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Modulation of NF-κB/Nrf2 signaling by nobiletin mitigates airway inflammation and oxidative stress in PM2.5-exposed asthmatic mice

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ABSTRACT

Exposure to fine particulate matter ($PM_{2.5}$) is a significant risk factor for asthma, promoting airway inflammation and oxidative stress. This study evaluates Nobiletin's (NOB) efficacy in mitigating airway inflammation and oxidative lung damage in asthma-induced mice exposed to $PM_{2.5}$. Using an ovalbumin (OVA) plus $PM_{2.5}$-induced asthma model in BALB/c mice, we investigated the therapeutic impacts of NOB compared to dexamethasone (DEX). NOB significantly moderated lung index values and inflammatory markers without affecting body weight. Notably, NOB enhanced Nrf2 expression and decreased NF-κB-p65, IKK, and Keap-1 levels, aligning with reductions in malondialdehyde (MDA) and reactive oxygen species (ROS) while increasing superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities. These findings suggest that NOB can effectively reduce airway inflammation and oxidative lung damage by modulating the NF-κB/Nrf2 signaling pathways.

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KEYWORDS
Nobiletin; asthma; PM2.5; airway inflammation; oxidative stress

Introduction

Asthma is a chronic inflammatory respiratory disease characterized by increased inflammatory cells, enhanced airway hyperresponsiveness (AHR), elevated mucus production, and bronchoconstriction (Guilbert et al. 2014). Particulate matter (PM), specifically those particles with a diameter of less than 2.5 micrometers – referred to as $PM_{2.5}$—is a complex pollutant known to exacerbate these symptoms (Xing et al. 2016). Epidemiological studies consistently demonstrate a positive correlation between the ambient concentration of $PM_{2.5}$ and the incidence of asthma, suggesting that $PM_{2.5}$ exposure may intensify airway inflammation (Delfino et al. 2014). Compared with healthy subjects, the level of IL-33 in asthma patients is increased, and IL-33 can promote the production of IL-13 in mast cells to enhance AHR (Kaur et al. 2015). Experimental research further supports this association, indicating that the severity of airway inflammation in asthmatic rats is dose-dependent on $PM_{2.5}$ exposure (Zhang et al. 2015; Yu et al. 2019). The mechanisms involved may be related to the following factors. IL-17 secreted by Th17 cells induces AHR through

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activation of the MAPK and NF-κB signaling pathways (Xu and Cao 2010). IL-8 directly stimulates airway smooth muscle cell contraction and migration, promotes neutrophil degranulation, and enhances lymphocyte and eosinophil chemotaxis (Nakagome and Nagata 2018). IL-1β, IL-6, and TNF-α promote airway inflammation and disrupt immune responses to promote AHR (Sakai et al. 2017; Kim et al. 2017; Mahmutovic Persson et al. 2018).

Reactive oxygen species (ROS) and oxidative stress are integral to the pathogenesis and progression of asthma, with oxidative stress identified as a critical determinant of asthma severity (Erzurum 2016). It has been established that oxidative stress plays a pivotal role in the development of $PM_{2.5}$-mediated asthma complications (Liu et al. 2022; El Tabaa et al. 2023; Fan et al. 2023, Li et al. 2018a; Moazamiyanfar et al. 2023; Qin et al. 2024). ROS can activate multiple signaling pathways and affect gene transcription and expression, leading to an imbalance between the oxidative and antioxidant systems in the body and damage to airway epithelial cells.

NOB has demonstrated potent anti-inflammatory and antioxidant effects in various models. For instance, in lipopolysaccharide-induced acute lung injury in mice and A549 cell inflammation models, NOB effectively inhibited NF-κB activation, which reduced inflammatory responses and protected lung tissues from damage. It also decreased the levels of pro-inflammatory cytokines TNF-α, and IL-6 (Li et al. 2018a), and in models using nematodes, NOB lowered ROS and MDA levels, boosted antioxidant enzyme activity, and extended lifespan under stress conditions such as high temperatures and UV exposure (Yang et al. 2020).

This study aims to explore the protective effects of NOB against airway inflammation and oxidative lung damage in asthma models induced by $PM_{2.5}$ exposure. By examining its therapeutic mechanisms, this research seeks to provide a deeper understanding of how NOB can modulate the NF-κB/Nrf2 signaling pathways to alleviate these conditions.

Materials and methods

Chemicals and reagents

Nobiletin (NOB) with 99% purity was sourced from the China Institute for Pharmaceutical and Biological Products Inspection (NICPBP, Beijing, China), lot number: 10236-47-2. Aluminum hydroxide [Al(OH)3] was procured from Thermo Fisher Scientific (Waltham, MA, USA), lot number: 21645-51-2, and Ovalbumin (OVA) was obtained from Sigma Chemical Co. (St. Louis, MO, USA), lot number: 138831-86-4. The ELISA kits for IgE, IL-1β, IL-6, and TNF-α were purchased from Beijing Solarbio Technology Co., Ltd. (Solarbio, Beijing, China), lot number: SEKM-0007, SEKM-0002, SEKM-0138, and SEKM-0345. Assay kits for Reactive oxygen (ROS), Superoxide Dismutase (SOD), Malondialdehyde (MDA), and Glutathione Peroxidase (GPx) were acquired from Nanjing Jiancheng Bioengineering Institute (Njjcbio, Nanjing, China), lot number: E004-1-1, A001-3-2, A003-1-2, and A005-1-2. Nrf2, Keap-1, p65, and IKK antibodies were sourced from Cell Signaling Technologies (Danvers, MA, USA), lot number: 12721S, 8047S, 8242S, 2697S. Hematoxylin-eosin stain was purchased from Nanjing Jiancheng Bioengineering Institute (Njjcbio, Nanjing, China), lot number: D006-1-1. Liquid paraffin, xylene, anhydrous ethanol, and lithium carbonate were purchased from Macklin Co., Ltd. (Macklin, Shanghai, China), lot number: 8042-47-5, 1330-20-7, 64-17-5, 554-13-2.

Drug preparation and $PM_{2.5}$ collection

$PM_{2.5}$ Collection and Preparation: Air sampling was conducted from October to December 2020 at the south gate of Shenyang Medical College, Huanghe North Street, Shenyang City, Liaoning Province, China. Collected samples were transferred to a sterile environment where the filter

membrane was removed, segmented into 1 cm^2 pieces, and soaked in 10 ml of ultrapure water. These were subjected to ultrasonic oscillation for 30–40 minutes, followed by filtration through six layers of sterile gauze. The filtrate was then freeze-dried under vacuum at low temperatures to yield a powdered form. This powder was stored at −80°C in sterile conditions. Before use, the $PM_{2.5}$ powder was reconstituted in sterile saline to 100 mg/ml.

Preparation of OVA solution

A 100 mg OVA and 100 mg aluminum hydroxide solution in 1 mL of normal saline was prepared under aseptic conditions to ensure homogeneity, yielding a 10% OVA-sensitized solution.

Preparation of NOB suspension

NOB powder (7.2 g) was combined with 100 mL of normal saline. The mixture was subjected to ultrasonication at a power setting of 250 W for 15 minutes to ensure thorough mixing. This procedure produced a 72 mg/mL NOB suspension, stored at 4°C shielded from light. Ultrasonic agitation was performed before each use to maintain suspension consistency and minimize variability.

Animal treatment and experimental groups

Forty-two 6-week-old female BALB/c mice weighing 18-22 g were procured from Liaoning Changsheng Biotechnology Co., LTD. All procedures involving animals were approved by the Shenyang Medical College Animal Ethics Committee (Approval No: SYYXY 2,021,061,502). The mice were accommodated in specific pathogen-free (SPF) facilities at 22–24°C with 50%-60% humidity and maintained on a 12-hour light-dark cycle. Adaptation to these conditions lasted for one week. The study used a modified version of the established ovalbumin (OVA)-induced asthma model to incorporate $PM_{2.5}$ exposure (Cui et al. 2019; Bai et al. 2022). Mice were randomly divided into seven groups ($n = 6$ per group): a control group, a $PM_{2.5}$ group, a $PM_{2.5}$ + OVA group, three NOB treatment groups (1.8, 3.6, and 7.2 mg/kg), and a dexamethasone (DEX) group. Mice in the NC group were exposed to saline instead of $PM_{2.5}$ suspension, and the $PM_{2.5}$ model group was only exposed to $PM_{2.5}$. The remaining five groups of mice were sensitized by intraperitoneal and subcutaneous injections of 10% OVA solution from day 1 to 7, respectively. From day 8 to 14, mice in the experimental groups were sensitized using a single-concentration oronasal exposure system, in which mice were first disinfected inside and outside their nostrils and mouths with a cotton swab moistened with 75% alcohol, followed by oronasal exposure for 4 h at a concentration of 750 μg/m^3. $PM_{2.5}$ was combined with 5% OVA for exposure from day 15 to day 38. From day 30 to 45, mice in the NOB/DEX intervention group were gavaged with 1.8 mg/3.6 mg/7.2 mg/kg of NOB or 0.75 mg/kg of DEX, respectively, 30 min prior to the double-exposure stimulus, whereas mice in the $PM_{2.5}$+OVA model group were given equal doses of saline.

Serum, bronchoalveolar lavage fluid (BALF), and tissue collection

Serum collection

Twenty-four hours after the final OVA challenge, mice were anesthetized, and blood samples were collected via puncture of the retro-orbital venous plexus using a sterile needle. The samples were centrifuged at 1500 g(rpm) for 20 minutes at 4°C. The supernatant was transferred to new EP tubes and stored at −80°C for subsequent analyses.

BALF collection

Following serum collection, mice were euthanized by cervical dislocation. Each mouse was then placed supine on a pre-cooled platform to dissect the neck and chest, exposing the trachea and lungs. A suture was placed under the trachea for stabilization, and the thoracic cavity was

opened. The right main bronchus was tied off, and a modified size 7 needle was inserted into the trachea to secure the opening. Lavage was performed using 0.3 mL of pre-cooled PBS and repeated five times to ensure thorough sampling. The BALF was collected and centrifuged at 1000 rpm for 5 minutes at 4°C, and the supernatant was stored at −80°C for inflammation marker analysis.

Tissue collection

The non-lavaged right lung and trachea were excised and rinsed with chilled saline, and a portion was fixed in 4% paraformaldehyde for subsequent histological examination, including HE staining and immunohistochemical analysis.

Body weight and lung index measurement

Body weights were recorded at predetermined intervals: on days 0, 10, 20, 30, 40, and 45. Lung tissues were harvested post-euthanasia and weighed, and the lung index was calculated using the following formula:

$$\text{Lung Index} = \frac{\text{Lung Weight(g)}}{\text{Body Weight(kg)}} \times 100\%$$

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IgE, IL-1β, IL-6, and TNF-α in serum and bronchoalveolar lavage fluid (BALF) were quantified using specific ELISA kits according to the manufacturer's instructions. Absorbance was measured at 450 nm. To ensure accuracy and reproducibility, all samples were assayed in triplicate.

Hematoxylin and eosin (H&E) staining

Paraffin sections of tracheal and lung tissues were deparaffinized in xylene for 10 min, then transferred to anhydrous ethanol, 90% ethanol, and 80% ethanol for 2 min, and rinsed with water for 2 min. The deparaffinized sections were stained in hematoxylin solution for 10 min, washed with water to remove the hematoxylin, and then transferred to differentiation solution for 2 min, then transferred to saturated aqueous lithium carbonate to return to the blue for 2 min, and rinsed with water for 15 min. The tissue sections were re-stained in 0.5% eosin solution for 2 min and rinsed with water for 15 min, then transferred to 80% ethanol, 90% ethanol and anhydrous ethanol for 2 min each until transparent, and then transferred to xylene for 10 min. Finally, the sections were sealed with gum and examined with a microscope.

Immunohistochemistry (IHC) staining

Sections (4 μm thick) of paraffin-embedded trachea and lung tissues were deparaffinized. Following the IHC kit protocol, sections were incubated with a p65 antibody at a dilution recommended by the manufacturer (1:800) at 4°C overnight. After washing, sections were incubated with anti-rabbit IgG-HRP (1:1000) and developed using diaminobenzidine. Immunostaining was quantified using imaging software to analyze the distribution of protein expression.

Measurement of SOD, GPx, and MDA activity

Lung tissues were homogenized, and the activities of Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), and the concentration of Malondialdehyde (MDA) were measured using assay kits with readings taken at optical densities of 550 nm for SOD, 450 nm for GPx, and 523 nm for MDA. These measurements assessed the oxidative stress levels in the lung tissues.

Measurement of ROS levels

Lung tissues were stained with the DCHF-DA fluorescent probe for detection of reactive oxygen species (ROS). Stained tissues were examined under a fluorescence microscope, and the intensity of ROS fluorescence was quantified for statistical analysis.

Western blot analysis

The laboratory temperature is maintained at 21°C. Lung tissues were homogenized in RIPA lysis buffer containing protease inhibitors, and the homogenates were centrifuged to collect the supernatant. Protein concentrations were quantified using the bicinchoninic acid (BCA) protein assay. Upon completion of the assay, the sample volume was calculated based on the results of the assay to ensure that each well contained 20 μg of protein. Proteins were then separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked at room temperature for 1 hour, then incubated with primary antibodies (dilution ratio: 1:1000) overnight at 4°C. After three washes with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (dilution ratio: 1:10000) for 1 hour at room temperature. Chemiluminescence was detected using an enhanced chemiluminescence (ECL) reagent, and images were captured and analyzed with Image J software.

Statistical analysis

Data were analyzed using SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA) and are presented as mean ± standard error of the mean (SEM). Each experiment was conducted in triplicate. Differences among groups were evaluated using one-way ANOVA, with Tukey's post hoc test applied for pairwise comparisons and Dunnett's test used for comparisons with the control group. Graphs were created using GraphPad Prism software version 5.0 (GraphPad Software, La Jolla, CA, USA). A p-value of less than 0.05 was considered statistically significant.

Results

Establishment of an asthma model with combined exposure to OVA and $PM_{2.5}$

During the nebulization stimulation phase, mice exposed to $PM_{2.5}$ and OVA exhibited noticeable clustering, reduced vitality, and symptoms including wheezing, sneezing, and whistling sounds akin to wheezing, confirming the successful establishment of the asthma model. Notably, mice receiving Nobiletin (NOB) and Dexamethasone (DEX) treatments displayed varying degrees of symptomatic relief.

Effect of NOB on body weight and lung index

Body weight measurements on days 0, 10, 20, 30, 40, and 45 showed no significant differences across all groups (Figure 1a). Lung indices, which reflect lung function and health status, indicated more severe lung damage with higher values. As illustrated in Figure 1b, lung indices were elevated in the $PM_{2.5}$ and $PM_{2.5}$+OVA groups ($p < 0.05$). In contrast, indices in the NOB medium dose ($PM_{2.5}$+OVA+$NOB_{3.6}$),

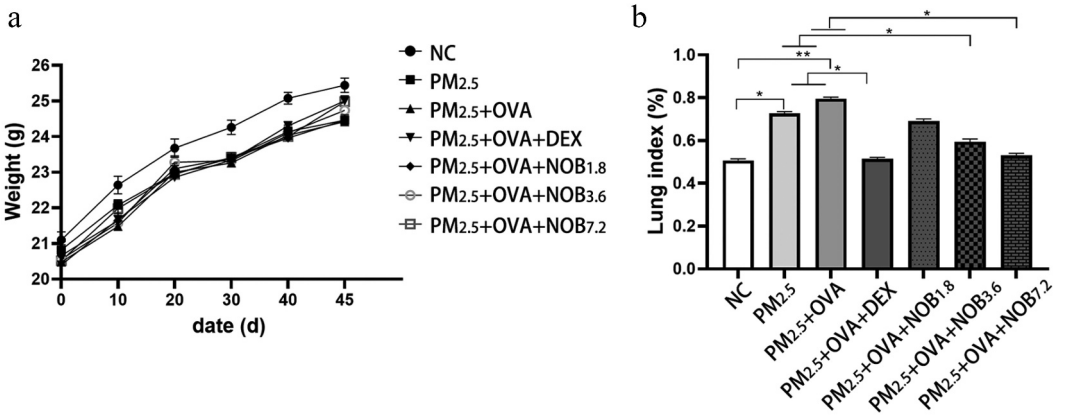

Figure 1. The impact of NOB on body weight and lung damage in mice exposed to During the experiment, the mice were observed for their condition from 0 to 45 days, weighed, and analyzed. The mice's lung tissues were collected, weighed, and analyzed after calculation. A: The changes in body weight; B: The changes in lung index. ($N = 6$) *$p < 0.05$, **$p < 0.01$.

high dose ($PM_{2.5}$+OVA+$NOB_{7.2}$), and DEX ($PM_{2.5}$+OVA+DEX) treatment groups showed significant reductions ($p < 0.05$). The low-dose NOB group ($PM_{2.5}$+OVA+$NOB_{1.8}$) also experienced a reduction, though it did not reach statistical significance.

Impact of NOB on BALF and serum IgE levels

The concentration of IgE in BALF and serum was assessed and presented in Figure 2(a,b). The $PM_{2.5}$ group did not show a significant change in IgE levels compared to the control (NC), whereas a significant increase was observed in the $PM_{2.5}$+OVA group ($p < 0.01$). Treatment with NOB and DEX resulted in a notable reduction in IgE levels in the medium and high NOB dose groups ($PM_{2.5}$+OVA+$NOB_{3.6}$ and $PM_{2.5}$+OVA+$NOB_{7.2}$) and the DEX

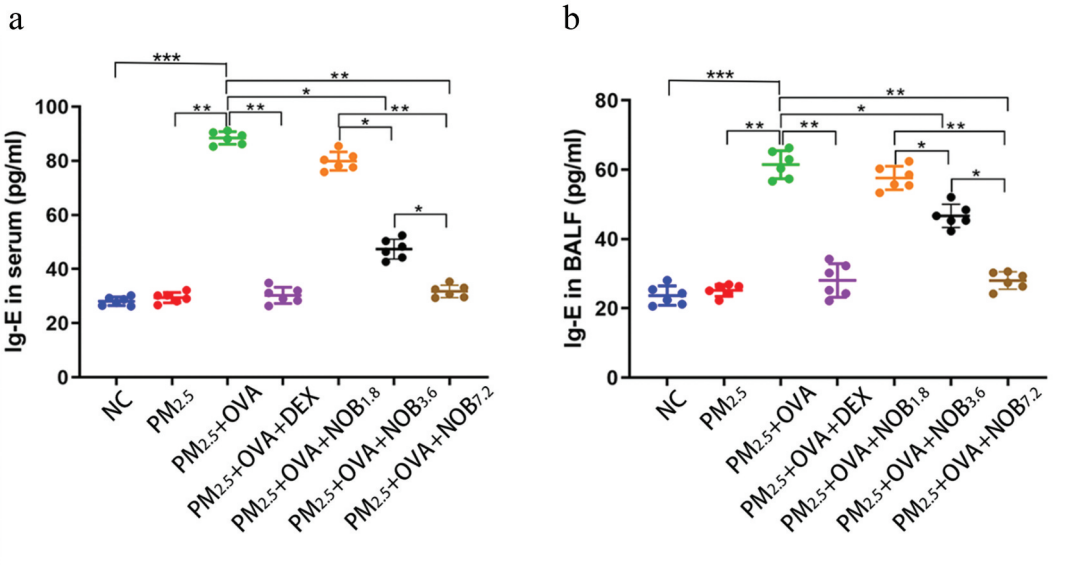

Figure 2. NOB reversed the IgE levels in the serum and BALF of mice. The ELISA kit detected the level of IgE. a: The changes of IgE levels in serum; b: The changes of IgE levels in BALF; ($N = 6$) *$p < 0.05$, **$p < 0.01$.

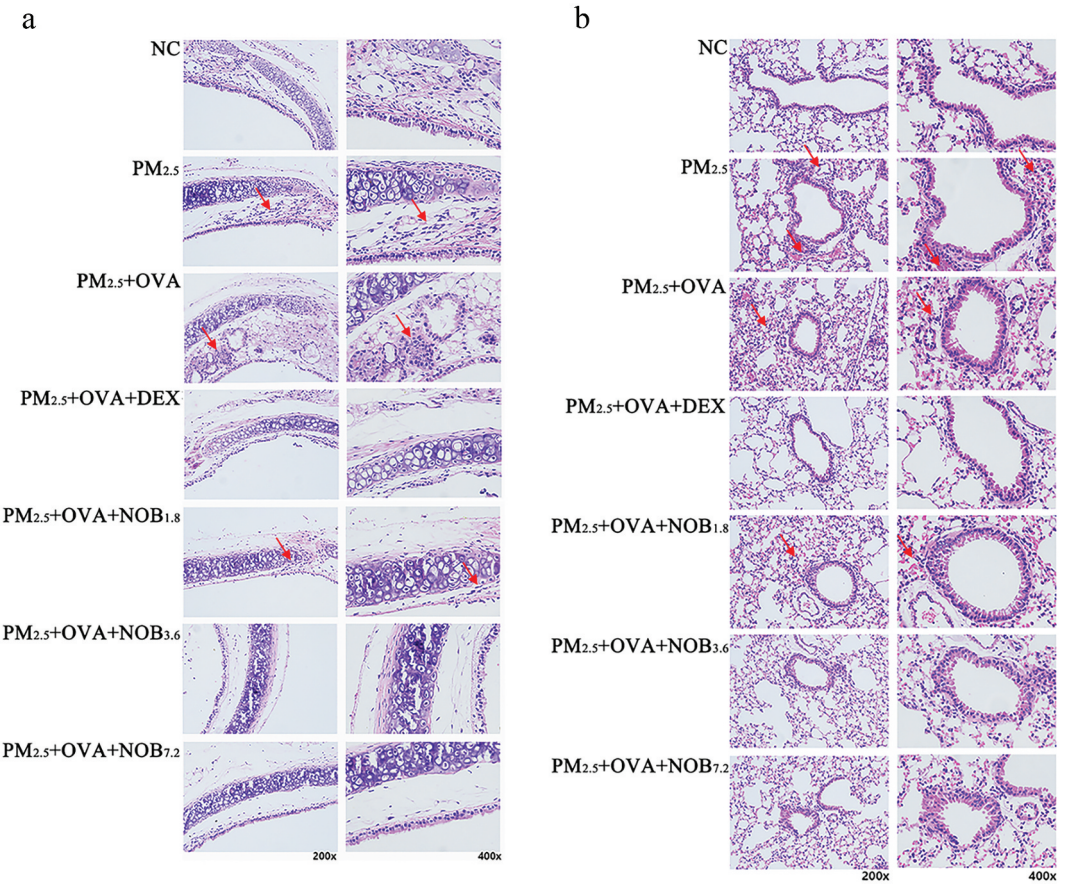

Figure 3. The pathological change of tracheal and lung tissues in mice. Pathological changes were observed through H&E staining. a: Pathological changes in tracheal tissues; b: Pathological changes in lung tissues. ($N = 6$) *$p < 0.05$, **$p < 0.01$.

group ($PM_{2.5}$+OVA+DEX), all showing significant decreases ($p < 0.01$). The low-dose NOB group ($PM_{2.5}$+OVA+$NOB_{1.8}$) exhibited a slight reduction, which was not statistically significant.

Effects of NOB on airway histopathological changes

Histopathological analyses of the trachea and lung tissues were visualized in Figure 3(a,b). In the control group (NC), mice displayed intact bronchial and alveolar structures without noticeable inflammatory secretions. In contrast, the $PM_{2.5}$ and $PM_{2.5}$+OVA groups exhibited bronchial congestion, inflammatory exudates, significant thickening of the tracheal walls, epithelial disruption, and mucosal muscle layer deterioration. These groups also showed loss of normal alveolar architecture, formation of merged pulmonary bullae, and inflammatory infiltrates, with notably dilated, congested, and thickened interstitial blood vessels. The severity of these symptoms was greater in the $PM_{2.5}$+OVA group than in the $PM_{2.5}$ group. Treatment with medium ($PM_{2.5}$+OVA+$NOB_{3.6}$) and high ($PM_{2.5}$+OVA+$NOB_{7.2}$) doses of NOB, as well as DEX ($PM_{2.5}$+OVA+DEX), resulted in significant alleviation of these pathological changes, whereas the low dose NOB group ($PM_{2.5}$+OVA+$NOB_{1.8}$) exhibited only slight improvement.

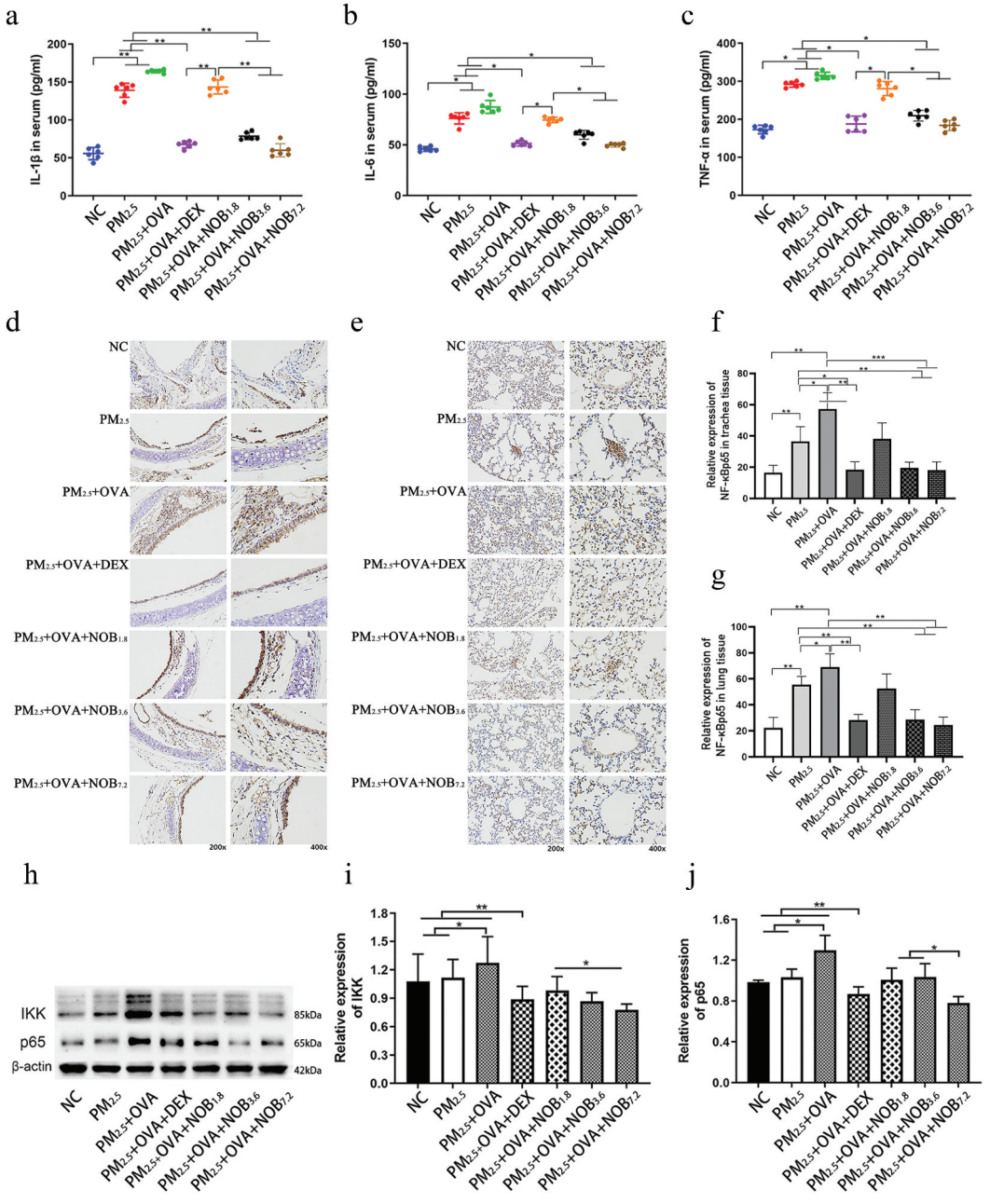

Figure 4. NOB reversed the inflammatory response in mice. ELISA assay kits assessed the levels of inflammatory factors in the serum; protein expression levels of inflammatory markers in lung and tracheal tissues were analyzed through Western blot and IHC. (a-c) the changes of inflammatory factors (IL-1β, tnf-α and IL-6) in serum; (d-g) the protein expression of p65 and IKK in lung tissue and the corresponding statistical graphs. (h-j) the changes in p65 protein expression in trachea and lung tissues and the corresponding statistical graphs. (N = 6)*$p < 0.05$, **$p < 0.01$; *$p < 0.005$.

Effects of NOB on inflammatory response

The inflammatory cytokines IL-1β, IL-6, and TNF-α were quantified using ELISA kits, as shown in Figure 4(a–c). Compared to the control group (NC), the $PM_{2.5}$ and $PM_{2.5}$+OVA

groups showed significant increases in the levels of these cytokines (IL-1β, $p < 0.01$; IL-6 and TNF-α, $p < 0.05$). Following treatment with NOB and DEX, these indicators were significantly reduced in the medium and high NOB dose groups ($PM_{2.5}$+OVA+$NOB_{3.6}$ and $PM_{2.5}$ +OVA+$NOB_{7.2}$), as well as in the DEX group ($PM_{2.5}$+OVA+DEX). The expression of p65 protein, localized in the epithelial cell layer of tracheal tissues and interstitial cells of lung tissues, was notably increased in the $PM_{2.5}$ and $PM_{2.5}$+OVA groups compared to the control (NC) ($p < 0.01$), with a significant reduction observed in the treatment groups (Figure 4(d–g)).

Western blot analyses further confirmed these findings, showing elevated levels of IKK and p65 proteins in the $PM_{2.5}$ and $PM_{2.5}$+OVA groups compared to the control ($p < 0.01$). Introduction of NOB ($PM_{2.5}$+OVA+$NOB_{1.8}$, $PM_{2.5}$+OVA+$NOB_{3.6}$, $PM_{2.5}$+OVA+$NOB_{7.2}$) led to a decrease in the expression of these proteins, indicative of NOB's inhibitory effect on the NF-κB pathway, although the lowest dose group ($PM_{2.5}$+OVA+$NOB_{1.8}$) showed inhibition of IKK protein expression without reaching statistical significance (Figure 4(h–j)).

These results suggest that NOB effectively reduces inflammatory damage in asthma models exacerbated by $PM_{2.5}$ and OVA exposure by modulating key inflammatory mediators.

The effect of NOB on oxidative stress

Biochemical assays and fluorescent probe methodologies were employed to evaluate oxidative stress markers in the lung tissues of mice. As depicted in Figure 5(a–d), significant reductions in the levels of Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) were observed in the $PM_{2.5}$ and $PM_{2.5}$+OVA groups compared to the control (NC) group ($p < 0.05$). Conversely, levels of Malondialdehyde (MDA) and Reactive Oxygen Species (ROS) were significantly increased in these groups ($p < 0.05$).

Treatment with Nobiletin (NOB) and Dexamethasone (DEX) resulted in marked improvements. In the $PM_{2.5}$+OVA+$NOB_{3.6}$, $PM_{2.5}$+OVA+$NOB_{7.2}$, and $PM_{2.5}$+OVA+DEX groups, there were significant increases in SOD and GPx levels, alongside notable decreases in MDA and ROS levels compared to the $PM_{2.5}$+OVA group ($p < 0.05$). These changes indicate effective mitigation of oxidative stress by these treatments.

Western blot analysis further quantified the effects on oxidative stress-related proteins, Nrf2 and Keap-1 (Figure 5(e–g)). While there was no significant change in Nrf2 expression in the $PM_{2.5}$ and $PM_{2.5}$+OVA groups compared to NC, an increase in Keap-1 levels was observed. Notably, NOB treatment led to a dose-dependent increase in Nrf2 levels and a decrease in Keap-1 levels in the medium and high-dose NOB groups ($PM_{2.5}$+OVA+$NOB_{3.6}$, $PM_{2.5}$+OVA+$NOB_{7.2}$) and the DEX group ($PM_{2.5}$+OVA+DEX), enhancing the antioxidant defense pathway. However, the high-dose NOB group ($PM_{2.5}$+OVA+$NOB_{7.2}$) showed only a weak inhibitory effect on Keap-1, which was not statistically significant.

Overall, these results demonstrate that NOB significantly reduces oxidative stress markers such as ROS and MDA while enhancing antioxidant enzymes (SOD, GPx), and modulating the Nrf2/Keap-1 pathway, thereby alleviating oxidative lung damage induced by combined exposure to $PM_{2.5}$ and OVA.

Discussion

Asthma is a chronic inflammatory disease that significantly impacts the quality of life and imposes a substantial economic burden on affected families, persisting throughout the year for many patients (Mortimer et al. 2022). Exposure to particulate matter less than 2.5 micrometers in diameter ($PM_{2.5}$) is a critical environmental factor that exacerbates asthma symptoms by triggering and intensifying airway inflammation (Stern et al. 2020). Despite ongoing research, asthma remains incurable, with current treatments often failing to fully alleviate symptoms. This study represents

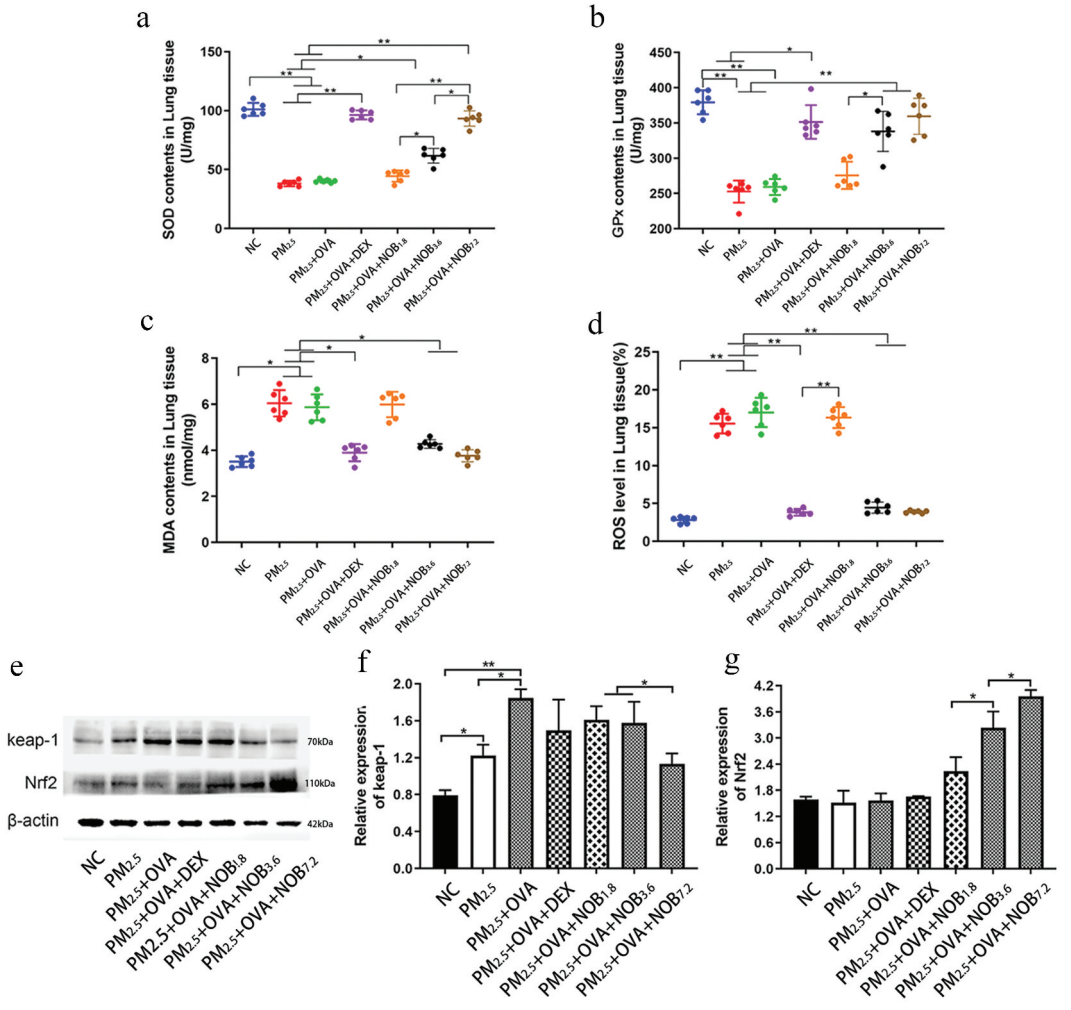

Figure 5. NOB alleviated oxidative lung damage in mice. The levels of SOD, ROS, MDA, and GPx were detected using specific biochemical assay kits and fluorescent probes, while the levels of Nrf2/Keap-1 were assessed through Western blot analysis. A-D: The changes of SOD/GPx/MDA/ROS levels in lung tissues; E-G: Expression of Keap-1/Nrf2 proteins in mouse lung tissues and the corresponding statistical graphs; ($N = 6$) $^{*}p < 0.05$, $^{**}p < 0.01$.

the first systematic investigation into the protective effects of Nobiletin (NOB) against airway inflammation and oxidative damage in asthma induced by $PM_{2.5}$ exposure.

IgE plays a pivotal role in airway inflammation as both a mediator and a biomarker of allergic inflammatory reactions (Rosenwasser 2011; Shamji et al. 2021). Previous studies have shown that pharmacological blockade of IgE can reduce asthma symptoms (Mitchell et al. 2017). Our results further corroborate these findings, demonstrating increases in IgE levels in BALF and serum following exposure to $PM_{2.5}$ and ovalbumin (OVA), thus validating the efficacy of our asthma model. Interestingly, mice exposed solely to $PM_{2.5}$ did not show elevated IgE levels immediately, suggesting a potentially delayed allergic response induced by $PM_{2.5}$.

Inflammation is fundamentally linked to the development and exacerbation of asthma. $PM_{2.5}$ exposure disrupts the cytokine network, leading to enhanced inflammatory responses that may contribute to structural changes within the airways, such as thickening of airway smooth muscles, epithelial hyperplasia, and collagen deposition (He et al. 2017). These pathological alterations can culminate in airway remodeling, a critical feature of chronic asthma (Hirota and

Martin 2013). Key cytokines, including TNF-α, IL-1β, and IL-6, play crucial roles in the inflammatory pathways associated with asthma pathogenesis (Charrad et al. 2016; Pavón-Romero et al. 2021). Inhalation of $PM_{2.5}$ prompts alveolar macrophages to engulf this particulate matter, initiating a cascade of events that produces substantial amounts of reactive oxygen species (ROS) and releases pro-inflammatory cytokines, thereby driving the pulmonary inflammatory response (He et al. 2017).

Our findings indicate that NOB effectively mitigates these inflammatory and oxidative processes through its modulation of the NF-κB/Nrf2 signaling pathways. Reducing key pro-inflammatory cytokines and oxidative stress markers in NOB-treated groups highlights its potential as a therapeutic agent for asthma-exacerbating environmental pollutants like $PM_{2.5}$. The anti-inflammatory and antioxidant mechanisms of NOB are similar to those of corni fructus and ellagic acid (Chagas et al. 2022). This study contributes to our understanding of the molecular interactions involved in $PM_{2.5}$-induced asthma and opens avenues for developing novel therapeutic strategies based on antioxidant and anti-inflammatory agents such as NOB.

The IKK/NF-κB signaling pathway regulates inflammation and immune responses (Barnes and Adcock 1997; Dagher et al. 2007). Under resting conditions, NF-κB is sequestered in the cytoplasm by its inhibitor IκB. Activation of the IKK complex leads to IκB degradation, freeing NF-κB to enter the nucleus and activating the transcription of genes involved in inflammation and immunity (Yoon et al. 2010; Dai et al. 2021). The NF-κB pathway primarily functions through a heterodimer composed of p50 and p65 subunits, with the p65 subunit recognized as a key mediator of pro-inflammatory responses (Ghosh et al. 2012). Targeted inhibition of p65 has demonstrated significant potential in reducing asthma-related inflammation (Pang et al. 2019; Dai et al. 2021). Furthermore, disruptions in the balance between oxidative and antioxidant pulmonary mechanisms can activate NF-κB, linking oxidative stress directly to inflammatory processes (Henderson et al. 2002).

A robust pulmonary antioxidant defense involves enzymatic and non-enzymatic pathways, crucial for mitigating oxidative stress. Key enzymes such as SOD and GPX play vital roles in neutralizing ROS (Zelko et al. 2002; Zhang et al. 2013; Kleniewska and Pawliczak 2017; Tang et al. 2019). Enhancing the activity of these enzymes has been shown to alleviate oxidative damage in asthma models, highlighting the therapeutic potential of boosting antioxidant defenses (Arthur 2000; Ho et al. 2012; Chen et al. 2014). MDA, a byproduct of lipid peroxidation, is a biomarker for oxidative stress, indicating the extent of lipid damage within cells. Excessive ROS levels can overwhelm the natural antioxidant capacity of the lungs, leading to tissue damage (Hybertson et al. 2011). This same phenomenon has been found in previous studies (Lu et al. 2023a, 2023b, 2024).

The Keap-1/Nrf2 pathway is an essential cellular defense mechanism against oxidative stress. Under normal conditions, Nrf2 is bound to Keap-1 in the cytoplasm, inhibiting its activity (Yu and Xiao 2021; Hirose et al. 2022). Oxidative stress leads to modifications in Keap-1 that trigger the release of Nrf2, which then moves to the nucleus to stimulate the expression of antioxidant genes by binding to antioxidant response elements (Li et al. 2018b; Ulasov et al. 2022; Wu et al. 2022). This process significantly enhances the cell's ability to combat oxidative stress, making it a key target for therapeutic intervention.

In this context, our study underscores the dual regulatory roles of NOB in modulating these critical pathways. By diminishing NF-κB activation and promoting Nrf2-driven antioxidant responses, NOB mitigates inflammatory responses and enhances the pulmonary antioxidant defense, thereby providing comprehensive protection against the exacerbating effects of $PM_{2.5}$ on asthma.

Our investigations focused on the respiratory damage caused by combined exposure to $PM_{2.5}$ and OVA. This dual exposure led to the release of inflammatory cytokines such as IL-1β, IL-6, and TNF-α, which contributed to extensive inflammatory infiltration in lung and tracheal tissues and increased the expression of IKK and p65 proteins, thereby intensifying airway inflammation. Concurrently, this exposure scenario resulted in elevated levels of ROS and MDA, decreased activity

of antioxidants like SOD and GPx, upregulated Keap-1, and downregulated Nrf2 expression, exacerbating oxidative damage in lung tissues.

In our asthma model exacerbated by $PM_{2.5}$ and OVA, NOB significantly reduced airway inflammation by inhibiting key inflammatory markers and proteins, including IgE, IL-1β, IL-6, TNF-α, IKK, and p65. It also mitigated oxidative lung damage by suppressing ROS and MDA levels while enhancing SOD and GPx activities and modulating Keap-1 and Nrf2 pathways. However, our study did not extensively explore the dynamic interactions and mechanisms involving IKK, p65, Keap-1, and Nrf2 within the lung tissues, suggesting avenues for further research.

Although this study has been optimized several times, there are shortcomings. The first one is that the sample size of this study is small, which cannot exclude the random phenomenon in the determination results. The second one is that the determination of Nrf2/NF-κB signaling pathway in this study was only limited to four proteins, IKK, p65, Keap-1 and Nrf2. More proteins upstream or downstream should be investigated. In the next step, we propose to explore the above issues in depth.

Conclusion

Despite these limitations, our findings underscore the therapeutic potential of NOB in ameliorating respiratory ailments triggered by environmental pollutants ($PM_{2.5}$). This research contributes to a deeper understanding of the molecular pathways involved and supports the development of NOB as a viable intervention to combat the inflammatory and oxidative challenges presented in asthma exacerbated by $PM_{2.5}$ exposure.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are available on request from the corresponding authors.

References

Arthur JR. 2000. The glutathione peroxidases. Cell Mol Life Sci. 57(13–14):1825–1835. doi: 10.1007/pl00000664.

Bai DH, Sun TX, Lu F, Shen YC, Zhang Y, Zhang B, Yu GL, Li HH, Hao JJ. 2022. Eupatilin suppresses OVA-induced asthma by inhibiting nf-κB and MAPK and activating Nrf2 signaling pathways in mice. Int J Mol Sci. 23(3):1582. doi: 10.3390/ijms23031582.

Barnes PJ, Adcock IM. 1997. Nf-kappa B: a pivotal role in asthma and a new target for therapy. Trends Pharmacol Sci. 18(2):46–50. doi: 10.1016/s0165-6147(97)89796-9.

Chagas MDSS, Behrens MD, Moragas-Tellis CJ, Penedo GXM, Silva AR, Gonçalves-de-Albuquerque CF. 2022. Flavonols and flavones as potential anti-inflammatory, antioxidant, and antibacterial compounds. Oxidative Med Cellular Longevity. 2022:9966750. doi: 10.1155/2022/9966750.

Charrad R, Berraïes A, Hamdi B, Ammar J, Hamzaoui K, Hamzaoui A. 2016. Anti-inflammatory activity of IL-37 in asthmatic children: Correlation with inflammatory cytokines tnf-α, il-β, IL-6 and IL-17A. Immunobiology. 221 (2):182–187. doi: 10.1016/j.imbio.2015.09.009.

Chen X, Huang Y, Feng J, Jiang XF, Xiao WF, Chen XX. 2014. Antioxidant and anti-inflammatory effects of Schisandra and Paeonia extracts in the treatment of asthma. Exp Ther Med. 8(5):1479–1483. doi: 10.3892/etm.2014.1948.

Cui J, Xu F, Tang Z, Wang W, Hu LL, Yan C, Luo QL, Gao HJ, Wei Y, Dong JC. 2019. Bu-Shen-Yi-Qi formula ameliorates airway remodeling in murine chronic asthma by modulating airway inflammation and oxidative stress in the lung. Biomed Pharmacother. 112:108694. doi: 10.1016/j.biopha.2019.108694.

Dagher Z, Garçon G, Billet S, Verdin A, Ledoux F, Courcot D, Aboukais A, Shirali P. 2007. Role of nuclear factor-kappa B activation in the adverse effects induced by air pollution particulate matter (PM2.5) in human epithelial lung cells (L132) in culture. J Appl Toxicol. 27(3):284–290. doi: 10.1002/jat.1211.

Dai R, Niu M, Wang N, Wang Y. 2021. Syringin alleviates ovalbumin-induced lung inflammation in BALB/c mice asthma model via nf-κB signaling pathway. Environ Toxicol. 36(3):433–444. doi: 10.1002/tox.23049.

Delfino RJ, Wu J, Tjoa T, Gullesserian SK, Nickerson B, Gillen DL. 2014. Asthma morbidity and ambient air pollution: effect modification by residential traffic-related air pollution. Epidemiology. 25(1):48–57. doi: 10.1097/ede.0000000000000016.

El Tabaa MM, El Tabaa MM, Elgharabawy RM, Abdelhamid WG. 2023. Suppressing NLRP3 activation and PI3K/AKT/mTOR signaling ameliorates amiodarone-induced pulmonary fibrosis in rats: a possible protective role of nobiletin. Inflammopharmacology. 31(3):1373–1386. doi: 10.1007/s10787-023-01168-2.

Erzurum SC. 2016. New insights in oxidant biology in asthma. Ann Am Thorac Soc. 13(Suppl 1):S35–39. doi: 10.1513/AnnalsATS.201506-385MG.

Fan CW, Ling-Hu A, Sun DL, Gao WM, Zhang CF, Duan XQ, Li HY, Tian WY, Yu Q, Ke ZL. 2023. Nobiletin ameliorates hepatic lipid deposition, oxidative stress, and inflammation by mechanisms that involve the Nrf2/NF-κB axis in nonalcoholic fatty liver disease. J Agric Food Chem. 71(50):20105–20117. doi: 10.1021/acs.jafc.3c06498.

Ghosh G, Wang VY, Huang DB, Fusco A. 2012. Nf-κB regulation: lessons from structures. Immunol Rev. 246 (1):36–58. doi: 10.1111/j.1600-065X.2012.01097.x.

Guilbert TW, Mauger DT, Lemanske RF. 2014. Childhood asthma-predictive phenotype. J Allergy Clin Immunol Pract. 2(6):664–670. doi: 10.1016/j.jaip.2014.09.010.

He M, Ichinose T, Yoshida Y, Arashidani K, Yoshida S, Takano H, Sun G, Shibamoto T. 2017. Urban PM2.5 exacerbates allergic inflammation in the murine lung via a TLR2/TLR4/MyD88-signaling pathway. Sci Rep. 7 (1):11027. doi: 10.1038/s41598-017-11471-y.

Henderson WR, Chi EY, Teo JL, Nguyen C, Kahn M. 2002. A small molecule inhibitor of redox-regulated nf-kappa B and activator protein-1 transcription blocks allergic airway inflammation in a mouse asthma model. J Immunol. 169(9):5294–5299. doi: 10.4049/jimmunol.169.9.5294.

Hirose W, Oshikiri H, Taguchi K, Yamamoto M. 2022. The KEAP1-NRF2 system and esophageal cancer. Cancers (Basel). 14(19):4702. doi: 10.3390/cancers14194702.

Hirota N, Martin JG. 2013. Mechanisms of airway remodeling. Chest. 144(3):1026–1032. doi: 10.1378/chest.12-3073.

Ho WE, Cheng C, Peh HY, Xu F, Tannenbaum SR, Ong CN, Wong WS. 2012. Anti-malarial drug artesunate ameliorates oxidative lung damage in experimental allergic asthma. Free Radic Biol Med. 53(3):498–507. doi: 10.1016/j.freeradbiomed.2012.05.021.

Hybertson BM, Gao B, Bose SK, McCord JM. 2011. Oxidative stress in health and disease: the therapeutic potential of Nrf2 activation. Mol Aspects Med. 32(4–6):234–246. doi: 10.1016/j.mam.2011.10.006.

Kaur D, Gomez E, Doe C, Berair R, Woodman L, Saunders R, Hollins F, Rose FR, Amrani Y, May R, et al. 2015. IL-33 drives airway hyper-responsiveness through IL-13-mediated mast cell: airway smooth muscle crosstalk. Allergy. 70 (5):556–567. doi: 10.1111/all.12593.

Kim RY, Pinkerton JW, Essilfie AT, Robertson AAB, Baines KJ, Brown AC, Mayall JR, Ali MK, Starkey MR, Hansbro NG, et al. 2017. Role for NLRP3 Inflammasome-mediated, IL-1β-dependent responses in severe, steroid-resistant asthma. Am J Respir Crit Care Med. 196(3):283–297. doi: 10.1164/rccm.201609-1830OC.

Kleniewska P, Pawliczak R. 2017. The participation of oxidative stress in the pathogenesis of bronchial asthma. Biomed Pharmacother. 94:100–108. doi: 10.1016/j.biopha.2017.07.066.

Li WF, Zhao RQ, Wang XM, Liu F, Zhao JM, Yao Q, Zhi WB, He ZH, Niu XF. 2018a. Nobiletin-ameliorated lipopolysaccharide-induced inflammation in acute lung injury by suppression of nf-κB pathway in vivo and vitro. Inflammation. 41(3):996–1007. doi: 10.1007/s10753-018-0753-3.

Li XN, Ma LY, Ji H, Qin YH, Jin SS, Xu LX. 2018b. Resveratrol protects against oxidative stress by activating the Keap-1/Nrf2 antioxidant defense system in obese-asthmatic rats. Exp Ther Med. 16(6):4339–4348. doi: 10.3892/etm.2018.6747.

Liu K, Hua S, Song L. 2022. PM2.5 exposure and asthma development: The key role of oxidative stress. Oxid Med Cell Longev. 2022:3618806. doi: 10.1155/2022/3618806.

Lu C, Liu Q, Deng M, Liao H, Yang X, Ma P. 2023a. Interaction of high temperature and NO2 exposure on asthma risk: In vivo experimental evidence of inflammation and oxidative stress. The Sci Total Environ. 869:161760. doi: 10.1016/j.scitotenv.2023.161760.

Lu C, Liu Q, Qiao Z, Yang X, Baghani AN, Wang F. 2024. High humidity and NO2 co-exposure exacerbates allergic asthma by increasing oxidative stress, inflammatory and TRP protein expressions in lung tissue. Environ Pollut. 353:124127. doi: 10.1016/j.envpol.2024.124127.

Lu C, Wang F, Liu Q, Deng M, Yang X, Ma P. 2023b. Effect of NO2 exposure on airway inflammation and oxidative stress in asthmatic mice. J Hazard Mater. 457:131787. doi: 10.1016/j.jhazmat.2023.131787.

Mahmutovic Persson I, Menzel M, Ramu S, Cerps S, Akbarshahi H, Uller L. 2018. IL-1β mediates lung neutrophilia and IL-33 expression in a mouse model of viral-induced asthma exacerbation. Respir Res. 19(1):16. doi: 10.1186/s12931-018-0725-z.

Mitchell PD, Ai E-G, O'Byrne PM. 2017. Anti-IgE and biologic approaches for the treatment of asthma. Handb Exp Pharmacol. 237:131–152. doi: 10.1007/164_2016_65.

Moazamiyanfar R, Rezaei S, AliAshrafzadeh H, Rastegar-Pouyani N, Jafarzadeh E, Mouludi K, Khodamoradi E, Zhaleh M, Taeb S, Najafi M. 2023. Nobiletin in cancer therapy; mechanisms and therapy perspectives. Curr Pharm Des. 29(22):1713–1728. doi: 10.2174/1381612829666230426115424.

Mortimer K, Lesosky M, García-Marcos L, Asher MI, Pearce N, Ellwood E, Bissell K, El Sony A, Ellwood P, Marks GB, et al. 2022. The burden of asthma, hay fever and eczema in adults in 17 countries: GAN Phase I study. Eur Respir J. 60(3). doi: 10.1183/13993003.02865-2021.

Nakagome K, Nagata M. 2018. Involvement and possible role of eosinophils in asthma exacerbation. Front Immunol. 9:2220. doi: 10.3389/fimmu.2018.02220.

Pang L, Zou S, Shi Y, Mao Q, Chen Y. 2019. Apigenin attenuates PM2.5-induced airway hyperresponsiveness and inflammation by down-regulating nf-κB in murine model of asthma. Int J Clin Exp Pathol. 12(10):3700–3709. PMCID: PMC6949738.

Pavón-Romero GF, Serrano-Pérez NH, García-Sánchez L, Ramírez-Jiménez F, Terán LM. 2021. Neuroimmune pathophysiology in asthma. Front Cell Dev Biol. 9:663535. doi: 10.3389/fcell.2021.663535.

Qin Y, Yang J, Li H, Li J. 2024. Recent advances in the therapeutic potential of nobiletin against respiratory diseases. Phytomedicine. 128:155506. doi: 10.1016/j.phymed.2024.155506.

Rosenwasser LJ. 2011. Mechanisms of IgEinflammation. Curr Allergy Asthma Rep. 11(2):178–183. doi: 10.1007/s11882-011-0179-6.

Sakai H, Suto W, Kai Y, Chiba Y. 2017. Mechanisms underlying the pathogenesis of hyper-contractility of bronchial smooth muscle in allergic asthma. J Smooth Muscle Res. 53:7–47. doi: 10.1540/jsmr.53.37.

Shamji MH, Valenta R, Jardetzky T, Verhasselt V, Durham SR, Würtzen PA, van Neerven RJJ. 2021. The role of allergen-specific IgE, IgG and IgA in allergic disease. Allergy. 76(12):3627–3641. doi: 10.1111/all.14908.

Stern J, Pier J, Litonjua AA. 2020. Asthma epidemiology and risk factors. Semin Immunopathol. 42(1):5–15. doi: 10.1007/s00281-020-00785-1.

Tang D, Kang R, Berghe TV, Vandenabeele P, Kroemer G. 2019. The molecular machinery of regulated cell death. Cell Res. 29(5):347–364. doi: 10.1038/s41422-019-0164-5.

Ulasov AV, Rosenkranz AA, Georgiev GP, Sobolev AS. 2022. Nrf2/Keap1/ARE signaling: Towards specific regulation. Life Sci. 291:120111. doi: 10.1016/j.lfs.2021.120111.

Wu SJ, Liao XY, Zhu ZJ, Huang R, Chen MF, Huang AH, Zhang JM, Wu QP, Wang J, Ding Y. 2022. Antioxidant and anti-inflammation effects of dietary phytochemicals: The Nrf2/NF-κB signalling pathway and upstream factors of Nrf2. Phytochemistry. 204:113429. doi: 10.1016/j.phytochem.2022.113429.

Xing YF, Xu YH, Shi MH, Lian YX. 2016. The impact of PM2.5 on the human respiratory system. J Thorac Dis. 8(1):E69–74. doi: 10.3978/j.issn.2072-1439.2016.01.19.

Xu S, Cao X. 2010. Interleukin-17 and its expanding biological functions. Cellular & Mol Immunol. 7(3):164–174. doi: 10.1038/cmi.2010.21.

Yang X, Wang H, Li T, Chen L, Zheng B, Liu RH. 2020. Nobiletin delays aging and enhances stress resistance of Caenorhabditis *elegans*. Int J Mol Sci. 21(1):341. doi: 10.3390/ijms21010341.

Yoon WJ, Moon JY, Song G, Lee YK, Han MS, Lee JS, Ihm BS, Lee WJ, Lee NH, Hyun CG. 2010. Artemisia fukudo essential oil attenuates lps-induced inflammation by suppressing nf-kappaB and MAPK activation in RAW 264.7 macrophages. Food Chem Toxicol. 48(5):1222–1229. doi: 10.1016/j.fct.2010.02.014.

Yu C, Xiao JH. 2021. The Keap1-Nrf2 system: A mediator between oxidative stress and aging. Oxid Med Cell Longev. 2021(1):6635460. doi: 10.1155/2021/6635460.

Yu PF, Pang LL, Mao QS, Zou SC, Shi Y, Lin DJ. 2019. Dose dependency PM2.5 aggravated airway inflammation in asthmatic mice via down-regulating expression of ITGB4. Eur Rev Med Pharmacol Sci. 23(4):1688–1697. doi: 10.26355/eurrev_201902_17131.

Zelko IN, Mariani TJ, Folz RJ. 2002. Superoxide dismutase multigene family: a comparison of the CuZn-sod (SOD1), Mn-sod (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic Biol Med. 33(3):337–349. doi: 10.1016/s0891-5849(02)00905-x.

Zhang XL, Zhong WQ, Meng QQ, Lin QW, Fang C, Huang XL, Li CY, Huang YG, Tan JX. 2015. Ambient PM2.5 exposure exacerbates severity of allergic asthma in previously sensitized mice. J Asthma. 52(8):785–794. doi: 10.3109/02770903.2015.1036437.

Zhang YX, Wang L, Xiao EL, Li SJ, Chen JJ, Gao B, Min GN, Wang ZP, Wu YJ. 2013. Ginsenoside-Rd exhibits anti-inflammatory activities through elevation of antioxidant enzyme activities and inhibition of JNK and ERK activation in vivo. Int Immunopharmacol. 17(4):1094–1100. doi: 10.1016/j.intimp.2013.10.013.