Resveratrol protects against asthma-induced airway inflammation and remodeling by inhibiting the HMGB1/TLR4/NF-κB pathway

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Abstract. The aim of the present study was to explore the protective role of resveratrol (RES) in asthma-induced airway inflammation and remodeling, as well as its underlying mechanism. An asthma rat model was induced by ovalbumin (OVA) treatment. Rats were randomly assigned into sham, asthma, 10 μmol/l RES and 50 μmol/l RES groups. The amount of inflammatory cells in rat bronchoalveolar lavage fluid (BALF) was detected. Pathological lesions in lung tissues were accessed by hematoxylin and eosin (H&E), and Masson's trichrome staining. Levels of inflammatory factors in lung homogenate were detected via ELISA. The blood serum of asthmatic and healthy children was also collected for analysis. Reverse transcription-quantitative polymerase chain reaction was performed to detect high mobility group box 1 (HMGB1), Toll-like receptor 4 (TLR4), myeloid differentiation primary response gene 88 (MyD88) and NF-κB expression in asthmatic and healthy children, as well as rats of the different groups. H&E staining demonstrated that multiple inflammatory cell infiltration into the rat airway epithelium of the asthma group occurred whilst the 50 μmol/l RES group displayed alleviated pathological lesions. Masson staining indicated that there was an increased airway collagen deposition area in the asthma and 10 μmol/l RES groups compared with the 50 μmol/l RES group. The number of inflammatory cells in BALF extracted from rats of the asthma and 10 μmol/l RES groups was higher compared with the 50 μmol/l RES group. Treatment with 50 μmol/l RES significantly decreased the thicknesses of the airway wall and smooth muscle. ELISA results illustrated that interleukin (IL)-1, IL-10 and tumor necrosis factor-α (TNF-α) levels were elevated, whereas IL-12 level was reduced in lung tissues of the asthma and 10 μmol/l RES groups whilst the 50 μmol/l RES group demonstrated the opposite trend. HMGB1, TLR4, MyD88 and NF-κB mRNA levels were remarkably elevated in rats of the asthma and 10 μmol/l RES groups compared with the 50 μmol/l RES group. Serum levels of IL-1, IL-10 and TNF-α were elevated, whereas IL-12 was reduced in asthmatic children compared with healthy children. The present results demonstrated that a large dose of RES alleviated asthma-induced airway inflammation and airway remodeling by inhibiting the release of inflammatory cytokines via the HMGB1/TLR4/NF-κB pathway.

Introduction

Asthma is a common chronic respiratory disease in children. Its main pathological features are airway inflammation and airway remodeling. Eosinophils, neutrophils and other inflammatory cells participate in asthma-induced airway remodeling (1-3). As a chronic inflammatory disease, asthma may progress to irreversible airway remodeling without the appropriate treatment (4). Research has identified that asthma pathogenesis involves multiple mechanisms, including genetic mechanisms, the immune response, chronic airway inflammation, airway hyper responsiveness, airway neuromodulation disorders and neural signaling pathway (5-7). The immune response is the leading pathogenic factor.

High mobility group box 1 (HMGB1) is a highly conserved nuclear protein that is released by mononuclear cells, macrophages and other immune cells following stimulation by lipopolysaccharide, tumor necrosis factor-α (TNF-α) or interleukin (IL)-1. HMGB1 is also passively released from damaged and necrotic tissue cells to further promote the secretion of a number of inflammatory factors. As a vital endogenous pro-inflammatory factor and inflammatory mediator, HMGB1 participates in the pathological processes of sepsis, pneumonia, arthritis and other diseases (8,9). Extracellular HMGB1 promotes cytokine release via the mitogen-activated protein kinase (MAPK), ERK1/2 and NF-κB pathways (10). HMGB1 also activates Toll-like receptor (TLR) 2 and TLR4, which in turn leads to a downstream inflammatory response via targeting myeloid differentiation primary response gene 88 (MyD88) and NF-κB (11). It has been demonstrated that TLR4 is an essential receptor in HMGB1-induced inflammation (12). HMGB1 exerts its pro-inflammatory role via binding to TLR4 leading to release of inflammatory cytokines

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such as IL-β, IL-10, IL-12 and TNFα (13,14). The present study hypothesized that the HMGB1/TLR4/NF-κB pathway may be of great significance in the pathogenesis of asthma.

In recent years, resveratrol (RES) has attracted research focus due to its low toxicity and wide range of action. Beneficial effects of RES have been demonstrated in pulmonary fibrosis, chronic obstructive pulmonary disease, pulmonary hypertension and other lung diseases (15-17). RES can effectively inhibit eosinophil activation and degranulation (18), and alleviate airway inflammation and airway hyper-responsiveness in an acute asthma model (19). In addition, RES regulates the differentiation of type 1 T helper (Th1)/Th2 cells through Tbet/GATA binding protein 3 pathway, suggesting that RES may have a role in protecting against bronchial asthma (20). However, the specific mechanisms of RES in regards to asthma remains not fully understood. The present study first constructed an asthma rat model. Following intervention with different doses of RES, the alterations in rat airway remodeling and the HMGB1/TLR4/NF-κB pathway were observed. These findings provided a theoretical basis for improving clinical asthma outcomes in children.

Materials and methods

Animal procedures. A total of 40 male Sprague Dawley rats (age, 4 weeks; weight, 80±15 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed at 24±3˚C and humidity of 40-60% on a 12-h light/dark cycle (lights on at 06:00 am). The rats were provided food and water *ad libitum*. Rats were acclimated for 1 week and subsequently randomly assigned into the sham, asthma, 10 μmol/l RES or 50 μmol/l RES groups (n=10).

Asthma rat model construction. A total of 0.2 ml of mixed antigen [2 mg ovalbumin (OVA) + 40 mg aluminum hydroxide] solution (Sigma-Aldrich; Merck KGaA) was intraperitoneally injected into rats of the asthma group, 10 μmol/l RES (Sigma-Aldrich; Merck KGaA) group and 50 μmol/l RES group on day 1, 8 and 15, respectively. Rats in the sham group received intraperitoneal injection of 0.2 ml of saline. On day 22, rats in the asthma group, 10 μmol/l RES group and 50 μmol/l RES group received atomization inhalation of 1% OVA to induce the asthma model. For the RES group rats, 30 min before inhalation of OVA, 0.2 ml of either 10 μmol/l or 50 μmol/l RES was injected intraperitoneally into rats. Rats in the sham group and asthma group were injected with 0.2 ml of saline. The experiment lasted until day 35.

Bronchoalveolar lavage fluid (BALF) sample collection. Rats were anesthetized with 1% sodium pentobarbital (40 mg/kg) intraperitoneally prior to collection of BALF. In brief, 1 ml of saline was injected into rats then repeat suction was performed three times. BALF was recycled and centrifuged at 400 x g for 15 min at 4˚C. A sample was considered as suitable for experimentation when the recycled amount was >0.8 ml. Cell counting was performed within 1 h. Cell sedimentation was resuspended with PBS solution, then 10 μl was used for measurement of total cell number. Then 0.1 ml was used for cell pellets smear. The cell pellets smear was fixed and stained with Wright's staining. Cells were differential counted in

three visual field and the values were averaged as previously described in the literature (19).

Hematoxylin and eosin (H&E) staining. The right lung tissue of rats was fixed with 10% paraformaldehyde (Sigma-Aldrich; Merck KGaA) at room temperature for 48 h. Then the tissue was dehydrated in an ascending series of ethanol, embedded in paraffin and sectioned (5 μm). Following deparaffinization in xylene and rehydration in a descending series of alcohol, lung tissues were stained with H&E. Inflammatory cell infiltration and airway epithelial injury were observed under a light microscope.

Masson staining. The aforementioned paraffin embedded slices (5 μm) were stained with Weigert solution (Sigma-Aldrich; Merck KGaA) for 5-10 min. After being fully washed, sections were treated with Ponceau fuchsin acid solution for 5-10 min, immersed in 2% acetic acid aqueous solution for 1 min, then differentiated in 1% phosphomolybdic acid aqueous solution for 3-5 min. Without washing with water, the sections were treated with aniline blue for 5 min then immersed in 0.2% acetic acid aqueous solution for 1 min. Slices were permeabilized with xylene and mounted with neutral resin.

Measurement of the thicknesses of airway wall and smooth muscle. The intact small bronchioles were identified using a light microscope. Three transverse sections were randomly selected in each rat. Basement membrane perimeter (Pbm), total bronchial wall area (Wat_1), bronchial luminal area (Wat_2), external smooth muscle area (Wam_1) and internal smooth muscle area (Wam_2) of each rat were accessed using Image Pro Plus v.6.0 software (Media Cybernetics, Inc.). The thickness of airway wall (Wan) and smooth muscle (Wat) were then calculated using the following formulas:

$$(1)\ Wan=(Wat_1-Wat_2)/Pbm$$
$$(2)\ Wat=(Wam_1-Wam_2)/Pbm$$

Determination of inflammatory factors in lung homogenate. Harvested rat lung tissue (0.5 g) was ground and centrifuged at 4,500 x g for 10 min at 4˚C for preparation of lung homogenate. Levels of IL-1, IL-10, IL-12 and TNF-α in rat lung homogenate were detected using the respective ELISA kits (Rat IL-1 ELISA kit, cat. no. RAB0272; Rat IL-10 ELISA kit, cat. no. RAB0246; Rat TNF-α ELISA kit cat. no. RAB0480; all from Sigma-Aldrich; Merck KGaA; and Rat IL-12 ELISA kit, cat. no. KRC0121; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Reverse transcription-quantitative PCR (RT-qPCR). The lung tissue (0.5 g) was cut and homogenized; TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA. Total RNA underwent reverse transcription according to the instructions of PrimeScript RT reagent Kit (Takara Bio, Inc.). qPCR was performed using SYBR®-Green Master Mix (Takara Bio, Inc.) according to the manufacturer's protocol; amplification was performed under the recommended parameters: Initial denaturation at 95˚C for 5 min, followed by 40 cycles of 95˚C for 5 sec, 60˚C for 15 sec and 72˚C for 15 sec, and a final extension at 94˚C for 15 sec. The expression level of the target gene was calculated using the $2^{-\Delta\Delta Cq}$ method (21). Primers are listed in Table I.

Table I. Primer sequences.

Gene name	PCR size (bp)	Primer sequence (5'→3')
HMGB1	356	F: CGGATGCTTCTGTCAACT R: TCAGCTTGGCAGCTTTCT
TLR4	310	F: GGTGAGAAATGAGCTGGTA R: TCTGCTAAGAAGGCGATA
MyD88	113	F: CGTCGCATGGTGGTGGTTGTTT R: GGGATCAGTCGCTTCTGTTGGA
NF-κB	425	F: GCGCATCCAGACCAACAATAAC R: GCCGAAGCTGCATGGACACT
GAPDH	526	F CCACTTGAAGGGTGGAGC R: TGAAGTCGCAGGAGACAA

HMGB1, high mobility group box 1; MyD88, myeloid differentiation primary response gene 88; TLR4, Toll-like receptor; R, reverse; F, forward.

Determination of inflammatory factors in serum of children. A total of 34 pediatric patients with asthma (age, 4-14 years; 19 male, 15 female), admitted to The Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University (Changzhou, China) from January 2018 to May 2018 were selected as the asthma group. The diagnosis of childhood asthma conforms to the diagnostic criteria in the Guidelines for the Diagnosis and Prevention of Childhood Bronchial Asthma, formulated by the Respiratory Group of the Chinese Medical Association in 2008. Exclusion criteria: i) Patients with other tracheal, bronchial or pulmonary diseases; ii) patients with severe multiple organ, nervous or psychiatric diseases. Subjects were all in the acute asthmatic stage regardless of the severity of the attack. For the control group, 24 healthy children (age, 4-12 years; 13 male, 11 female) were selected during the same period. From each subject, 5 ml of venous blood sample was collected for detecting serum levels of IL-1, IL-10, IL-12 and TNF-α by ELISA. Written informed consent was obtained from the legal guardians for all patients and healthy controls.

Statistical analysis. Data were analyzed by SPSS v.17.0 statistical software (SPSS Inc.). Quantitative data were presented as mean ± standard deviation. Student's t-test was used for comparing differences between two groups. One-way analysis of variance was performed for assessing differences amongst multiple groups, followed by Fisher's least significant difference analysis or Dunnett's test. P<0.05 was considered to indicate statistical significance.

Results

High dose RES treatment attenuates pathological lesions in a rat asthma model. Pronounced pathological lesions were observed in the lung tissues of the asthma group, manifesting as edema and abscission of airway epithelium, airway constriction, multiple inflammatory cell infiltration and tracheal smooth muscle thickening (Fig. 1A). In addition, marked capillary congestion, alveolar fusion enlargement and alveolar septum widening were observed in asthma rats (Fig. 1A). Pathological changes were similar in the 10 µmol/l RES group. By contrast, pathological changes in lung tissues were less pronounced in the 50 µmol/l RES group compared with the asthma group (Fig. 1A).

High dose RES treatment decreases airway collagen deposition area. Masson staining is commonly used to stain collagen fibers, mucus and cartilage blue. Muscle fibers, cellulose and red blood cells are stained red, whilst cell nuclei are stained blue-black. Airway collagen deposition was more marked in the asthma group compared with the sham group (Fig. 1B). No significant difference in airway collagen deposition area was observed between the asthma group and 10 µmol/l RES group (Fig. 1B). By contrast, the airway collagen deposition area was markedly reduced in the 50 µmol/l RES group compared with the asthma group (Fig. 1B).

High dose RES treatment decreases inflammatory cell levels in the BALF of asthma model rats. Total amounts of inflammatory cells, eosinophils and lymphocytes were elevated in the asthma group and the 10 µmol/l RES group compared with the sham group (P<0.05; Fig. 2A-C). By contrast, the amounts of inflammatory cells, eosinophils and lymphocytes were significantly decreased in the 50 µmol/l RES group (P<0.05; Fig. 2A-C).

High dose RES treatment decreases airway wall and smooth muscle thickness in asthma model rats. Airway wall and smooth muscle thickness were increased in the asthma group and 10 µmol/l RES group compared to the sham group (P<0.05; Fig. 2D and E). Treatment with 50 µmol/l RES decreased airway wall and smooth muscle thickness (P<0.05; Fig. 2D and E).

High dose RES treatment decreases inflammatory factor levels in lung tissues of asthma model rats. ELISA results illustrated that IL-1, IL-10 and TNF-α levels in lung tissues of the asthma and 10 µmol/l RES groups were elevated, whereas IL-12 level was reduced (P<0.05; Fig. 3). In the 50 µmol/l RES group, IL-1, IL-10 and TNF-α levels were downregulated but IL-12 was upregulated in rat lung tissues following establishment of the asthma model (P<0.05; Fig. 3).

High dose RES treatment reduces HMGB1, TLR4, MyD88 and NF-κB expression in asthma model rats. HMGB1, TLR4, MyD88 and NF-κB mRNA expression levels were remarkably elevated in rats of the asthma and 10 µmol/l RES groups compared with the sham group (P<0.05; Fig. 4). By contrast, 50 µmol/l RES treatment significantly decreased HMGB1, TLR4, MyD88 and NF-κB mRNA expression in rat lung tissue following establishment of the asthma model (P<0.05; Fig. 4).

Inflammatory factor levels increase in serum samples of asthmatic children. Serum levels of IL-1, IL-10, IL-12 and TNF-α in asthma children and healthy children were analyzed. Asthmatic children had increased IL-1, IL-10 and TNF-α serum levels, as well as decreased IL-12 levels compared with healthy children (P<0.05; Fig. 5).
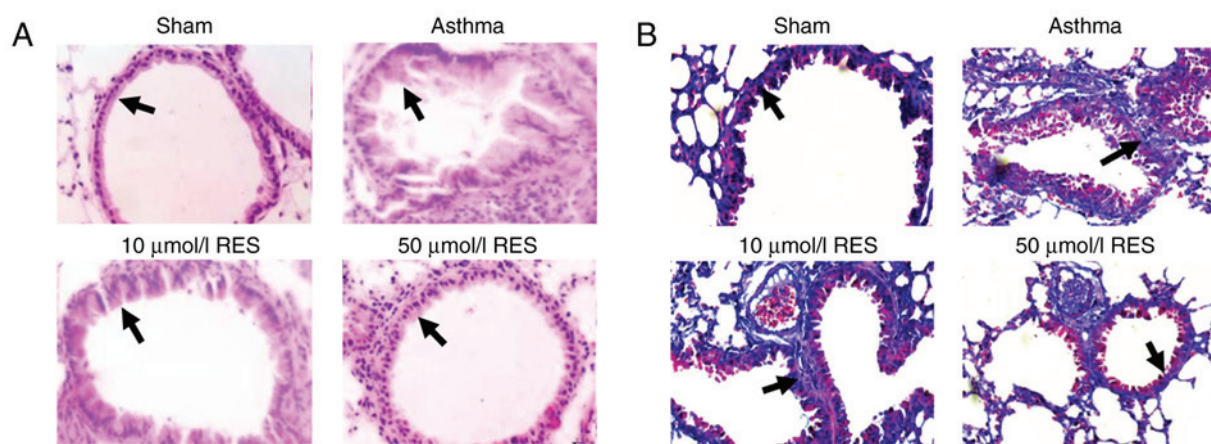
Figure 1. Hematoxylin and eosin, and 2 of rat lung tissues demonstrating pathological lesions and collagen deposition following establishment of an asthma rat model. (A) Pathological lesions in rat lung tissue of sham, asthma, 10 μmol/l RES and 50 μmol/l RES groups. (B) Alterations of airway collage deposition (blue staining) in rat lung tissues of sham, asthma, 10 μmol/l RES and 50 μmol/l RES groups (magnification, x200). RES, resveratrol.
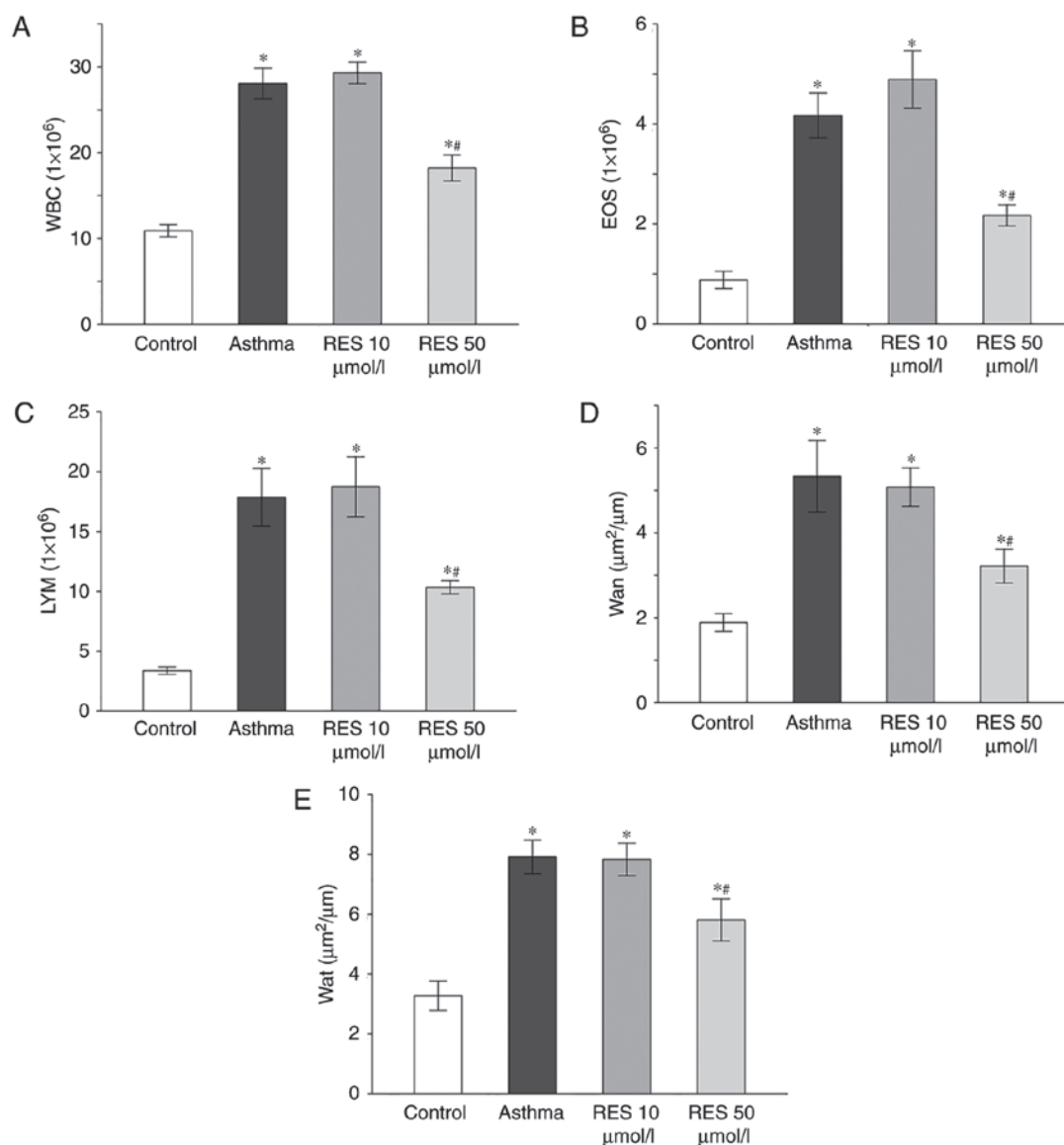

Figure 2. Inflammatory cell amount in BALF, and airway wall and smooth muscle thickness for sham, asthma, 10 μmol/l RES and 50 μmol/l RES groups. (A) Number of inflammatory cells in rat BALF for different groups. (B) Number of eosinophils for different groups. (C) Number of lymphocytes for different groups. (D) Thickness of airway wall for different groups. (E) Thickness of airway smooth muscle for different groups. *P<0.05 vs. sham group; $^\#$P<0.05 vs. asthma group. BALF, bronchoalveolar lavage fluid; RES, resveratrol; EOS, eosinophils; LYM, lymphocytes; Wan, the thickness of airway wall; Wat, the thickness of smooth muscle.
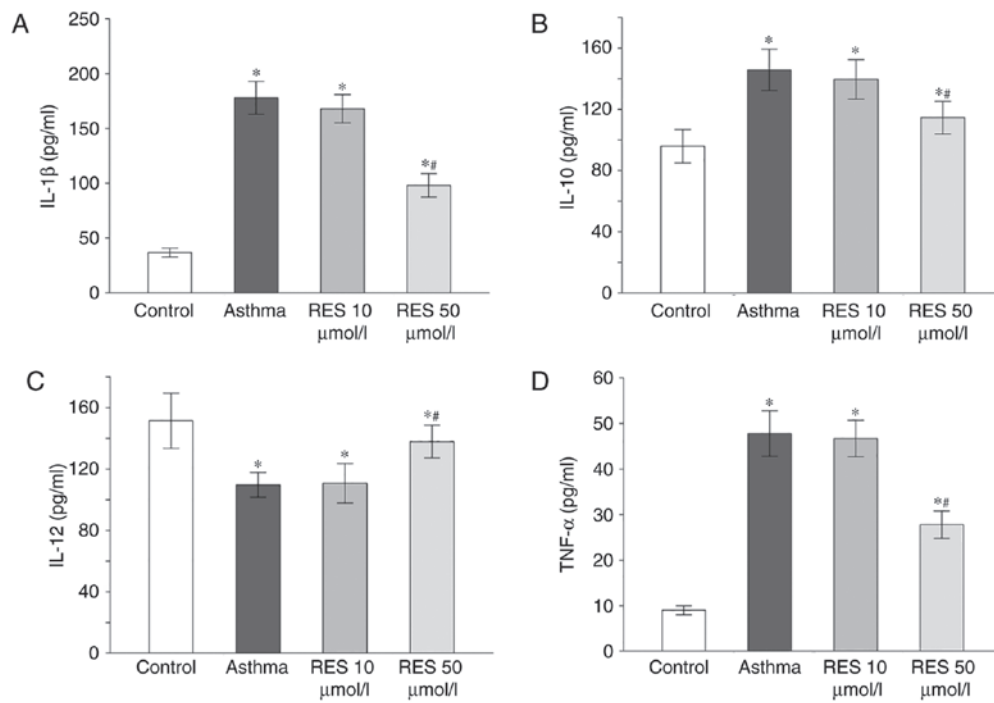
Figure 3. IL-1, IL-10, IL-12 and TNF-α levels in rat lung tissues for sham, asthma, 10 µmol/l RES and 50 µmol/l RES groups. (A) IL-1β, (B) IL-10, (C) IL-12, and (D) TNF-α levels in rat lung tissues from the different experimental groups. *P<0.05 vs. control; #P<0.05 vs. asthma group. IL, interleukin; TNF-α, tumor necrosis factor-α; RES, resveratrol.
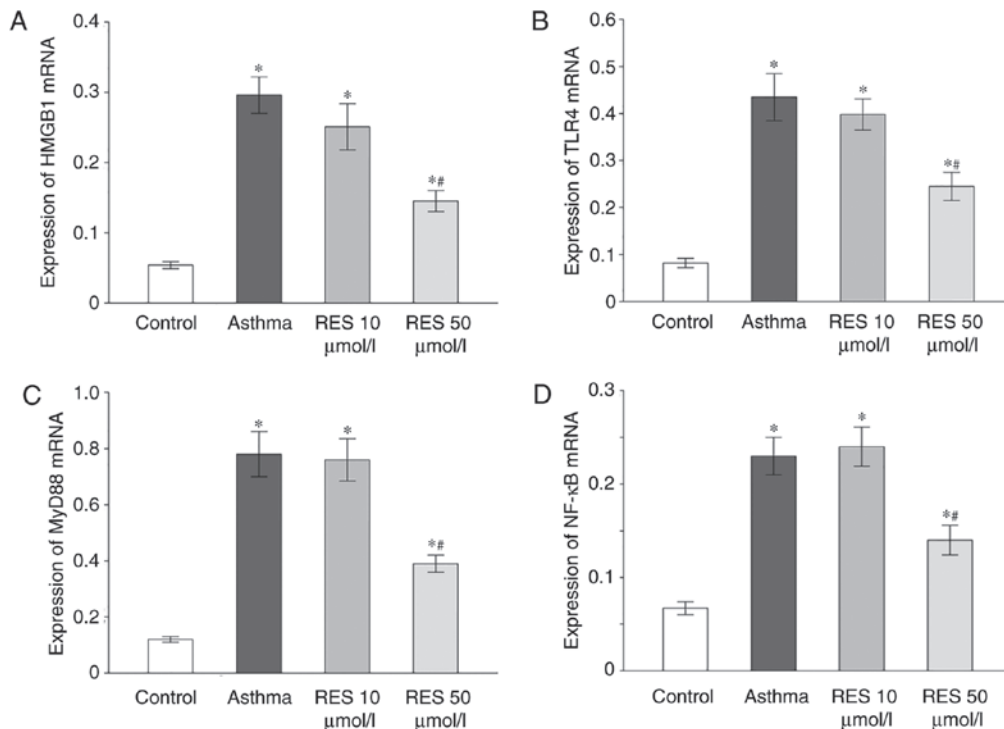

Figure 4. HMGB1, TLR4, MyD88 and NF-κB mRNA expression in rat lung tissues for sham, asthma, 10 µmol/l RES and 50 µmol/l RES groups. (A) HMGB1, (B) TLR4, (C) MyD88 and (D) NF-κB mRNA expression levels in rat lung tissues from the different experimental groups. *P<0.05 vs. control; #P<0.05 vs. asthma group. HMGB1, high mobility group box 1; TLR4, Toll-like receptor; MyD88, myeloid differentiation primary response gene 88; RES, resveratrol.

Discussion

Bronchial asthma in children is a common disease that can severely influence daily life (1). Asthma can progress to adulthood if proper and timely treatment is not received.

Pathologically, asthma is an airway chronic inflammatory disease involving a variety of cells and cellular components. Airway inflammation and remodeling are important pathological features of chronic asthma. IL-1β has an important role in the development of allergic asthma by inducing the formation
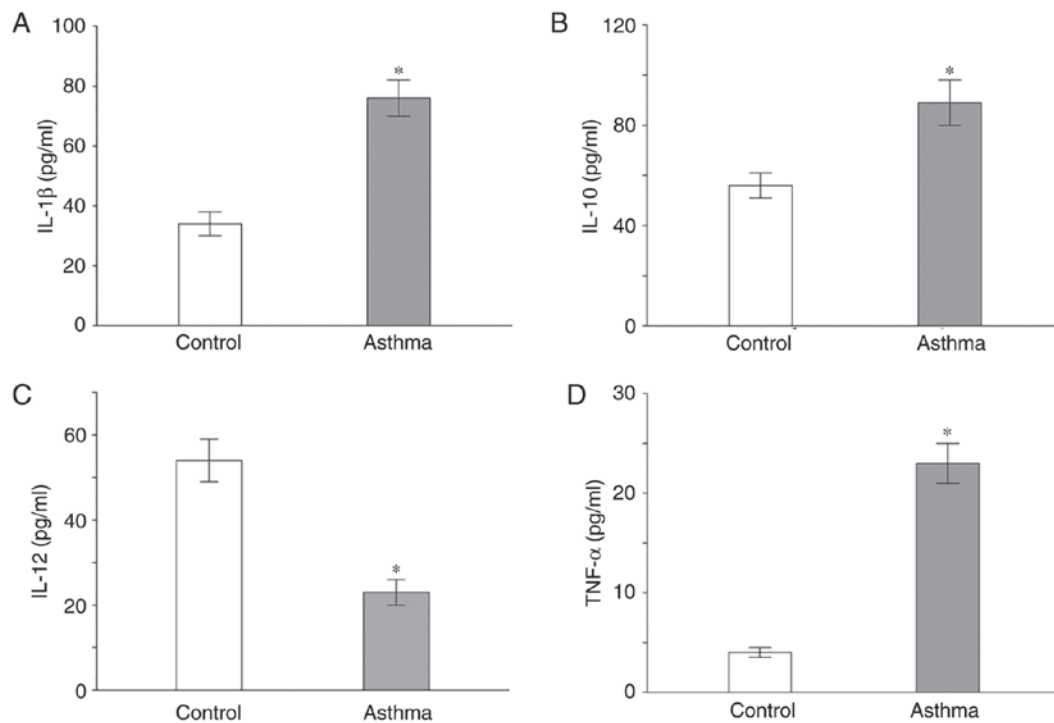
Figure 5. Inflammatory factor levels in serum samples of asthmatic and healthy children. (A) Serum levels of IL-1β, (B) IL-10, (C) IL-12 and (D) TNF-α. *P<0.05 vs. control. IL, interleukin; TNF-α, tumor necrosis factor-α.

of Th17 cells (22). IL-10 can regulate immune function and improve airway inflammatory response (23). IL-12 is a Th1 cytokine, which increases airway hyper responsiveness due to exogenous injury and decreases eosinophil aggregation around the airway (24). The present study determined that serum levels of IL-1, IL-10 and TNF-α in lung tissues of asthma model rats and serum samples of asthmatic children were significantly elevated. By contrast, IL-12 level was decreased. These results indicated that the inflammatory response is involved in the occurrence and progression of asthma.

HMGB1 is widely present in the cell nucleus and cytoplasm. Nuclear membrane permeability is increased by inflammation, which in turn promotes the translocation of HMGB1 to the cell cytoplasm where it exerts pro-inflammatory effects (14,25,26). HMGB1 stimulates mononuclear cell and macrophage-secreted inflammatory mediators, such as IL-1β, IL-10, IL-12 and TNF-α (27). In addition, HMGB1 promotes major histocompatibility complex II, CD80, CD83, CD86 and other costimulatory molecules in dendritic cells (DCs), inducing DC maturation to produce pro-inflammatory cytokines (28). Eosinophils are recruited by HMGB1 to the inflammatory sites further aggravating the inflammatory response (29,30). Recent studies have identified that HMGB2 deteriorates airway inflammation by upregulating TNF-α, VEGF, MMP-9 and TSLP expression. HMGB1 knockdown remarkably decreases relative indicators of airway inflammation, mucus secretion, collagen deposition, and airway smooth muscle thickness (31). TLR4 was the first TLR identified in mammals; it comprises an extracellular domain, a transmembrane domain and an intracellular domain (32,33). Relative studies have demonstrated that TLR4 is involved in the regulation of airway inflammation through regulating DC maturation and activation, antigen presentation and T cell

immune responses. TLR4 is overexpressed in airway smooth muscle cells by elastase stimulation, leading to thickening of the airway wall via activation of NF-κB (34) therefore it is evident that TLR4 participates in airway remodeling. NF-κB is a nuclear transcription factor with various biological activities. NF-κB pathway regulates genes involved in asthma-related airway inflammation (35). Inhibition of NF-κB activity alleviates the airway inflammatory response, thereafter improving airway remodeling. TLR4 activates NF-κB through MyD88-dependent and MyD88-independent ways, eventually stimulating the production of pro-inflammatory factors. HMBG1 induces the immune and inflammatory response after binding to its receptor TLR4 (36,37). It is hypothesized that the HMGB1/TLR4/NF-κB pathway may serve an important role in the development and progression of asthma.

RES is a plant polyphenolic substance present in grape skins, berries and nuts (38). Numerous studies have demonstrated that RES exerts anti-apoptosis, anti-inflammatory and anti-oxidative effects *in vitro* (39-43). RES also alleviates airway inflammation and airway hyper responsiveness *in vivo*, partially through inhibiting eosinophil activation and degranulation (18,19). The most pronounced effect of RES is inflammation alleviation, mainly via NF-κB pathway regulation (44,45). RES is capable of inhibiting TLR4 expression in cardiomyocytes with hypoxia-reoxygenation injury (46). However, the pathogenesis of asthma and the mechanism of RES on asthma are complex. In the present study, airway lesions were obvious in asthma model rats, manifesting as abundant inflammatory cell infiltration. Pathological lesions in rat airways were remarkably alleviated by treatment with 50 µmol/l RES. However, 10 µmol/l RES treatment did not present therapeutic effect on asthma-induced inflammation. HMGB1, TLR4, MyD88 and NF-κB mRNA levels in

50 μmol/l RES group were significantly lower compared with the asthma group. Treatment with 10 μmol/l RES did not affect HMGB1, TLR4, MyD88 and NF-κB mRNA levels following establishment of an asthma model. The results confirmed that HMGB1 and TLR4 were involved in asthma-induced airway inflammation. However, the present study used only RT-qPCR to detect HMGB1, TLR4, MyD88 and NF-κB levels; additional experimental methods should be investigated in future work. In addition, detection of the remodeling-associated proteins and verification of which cell type is targeted by RES require to be further elucidated. Finally, the relationship of the HMGB1/TLR4/MyD88/NF-κB axis and RES will be further studied in future work.

In conclusion, the present study demonstrated that large dose RES alleviated asthma-induced airway inflammation and airway remodeling by inhibiting the release of inflammatory cytokines via the HMGB1/TLR4/NF-κB pathway. The present results provided evidence for RES as a potential novel treatment for asthma.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HJ and WZ designed the study and performed the experiments. HJ, JD and KX established the animal models. HJ and KX collected the data. HJ and KX analyzed the data. HJ and WZ prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Experimental protocols involving the use of animals were approved by the Animal Ethics Committee of Nanjing Medical University Animal Center. Protocols involving the use of human samples were approved by the Ethics Committee of the Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University (Changzhou, China). Written informed consent was obtained from the legal guardians of all patients and healthy controls.

Patient consent for publication

Not applicable.

Competing interests

Not applicable.

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