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Research article

Nicotinamide adenine dinucleotide phosphate oxidase 2 deletion attenuates polyhexamethylene guanidine-induced lung injury in mice

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ABSTRACT

Inhalation of polyhexamethylene guanidine phosphate (PHMG) can cause pulmonary fibrosis. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox) are enzymes that produce reactive oxygen species, which may be involved in tissue damage in various lung diseases. To investigate whether the Nox2 isoform of Nox is involved in the progression of PHMG-induced lung damage, we studied the contribution of Nox2 in PHMG-induced lung injury in Nox2-deficient mice. We treated wild-type (WT) and Nox2 knockout mice with a single intratracheal instillation of 1.1 mg/kg PHMG and sacrificed them after 14 days. We analyzed lung histopathology and the number of total and differential cells in the bronchoalveolar lavage fluid. In addition, the expressions of cytokines, chemokines, and profibrogenic genes were analyzed in the lung tissues. Based on our results, Nox2-deficient mice showed less PHMG-induced pulmonary damage than WT mice, as indicated by parameters such as body weight, lung weight, total cell count, cytokine and chemokine levels, fibrogenic mediator expression, and histopathological findings. These findings suggest that Nox2 may have the potential to contribute to PHMG-induced lung injury and serves as an essential signaling molecule in the development of PHMG-induced pulmonary fibrosis by regulating the expression of profibrogenic genes.

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1. Introduction

Polyhexamethylene guanidine (PHMG) is a guanidine polymer commonly used in shampoos, wet wipes, and biocides in medicine, agriculture, and food industries owing to its broad-spectrum bactericidal activity and low toxicity in humans [1–3]. In addition, PHMG is used as a disinfectant on medical devices, as approved by the U.S. Food and Drug Administration [4]. In the Republic of Korea, PHMG-containing disinfectants prevent microbial growth in humidifiers. However, the inhalation of PHMG as a humidifier disinfectant has been linked to irreversible pulmonary damage, including pulmonary fibrosis, and an increased risk of fatalities, raising concerns about exposure to biocide aerosols in indoor environments [5–7]. However, the specific mechanisms underlying PHMG-induced lung injury remain unclear.

Reactive oxygen species (ROS) trigger fibrosis and can be produced by the mitochondria as a byproduct of metabolism and by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) family. The Nox family is believed to be responsible for the lung damage observed in several pulmonary pathologies. Among the Nox members, Nox2, the first discovered and most extensively-studied Nox isoform, is the most abundantly expressed in the phagocytes (neutrophils and macrophages) and lesser in structural cells (mesenchymal, smooth muscle, endothelial, and airway epithelial cells). Nox2-dependent ROS facilitate the antimicrobial activity of phagocytes and mediate inflammatory responses. Moreover, mounting evidence links excessive Nox-derived ROS to numerous potential chronic diseases (including fibrotic diseases). In this study, we investigated the role of the Nox2 isoform of Nox in the progression of PHMG-induced lung injury in Nox2-deficient mice to determine its contribution to PHMG-induced lung injury.

2. Material and methods

2.1. Animals

Seven-week-old male C57BL/6 wild-type (WT) mice were purchased from Orient Bio Inc. (Seongnam, Korea). Six-weed-old Nox2 knockout (KO) male mice (B6.129S-Cybbtm1Din/J) were purchased from Jackson Laboratories (Bar Harbor, USA). The animals were acclimated for 6 days before the study. Furthermore, the animals were housed in polycarbonate animal cages (135W × 3465L × 3200H mm) at a temperature of 22 ± 3 °C, relative humidity of 30–70%, light intensity of 150–300 Lux (12-h light/dark cycle), and a ventilation rate of 10–20 times/h. Finally, the mice were fed sterilized pellet food (PMI Nutrition International, Richmond, VA, USA) and tap water. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Toxicology (KIT) (IACUC approval No. 16-1-0339).

2.2. Experimental design

The mice were divided into two groups: WT and Nox2 KO mice. Each group was treated with phosphate-buffered saline (PBS) (vehicle control; VC) or PHMG. Forty male mice were randomly grouped (n = 5 per group) using Pristima System Ver. 6.4 (Xybion Medical Systems Co., USA) (Table 1).

Liquid PHMG (CAS No. 89697-78-9) was a gift from SK Chemicals (Seongnam, Korea). PHMG was dissolved in PBS. The mice were treated with single intratracheal instillation (ITI) of 1.1 mg/kg PHMG or PBS using an automatic video instillator [8], and the injection volume was 50 μL. Mice were sacrificed 14 days after a single ITI with PHMG. Clinical signs and mortality of the animals were examined and recorded daily during the study period. In addition, body weight was measured on days 0 (instillation), 1, 2, 4, 8, 11, and 14. After necropsy, the absolute and relative (lung-to-body weight ratios) lung weights were measured.

2.3. Bronchoalveolar lavage fluid (BALF) analysis

After euthanasia, the BALF was collected from the right lung with 0.7 mL of calcium- and magnesium-free PBS (pH 7.4). A syringe containing PBS was inserted into the incised airway and washed thrice to obtain the BALF. The BALF was centrifuged at 800×*g* for 10 min at 4 °C, and the supernatant was used to determine interleukin (IL)-6 and tumor necrosis factor (TNF)-α levels using a quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA). The cell pellets were resuspended in PBS to determine the total and differential cell counts. The total cell counts were measured by adding and mixing solution-18 (AO/DAPI, Chemometec, Gydevang, Denmark) to the cells using an automated cell analyzer (NucleoCounter® NC-250™, Chemometec, Gydevang, Denmark). A Diff-Quick Staining kit (Sysmex Inc., Kobe, Japan) was used for differential cell counting, and more than 200 cells per sample were counted under a microscope. Following the BALF sampling, the right lung of each animal was frozen in liquid nitrogen and stored in a cryo tube at −80 °C until further analysis.

Table 1
Experimental design of PHMG-treated WT and Nox2 KO mice.

Group		No. of Animals	Administration Volume (μL)	PHMG (mg/kg)
Wild Type	VC	5	50	0.00
	PHMG	5	50	1.10
Nox2	VC	5	50	0.00
KO	PHMG	5	50	1.10

2.4. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the lung tissues using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Subsequently, total RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Furthermore, complementary DNA was synthesized using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Moreover, qRT-PCR was performed using Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) on a StepOnePlus Real-Time PCR System (Applied Biosystems). The expression level of each transcript was normalized to that of the housekeeping gene (*Gapdh*). Finally, the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, where Ct is the threshold cycle. The primer sequences used for qRT-PCR are listed in Table 2.

2.5. Histopathological examination

To examine the histopathological alterations, the left lung tissues of mice were fixed in a 10% neutral buffered formalin solution, embedded in paraffin, and sectioned to 4-μm thick slices. The lung tissue sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome (MT) stains. All lung fields per section from each animal were analyzed at 200× magnification. The HE staining score was evaluated by a pathologist, and the assessment was performed in a double-blind fashion. It was rated the mean histopathological damage, including inflammation on a scale of 0 (normal) to 5 (severe).

2.6. Hydroxyproline assay

The collagen content of the right lung tissues was measured using the hydroxyproline colorimetric assay kit (Biovision Inc., Milpitas, CA, USA) according to the manufacturer's protocol, as described previously [9].

2.7. Immunohistochemistry (IHC) staining of 8-hydroxy-2′-deoxyguanosine (8-OHdG)

After processing the fixed tissue, it was embedded in paraffin. The paraffin block was then cut into sections with a thickness of 4 μm. The sections were deparaffinized and washed with distilled water. To eliminate the activity of endogenous peroxidase, Peroxide

Table 2
Primer sequences used for quantitative reverse transcription PCR (qRT-PCR).

Gene	Primer Sequence (5′ → 3′)	
Il-6	F	GTTTTCTGCAAGTGCATCATCG
	R	GGTTTCTGCAAGTGCATCATCG
Il-1β	F	GGGCCTCAAAGGAAAGAATC
	R	TACCAGTTGGGGAACTCTGC
Il-10	F	ATTTGAATTCCCTGGGTGAGAAG
	R	CACAGGGGAGAAATCGATGACA
Ccl2	F	TTGTCACCAAGCTCAAGAGAGA
	R	GAGGTGGTTGTGGAAAAGGTAG
Ccl6	F	AGGCTGGCCTCATACAAGAA
	R	TCCCCTCCTGCTGATAAAGA
Cxcl1	F	GCTGGGATTCACCTCAAGAA
	R	TGGGGACACCTTTTAGCATC
Mmp-12	F	CACAACAGTGGGAGAGAAAA
	R	AGCTTGAATACCAGATGGGATG
Fibronectin	F	CACGATGCGGGTCACTTG
	R	CTGCAACGTCCTCTTCATTCTTC
Timp-1	F	GTGGGAAATGCCGCAGAT
	R	GGGCATATCCACAGAGGCTTT
Cd68	F	TCCAAGCCCAAATTCAAATC
	R	ATATGCCCCAAGCCTTTCTT
Cd163	F	GCAAAAACTGGCAGTGGG
	R	GTCAAAATCACAGACGGAGC
Cd14	F	GGCTTGTTGCTGTTGCTTC
	R	CAGGGCTCCGAATAGAATCC
Nox1	F	TTCCCTGGAACAAGAGATGG
	R	CCAGCCAGTGAGGAAGAGAC
Nox3	F	ACTTTCCAAACTTGGCGATG
	R	ATATCAAAGGTGCGGACTGG
Nox4	F	GCATCTGCATCTGTCCTGAA
	R	CCGGCACATAGGTAAAAGGA
Gapdh	F	ATCACCATCTTCCAGGAGCGA
	R	AGGGGCCATCCACAGTCTT

F: Forward primer; R: Reverse primer.

Blocking (DAKO) was applied at room temperature for 10 min. After two washes with PBS, the sections were incubated with diluted (1:1000) the anti-8-OHdG antibody (ab48508, Abcam, Cambridge, UK) at 4 °C overnight. The sections were then washed with wash buffer (DAKO) and incubated with Envision + Mouse for 30 min. After washing again with Wash buffer (DAKO), the sections were stained with DAB (3,3-diaminibenzidine tetrahydrochloride) for approximately 3 min. The sections were neutralized with distilled water and counterstained with Mayer's hematoxylin. After rinsing with tap water, the sections were mounted following a clearing process.

2.8. Statistical analyses

The results are expressed as mean ± standard deviation (SD) or standard error of the mean (SEM). Data were analyzed using analysis of variance (ANOVA) tests, followed by Tukey–Kramer post hoc and Dunnett's multiple comparison tests. The statistical analyses were performed using SigmaStat (SPSS Inc., Chicago, Illinois, USA) and GraphPad Prism v.7 (GraphPad Software, San Diego, CA, USA). P-values <0.05 were considered statistically significant.

3. Results and discussion

3.1. Changes in body and lung weights of PHMG-treated mice

There were no mortality or distinct clinical observations after PHMG administration in WT and Nox2 KO mice. In WT and Nox2 KO mice, body weights were significantly decreased compared with those in the vehicle control (VC) group on day 1 after an ITI of 1.1 mg/kg PHMG. Body weight decreased on day 3 in the WT mice after PHMG exposure (Fig. 1A). The absolute and relative lung weights of the WT and Nox2 KO mice were increased. After PHMG exposure, the absolute and relative lung weights were significantly elevated in WT mice compared with those in the VC group (Fig. 1B and C).

3.2. Total and differential cell analysis in BALF of PHMG-treated mice

To evaluate the impact of PHMG on the respiratory system associated with the inflammatory response, we analyzed the total and differential cell counts in the BALF. We observed a significant increase in total cell count upon exposure to PHMG in the WT and Nox2 KO mice. Furthermore, PHMG exposure altered the composition of differential cells, including macrophages, neutrophils, and lymphocytes, with a predominant increase in macrophages (Fig. 2A and B). Moreover, in contrast to other cell types, neutrophil counts decreased in Nox2 KO mice after PHMG exposure. Overall, the extent of the increase observed upon PHMG exposure was lower in the Nox2 KO group than in the WT group. These findings suggest that PHMG exposure induces an inflammatory response in the respiratory system, particularly by increasing the macrophages. Furthermore, these results indicate that there could be a link between Nox2 and inflammatory response.

3.3. Expression of cytokines and chemokines in BALF from PHMG-exposed Nox2 KO mice

We found that PHMG exposure induces an inflammatory response in the lungs and that there is a correlation between Nox2 and inflammation. To determine whether the expression of cytokines and chemokines involved in the inflammatory response was altered, we analyzed their mRNA expression levels in BALF cells using qRT-PCR. The expression levels of cytokines (*Il-6, Il-1β, and Ifn-γ*) and chemokines (*Ccl2, Ccl6,* and *Cxcl1*) were altered in WT and Nox2 KO groups (Fig. 3A and B). We found similar gene expression patterns for *Il-6, Ccl2*, and *Ccl6* in response to PHMG exposure in WT and Nox2 KO mice. The relative mRNA expression levels of *Ccl6* were significantly increased in response to PHMG exposure in WT mice. However, Nox2 KO mice exhibited lower gene expression levels

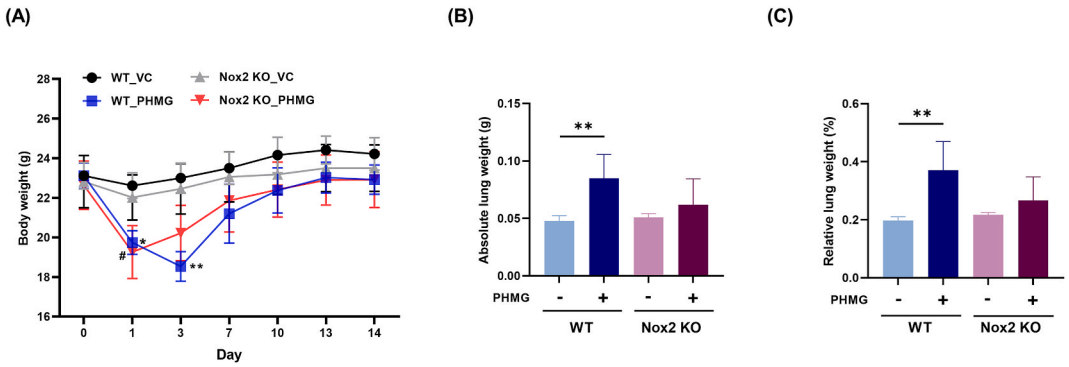

Fig. 1. (A) The changes in body weight after intratracheal instillation with PHMG in WT and Nox2 KO mice for 15 days. Data are shown as mean ± standard deviation (SD) (n = 5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ (WT-VC vs. WT-PHMG); $^{\#}P < 0.05$ (Nox2 KO-VC vs. Nox2 KO-PHMG). The (B) absolute and (C) relative lung weight of WT and Nox2 KO mice exposed to PHMG after 15 days.

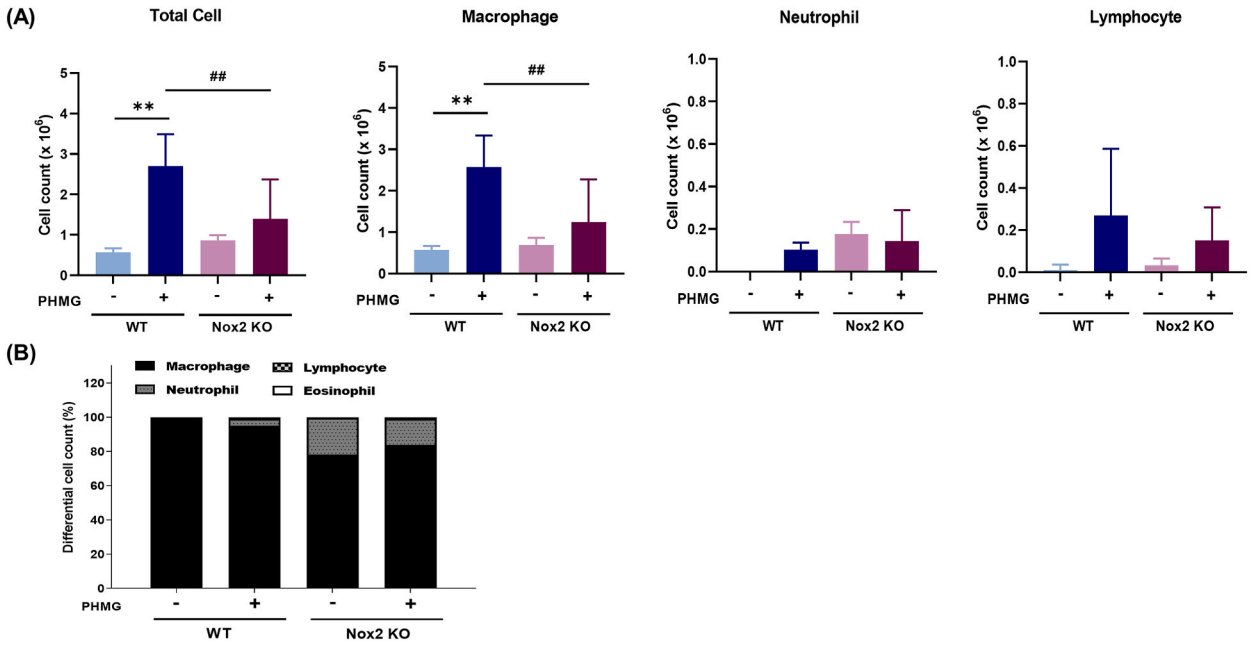

Fig. 2. (A) The counts of total cells, macrophages, neutrophils, and lymphocytes in the bronchoalveolar lavage fluid (BALF) of WT and Nox2 KO mice. Data are presented as mean ± SD (n = 5 per group). (B) The composition of differential cells in BALF. $^{**}P < 0.01$ (WT group); $^{\#\#}P < 0.01$ (PHMG-treated group).

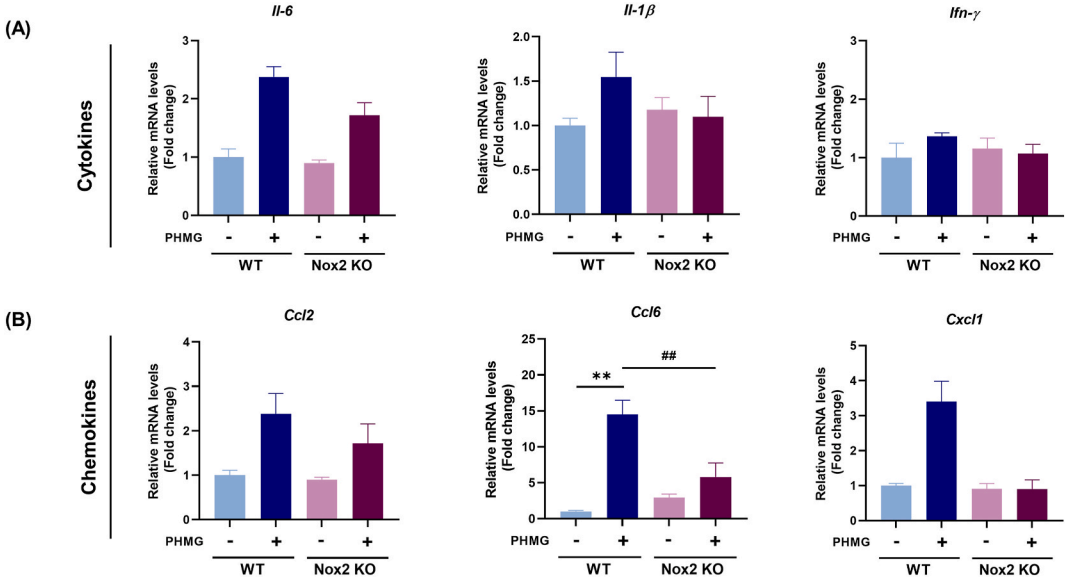

Fig. 3. Relative mRNA expression levels of (A) cytokines and (B) chemokines using qRT-PCR. The relative expression levels were calculated using the 2^-ΔΔCt method and normalized to the control. The data were shown as mean ± standard error of the mean (SEM) compared with the control. $^{**}P < 0.01$ (WT group); $^{\#\#}P < 0.01$ (Nox2 KO group).

than WT mice. Similarly, the gene expression levels of *Il-1β, Ifn-γ,* and *Cxcl1* changed insignificantly in Nox2 KO mice. Further studies are required to determine the underlying mechanisms associated with Nox2 and PHMG-induced inflammation. To determine whether Nox2 is involved in fibrotic-related process, the levels of IL-6 and TNF-α were measured in BALF using ELISA. The hydroxyproline content was also measured to evaluate the pulmonary fibrosis. The levels of IL-6, TNF-α, and hydroxyproline content were significantly higher in the PHMG-treated WT mice than in that of the VC group. However, this increase was not observed in Nox2 KO mice (Fig. S1).

3.4. Effects of PHMG-induced fibrogenesis in Nox2 KO mice

The expression levels of fibrotic mediators (*mmp-12, fibronectin*, and *timp-1*) were significantly higher in the lung tissues of WT mice in the PHMG group than in those of the VC group. However, the increased expression of these fibrotic mediators was less pronounced in the lung tissues of PHMG-treated Nox2 KO mice compared with those of the PHMG-treated WT mice. Additionally, the expression levels of fibrogenic mediators (*mmp-12, fibronectin*, and *timp-1*) were similar to those of inflammatory cytokines in the lung tissues of mice in all groups (Fig. 4). This suggests that the depletion of Nox2 attenuates the PHMG-induced upregulation of fibrosis-related genes.

3.5. Lung histopathological alterations following PHMG exposure

To determine histopathological alterations in PHMG-treated mice, we stained the lung sections with H&E. In addition, MT staining was performed to examine the collagen deposition. We observed that granulomatous inflammation and foamy macrophages were significantly induced (Fig. 5A), and collagen distribution and deposition, indicators of fibrosis, in the lung tissues of PHMG-treated WT and Nox2 KO mice (Fig. 5B). The Nox2 KO mice generally showed milder histopathological changes resulting from PHMG exposure than the WT mice. The histopathological scoring is summarized in Table 3.

3.6. Effects of PHMG-induced M1 and M2 markers in Nox2 KO mice

The recruitment of inflammatory macrophages is critical in the development of fibrosis. The expression of M1 macrophage markers, specifically *Cd68*, significantly increased in the lungs of PHMG-treated WT mice. However, this increase was not observed in the Nox2 KO mice (Fig. 6A). In contrast, the expression of M2 macrophage markers *Cd163* and *Cd14* either decreased or remained unchanged in the lungs of PHMG-treated WT mice. This indicates the predominance of M1 macrophages in the recruitment of inflammatory macrophages in PHMG-induced fibrosis and that there were no significant effects on the expression of M1 and M2 macrophages in Nox2 KO mice (Fig. 6B and C).

4. Discussion

This study aimed to investigate the effect of PHMG exposure on the respiratory system of mice and the potential role of Nox2 in PHMG-induced inflammatory and fibrotic responses. We found that PHMG exposure decreased body weight and increased lung weight in WT and Nox2 KO mice. Additionally, PHMG exposure induced an inflammatory response in the respiratory system, characterized by an increased total cell count and a shift in the composition of differential cells in BALF, with a predominant increase in macrophages. The results showed that Nox2 deficiency resulted in an insignificant increase in the expression of inflammatory cytokines and chemokines and a decrease in the production of pro-inflammatory cytokines, suggesting a possible association between Nox2 and PHMG-induced inflammation. Moreover, PHMG exposure upregulated the expression of fibrosis-related genes and increased collagen accumulation in the lung tissues of WT mice, whereas Nox2 deficiency attenuated the upregulation of these genes and collagen accumulation. Histopathological analysis revealed granulomatous inflammation, foamy macrophages, and collagen deposition in the lung tissues of PHMG-treated mice. Overall, these results suggest that Nox2-deficient mice are resistant to PHMG-induced lung injury and inflammation.

PHMG is a disinfectant used in various products owing to its bactericidal properties [2,3]. In addition, it is approved by the FDA as a disinfectant for use on medical devices [4]. However, an outbreak of the pulmonary disease in Korea in 2011 was linked to using PHMG-based humidifier disinfectants, which caused acute interstitial pneumonia, obstructive bronchitis, and pulmonary fibrosis, collectively known as humidifier disinfectant-induced lung injury. Similarly, pulmonary fibrosis was recently identified as the most common severe outcome of inhaled PHMG [9,10].

Nox is a vital enzyme complex involved in generating reactive oxygen species (ROS). It is crucial in various physiological processes,

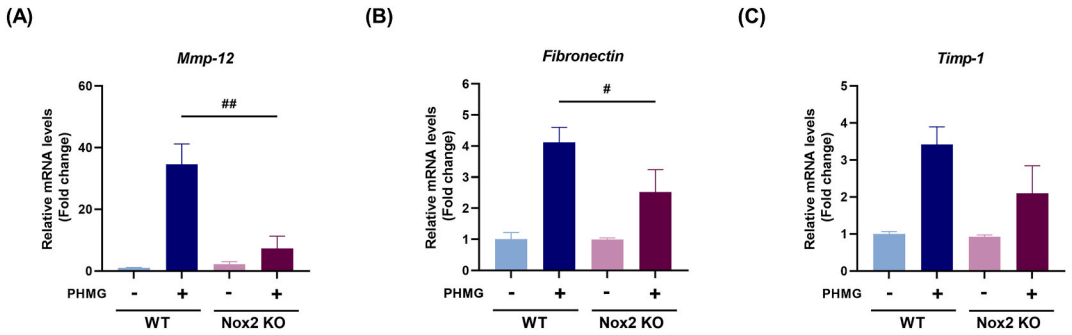

Fig. 4. The relative mRNA expression level mediators in fibrosis (A) *Mmp-12,* (B) *Fibronectin,* and (C) *Timp-1* were measured in BALF. Data are represented as mean ± SD ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$).

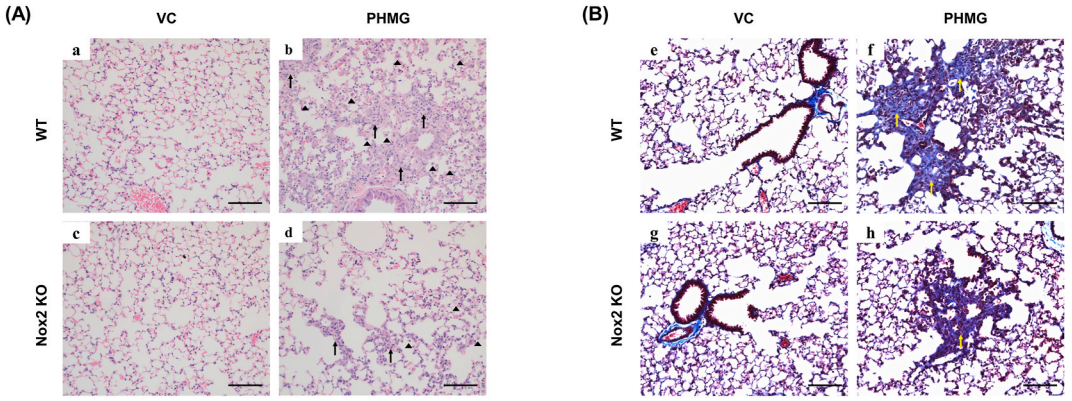

Fig. 5. Histopathological analysis of PHMG-treated mice. The lung sections stained with (A) H&E and (B) MT. The black arrows indicate inflammatory cell infiltration, and the black-filled triangles indicate foamy macrophages (b, d). The yellow arrows indicate collagen deposition (f, h) (scale bar = 50 μm).

Table 3
Histopathological scoring of hematoxylin and eosin (H&E)-stained lung tissues.

Group	Wild type		Nox2 KO	
	VC	PHMG	VC	PHMG
Number of animals	5	5	5	5
Granulomatous inflammation/fibrosis	(0)	(5)	(0)	(2)
Minimal	4	0	0	1
Slight	1	4	0	1
Moderate	0	1	0	0
Mean ± SD	0	2.20 ± 0.45**	0	0.60 ± 0.89$^{\#\#}$
Neutrophil infiltration	(0)	(0)	(0)	(0)
Minimal	0	0	0	0
Slight	0	0	0	0
Moderate	0	0	0	0
Mean ± SD	0	0	0	0
Foamy macrophages in alveolar space	(0)	(5)	(0)	(1)
Slight	0	2	0	1
Moderate	0	3	0	0
Mean ± SD	0	2.60 ± 0.55**	0	0.40 ± 0.89$^{\#\#}$

0, no symptoms; 1, minimal; 2, slight; 3, moderate; 4, marked; 5, severe. Data are presented as mean ± SD (n = 5 per group). $^{**}P < 0.01$ vs. WT VC group; $^{\#\#}P < 0.01$ vs. Nox2 KO VC group.

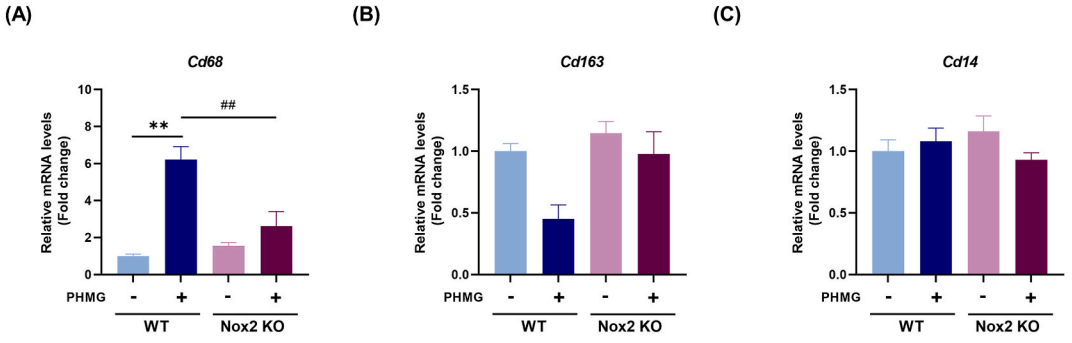

Fig. 6. mRNA expression of M1 and M2 macrophage markers (*Cd68, Cd163, and Cd14*) was determined using qRT-PCR in WT (n = 5) and Nox2 KO mice (n = 5 per group). The relative fold change of the target genes was calculated and presented as mean ± standard error of the mean (SEM) compared with the control. Statistical analysis revealed significant differences ($^{**}P < 0.01$, WT mice group; $^{\#\#}P < 0.01$, Nox2 KO mice group).

such as host defense against pathogens, signal transduction, and redox signaling [11]. Nox dysregulation has been implicated in several pathological conditions, including chronic inflammation, cardiovascular diseases, and cancer [12]. Nox is a complex of several subunits that generates superoxide anions ($O2^{-}$) and other ROS by transferring electrons from NADPH to molecular oxygen. It is regulated

in response to various stimuli such as growth factors, cytokines, and pathogens [13]. The production of ROS by Nox is critical in initiating and propagating inflammation [14]. Inflammatory cells, such as neutrophils and macrophages, express isoforms of Nox, including Nox1, Nox2, Nox3, Nox4, and Nox5. These isoforms differ in their expression patterns, enzymatic activities, and regulation [15]. In particular, Nox1, Nox2, and Nox4 are the major isoforms of Nox [16], central to the development of pulmonary fibrosis [17, 18]. Previous studies have reported that mice lacking Nox2 were protected from lung fibrosis induced by bleomycin or carbon nanotubes [17,19].

Several studies have shown that Nox is critical in PHMG-induced inflammation [20]. For example, treatment with PHMG induces the expression of Nox2 and Nox4 in lung epithelial cells and macrophages, leading to increased ROS production [21]. The generated ROS can activate various inflammatory cells, such as neutrophils and macrophages, leading to an inflammatory response [16].

ROS regulate cellular functions and signaling pathways by influencing gene expression [22–25]. However, under pathological conditions, excessive ROS levels can harm proteins, lipids, and DNA and activate harmful signaling pathways, contributing to the onset or progression of many diseases [26,27].

Therefore, in this study, the insignificant increase in total and inflammatory cells observed in the PHMG-treated Nox2 KO mice may be due to decreased ROS production compared with that in WT mice [16]. Moreover, to investigate the correlation between ROS, Nox2, and PHMG, we conducted the immunohistochemical analysis of 8-OHdG-stained lung tissues from both PHMG-treated WT and Nox2 KO mice (Fig. S2). In general, the ROS-induced elevation of 8-OHdG has been associated with exposure to various environmental factors such as chemical and biological agents [28]. We identified that there was an observed increase in the population of 8-OHdG positive cells in response to PHMG treatment and this increase in 8-OHdG expression was ameliorated in Nox2 KO mice compared to WT mice on day 1. There was no significant increase in the number of cells positive for 8-OHdG on the day 14 after exposure to PHMG. This is consistent with a previous paper showing that elevation of ROS by PHMG acts in the early stages of pulmonary fibrosis [29].

It also was known that neutrophil plays a key role in inflammatory response and Nox2 activation to generate large amounts of ROS upon stimulation [30]. In Fig. S3, the analysis of histopathological data on day 1 showed increased neutrophil infiltration in PHMG-treated WT and Nox2 KO mice. Additionally, we confirmed the upregulation of Cxcl1 gene expression, a chemokine associated with neutrophil infiltration, in the lungs of WT mice compared to Nox2 KO mice (Fig. S4) These findings reveled that neutrophil increased and ROS appears to have increased on day 1. Consequently, it suggests that Nox2 involved in regulation of neutrophil infiltration and ROS production in the PHMG-treated lungs. However, further studies are required to fully elucidate the underlying mechanisms.

Additionally, to confirm the interaction between PHMG treatment and Nox subunits in Nox2 KO mice, we evaluated the expression levels of Nox subunits (Nox1, Nox3, and Nox4). We found that PHMG treatment significantly decreased the mRNA levels of Nox subunits in the WT mice compared with those in the VC group, whereas no differences were observed in the Nox2 KO mice (Fig. S5). A previous study has shown that PHMG disrupts eukaryotic cell plasma membranes through ionic interactions between the cations of PHMG and anions in the cell membrane [31]. These results suggest that plasma membrane disruption by PHMG treatment induces a decrease in Nox1, Nox3, and Nox4 mRNA levels. Additional research is needed to understand better the connection between lung injury, Nox2-associated inflammation, inhaled PHMG, and the start of the fibrogenic process, which involves various mediators such as MMPs, fibronectin, and TIMPs. The absence of collagen deposition in Nox2 KO mice was associated with decreased expression of *mmp-12, fibronectin,* and *timp-1* in their lungs. These results suggest that ROS generated by Nox are involved in the progression of pulmonary fibrosis (Fig. 7).

5. Conclusions

These findings suggest that PHMG exposure can induce inflammatory and fibrotic responses in the respiratory system of mice, with Nox2 being potential in these phenomena. This study highlights the significance of further investigation to determine the underlying mechanisms associated with Nox2-and PHMG-induced inflammation and fibrosis, which could contribute to developing effective preventive and therapeutic strategies. In addition, future studies aimed at elucidating the mechanisms underlying PHMG-induced inflammation and the role of Nox in this process may facilitate developing novel therapeutic strategies.

Data availability statement

Data are available within the article. The data associated with this study have not been deposited into a publicly available repository, it will be made available upon request.

CRediT authorship contribution statement

Yoon Cho: Writing – review & editing, Writing – original draft, Data curation. **Seulgi Jeon:** Writing – review & editing, Writing – original draft, Data curation. **Sung-Hwan Kim:** Investigation, Formal analysis, Conceptualization. **Hyeon-Young Kim:** Investigation, Formal analysis. **Bumseok Kim:** Investigation. **Mi-Jin Yang:** Visualization, Validation, Investigation. **Jinhyung Rho:** Visualization, Validation, Investigation. **Moo-Yeol Lee:** Resources. **Kyuhong Lee:** Supervision, Project administration. **Min-Seok Kim:** Supervision, Project administration, Funding acquisition, Conceptualization.

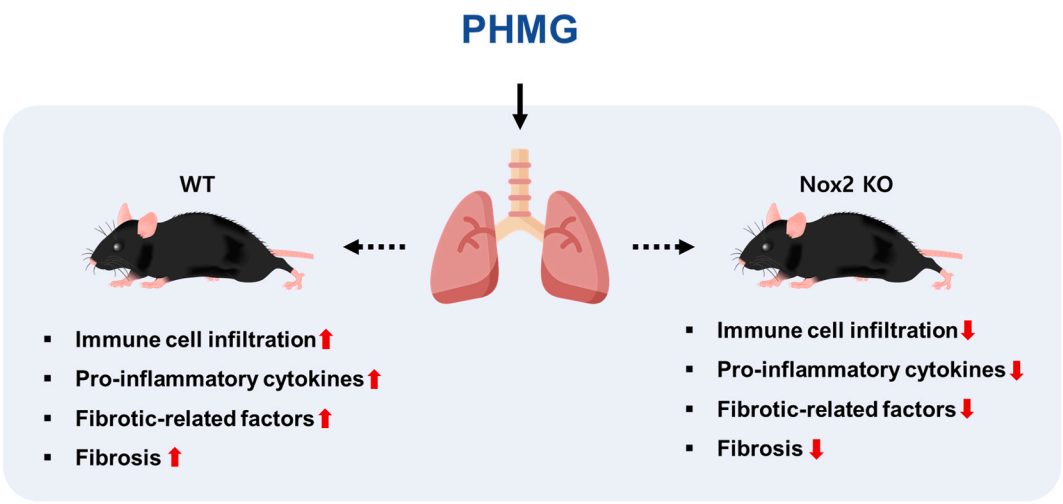

Fig. 7. Schematic diagram indicates the potential induction of an inflammatory and fibrotic response in the respiratory system by PHMG exposure associated with Nox2.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

NADPH	Nicotinamide adenine dinucleotide phosphate
Nox	NADPH oxidase
PHMG	polyhexamethylene guanidine
BALF	bronchoalveolar lavage fluid
WT	wild-type
KO	knockout
ROS	reactive oxygen species
VC	vehicle control

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e25045.

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