, NLRX1 has been shown to play a role in apoptosis through various pathways in different cell systems¹⁶. The mitochondrial apoptosis is known to be regulated by anti- and pro-apoptotic members of the Bcl-2 family³⁷. In particular, *Bax* and caspase-3 have a major role in the mitochondria apoptosis pathway³⁸. Similarly, in our study, we showed that levels of elements across the apoptosis pathways, from the Bcl-2 family to caspase cascade proteins, were increased in response to hyperoxia and reduced by NLRX1 deficiency. These data confirmed that NLRX1 acts as a pivotal regulator in hyperoxia-induced lung injury through the mitochondrial apoptotic pathway. Mortality is an important factor in hyperoxia as it is the end point of acute lung injury clinically. In previous research, *Bax* and *Bak* deficient mice had significantly less lung injury and increased long-term survival under hyperoxic conditions³⁹. In the present study, NLRX1-deficient mice showed dramatically reduced *Bax* and *Bak* expression and improved survival rate ($p = 0.0007$) as compared to WT mice. These results suggest that NLRX1 promotes alveolar epithelial cell apoptosis and induces lung tissue damage against oxygen toxicity and lethality via pathological apoptosis, suggesting that NLRX1 may have a critical role in the pathogenesis of hyperoxia.

The mitogen-activated protein kinase (MAPK) signaling pathways participate in a variety of biological processes, and regulate pro- and anti-apoptotic

mechanisms²¹. The key members of MAPK pathways are ERK 1/2, p38, and JNK, which modulate cell growth, proliferation, stress responses, and survival in hyperoxia²⁰. In addition, NLRX1 is known to modulate MAPK signaling^{27,40}. In an LC3-associated phagocytosis (LAP) model induced by *Histoplasma capsulatum*, NLRX1 deficiency attenuated MAPK signaling pathway²⁸. In our model, we also observed that phosphorylation levels of ERK 1/2, p38, and JNK were reduced in NLRX1^{-/-} mice than in WT mice. These results mean that NLRX1 plays a pivotal role in apoptosis under hyperoxic conditions by via the MAPK signaling pathway.

In conclusion, our current study revealed that NLRX1 deficiency relieves pulmonary hyperoxic acute injuries, such as inflammation and apoptosis, and ultimately mortality, through the MAPK pathway. These investigations suggest that NLRX1 might be a valuable therapeutic target for HALI treatment.

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ABSTRACT (IN KOREAN)

고농도 산소에 의해 유도된 폐 손상 병인 기전에서 NLRX1의 역할

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김 혜 린

고농도의 산소 투여는 흔히 급성 호흡 관련을 치료하기 위해 사용되어 왔으나, 이러한 치료법은 급성 폐 손상을 유발할 수 있다. Nucleotide-binding domain and leucine-rich-repeat-containing family member X1 (NLRX1)은 미토콘드리아에 위치하고 있으며, 활성산소종의 발생과, 염증, 세포사멸 등의 고농도 산소에 의해 유도된 폐 손상 특성들과 관련되어 있다. 그러나, 고농도 산소로 인한 폐 손상에서의 NLRX1의 규제와 기여에 대해서는 아직 밝혀진 바가 없다. 그러므로, 본 연구는 고농도의 산소에서의 NLRX1의 역할을 규명하고자 한다. 고농도 산소로 인해 유도된 급성 폐 손상 마우스 모델은 야생형 마우스 (WT)와 NLRX1 유

전자가 결핍된 마우스 (NLRX1^{-/-})에게 각각 95% 이상의 산소를 72시간 동안 투여하여서 제작하였다. 그 결과, 고농도의 산소에 노출되었던 마우스에서 NLRX1의 발현이 증가하였고, WT 마우스에 비해 NLRX1^{-/-} 마우스에서 염증 세포의 발생과 단백질 누출, 세포 독성, 염증 유발 사이토카인의 생성 등의 급성 폐 손상이 감소하였다. 생존 능력 시험에서도 NLRX1^{-/-} 마우스는 지속적인 고농도의 산소 투여 환경에서 더 높은 생존률을 나타냈으며, 세포 사멸과 카스파제 발현 및 활성화 또한 감소하였다. 더 나아가, MAPK 신호 전달 단백질인 ERK 1/2, JNK, 그리고 p38도 고농도의 산소에 노출된 WT 마우스보다 고농도의 산소에 노출된 NLRX1^{-/-} 마우스에서 발현이 감소하였다. 이번 연구를 통해 NLRX1의 유전적 결핍은 MAPK 신호전달 과정을 통해 고농도 산소로 인한 세포 사멸을 완화시킴을 밝혔다. 이는 고농도 산소로 인한 폐 손상에서 NLRX1이 중요한 조절 물질로 작용할 것임을 시사하고 있다.

핵심되는 말: 급성 폐 손상, 세포자연사, 고농도 산소, MAPK
신호 전달경로, NLRX1