Ethyl pyruvate attenuates murine allergic rhinitis partly by decreasing high mobility group box 1 release

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Abstract

High-mobility group box 1 (HMGB1) protein, a pro-inflammatory DNA-binding protein, mediates inflammatory responses through Toll-like receptor-4 signals and amplifies allergic inflammation by interacting with the receptor for advanced glycation end products. Previous studies have shown that HMGB1 is elevated in the nasal lavage fluids (NLF) of children suffering from allergic rhinitis (AR) and is associated with the severity of this disease. Furthermore, HMGB1 has been implicated in the pathogenesis of lower airway allergic diseases, such as asthma. Ethyl pyruvate (EP) has proven to be an effective anti-inflammatory agent for numerous airway diseases. Moreover, EP can inhibit the secretion of HMGB1. However, few studies have examined the effect of EP on AR. We hypothesized that HMGB1 plays an important role in the pathogenesis of AR and studied it using an AR mouse model. Forty BALB/c mice were divided into four groups: the control group, AR group, 50 mg/kg EP group, and 100 mg/kg EP group. The mice in the AR and EP administration groups received ovalbumin (OVA) sensitization and challenge, whereas those in the control group were given sterile saline instead of OVA. The mice in the EP administration group were given an intraperitoneal injection of EP 30 min before each OVA treatment. The number of nasal rubbings and sneezes of each mouse was counted after final treatment. Hematoxylin–eosin staining, AB-PAS staining, interleukin-4 and 13 in NLF, IgE, and the protein expression of HMGB1 were measured. Various features of the allergic inflammation after OVA exposure, including airway eosinophilia, Th-2 cytokine production, total IgE, and goblet cell hyperplasia were significantly inhibited by treatment with EP and the expression and release of HMGB1 were reduced after EP administration in a dose-dependent manner. These results indicate that HMGB1 is a potential therapeutic target of AR and that EP attenuates AR by decreasing HMGB1 expression.

Keywords: Mouse model, allergic rhinitis, ethyl pyruvate, high mobility group box 1, IgE

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Introduction

Allergic rhinitis (AR), a kind of allergic airway inflammation, is characterized by persistent symptomatic inflammation of the nasal mucosa. The symptoms include rhinorrhoea, nasal congestion, nasal itching, and sneezing.^{1–3} Mast cell responses induced by an IgE-mediated reaction and by late-phase responses of eosinophils and Th-2 cells are involved in the inflammatory response of nasal mucosa in AR.⁴ It is a common allergic disease throughout the world. The prevalence of AR in China ranges from 8.7 to 24.1%⁵ and has been increasing.⁶ Recent studies have shown that there are high levels of high-mobility group box 1 protein (HMGB1) in the nasal lavage fluids (NLF) of children with AR and that the level of HMGB1 is

associated with the severity of the disease.² There is evidence that HMGB1 plays a role in chronic rhinosinusitis with/without nasal polyps.^{7–9} Furthermore, previous studies^{10–12} have suggested that HMGB1 plays a role in asthma, a lower airway inflammation that is linked with AR.³ HMGB1 is involved in the pathogenesis of the disease. Airway hyperresponsiveness and pathological changes in either eosinophilic or neutrophilic asthma can be attenuated by blocking HMGB1 activity with an antibody or anti-inflammatory agent such as ethyl pyruvate (EP).^{11,13} However, the expression pattern and alteration of HMGB1 after ovalbumin (OVA) exposure in AR, an upper airway allergic disease, have not been studied.

HMGB1 was originally known as a non-histone DNA-binding protein that functions as a transcription

We hypothesized that HMGB1 is up-regulated and translocated after OVA exposure, and that EP administration can relieve AR by attenuating the expression of HMGB1. To test this hypothesis, we established a mouse AR model

according to the methods given in Saito's report²⁹ and examined the inhibitory effect of EP on the expression of HMGB1.

Methods and materials

Animals

Forty wild-type male BALB/c mice aged from 6 to 8 weeks were purchased from the Center for Animal Experiment, Wuhan University. The animals were kept in specific pathogen free animal facility. These mice were randomly divided into four groups, with 10 mice in each group: the control group, AR group, 50 mg EP group, and 100 mg EP group. Six mice from each group were used for the analysis of protein expression using the western blot technique, and the remaining mice were used for histological observation and immunochemistry. The experiments were conducted according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of Tongji Medical College, Huazhong University of Science and Technology (2013 IACUC Number: 305).

OVA sensitization and challenge

The establishment of mouse AR and the administration of EP were performed almost as described in Saito's research. The mice were injected intraperitoneally on the first, eighth, and 15th days of the study with a solution consisting of 40 µg OVA (Grade V, Sigma, MO, USA) dissolved in 200 µL PBS which was emulsified with 2 mg aluminum hydroxide. The OVA challenges were performed daily by intranasal instillation with 500 µg OVA in 20 µL PBS into the bilateral nasal cavity from the 22nd day and lasted for one week (Figure 1) in the AR and EP administration groups. Instead of the OVA

Figure 1 The protocol of mouse AR model establishment and EP administration. Mice in AR and EP groups were sensitized with 40 µg OVA and 2 mg aluminum hydroxide mix solution by intraperitoneal injection on days first, eighth, and 15th. OVA solution was replaced with sterile saline in control group. Mice in AR and EP groups were challenged with OVA by daily instillation from day 22nd to day 29th. Mice from EP groups received an intraperitoneal injection with EP at a dose of 50 or 100 mg/kg at half an hour before intranasal instillation, respectively. Mice from control and AR group received Ringer's solution instead of EP. Mice were sacrificed 24 h later after last OVA challenge. ID: intranasal drop; IP: intraperitoneal injection

solution, sterile saline was used in the control group. Accompanying the OVA challenge, EP in Ringer's solution was given by intraperitoneal injection 30 min before each OVA treatment at doses of 50 mg/kg body weight and 100 mg/kg body weight, respectively in the 50 and 100 mg EP groups. Because of the instability of EP in solution, the EP solutions were prepared just prior to administration.³⁰

Evaluation of nasal symptoms

On day 29 after the final intranasal administration, the mice ($n=6$) from each group were given 10 min of acclimatization in an observation cage before further experimentation. The number of sneezes and the nasal rubbings of each mouse was counted for 10 min by blinded observers.

Tissue preparation

All mice were deeply anesthetized by intraperitoneal injection with a ketamine-chlorpromazine mixture (10:1, 2 mL/kg body weight) 24 h after the last challenge. Nasal lavage was performed as Cho described,³¹ and NLFs were collected. Blood samples were collected via the inferior vena cava and serum samples were obtained after centrifugation and stored until use at -80°C . The mice were killed by decapitation and the nasal mucosa was collected from mice. These samples were also stored at -80°C until use.

Western blot analysis

The protein expression of HMGB1 was examined with a western blot. The total protein was extracted from the nasal mucosa samples using a RIPA Lysis Buffer (Beyotime, Haimen, Jiangsu, China) according to the manufacturer's instructions. Protein concentrations were detected by an Enhanced BCA Protein Assay Kit (Beyotime, Haimen, Jiangsu, China). Twenty micrograms of each protein lysate was separated using 12% SDS-polyacrylamide gels and transferred to polyvinylidenedifluoride membranes. The membranes were blocked for 40 min with 5% fat-free milk in a Tris-buffered saline mixed with 0.1% Tween (TBST) and incubated overnight at 4°C with the appropriate dilution of primary antibodies: anti-HMGB1 (Epitomics, Burlingame, CA, USA, diluted 1:10,000) and anti- β -actin (Millipore, Billerica, MA, USA, diluted 1:3000). After washing the membranes to remove any excess primary antibody, the membranes were incubated for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (diluted 1:3000). The protein bands were visualized by BeyoECL Plus (Beyotime, Haimen, Jiangsu, China). The Gel-Pro analyzer 4.0 software (Media Cybernetics, Inc., USA) was used for the relative quantification processing. β -actin was used as an internal control.

Enzyme linked immunosorbent assay

The level of total IgE in serum and those of IL-4 and IL-13 in NLF were determined using an ELISA kit (NeoBioscience, Shenzhen, Guangdong, China) in accordance with the manufacturer's instructions. Serum OVA-specific IgE, IgG1, and IgG2a were also detected using an ELISA kit (eBioscience, San Diego, CA, USA). To avoid assay

saturation, optimal dilutions were set as 1/50,000 for IgG1 and 1/100 for IgG2a.

Histological observation and immunohistochemistry

Four mice from each group were used for the analysis of the HMGB1 localization and inflammatory cell infiltration. The mice were humanely killed and immediately decapitated. The snouts were fixed with 4% formaldehyde for 12 h, decalcified with 10% ethylenediaminetetraacetic acid-2 Na in PBS for one month, dehydrated in a graded ethanol series (70, 80, 90, 95, and 100%), and embedded in paraffin. Finally, the snouts were sectioned at $5\mu\text{m}$ and collected on poly-L-lysine-coated glass slides.

For the immunohistochemical study, a REALTM EnvisionTM Detection Kit (Dako, Copenhagen, Denmark) was used, and all of the procedures were performed according to the manufacturer's instructions. After deparaffinization and dehydration in a graded ethanol series (70, 90, 95, and 100%), the tissue sections were subjected to heat-induced epitope retrieval. After being washed three times with PBS, the sections were incubated with 4% H_2O_2 for 10 min to block endogenous peroxidases. Bovine serum albumin protein was applied to saturate any excess protein-binding sites. The blocked sections were incubated with an antibody against HMGB1 (diluted 1:250) at 4°C overnight. A secondary antibody was applied to the sections for 50 min at 4°C , and the slides were again washed three times with PBS after incubation. Finally, the slides were stained with hematoxylin, mounted, and observed under a light microscope.

To observe the eosinophilic infiltration, hematoxylin and eosin staining was performed. Briefly, the tissue sections were deparaffinized, dehydrated, and then stained with hematoxylin and eosin for 30 and 5 s, respectively. The histological assessment was conducted under a light microscope. The number of eosinophils was counted in five high-power fields (magnification $400\times$). The count data came from the mean of the results from five randomly chosen areas.

To observe the goblet cell hyperplasia, the Alcian blue and periodic acid Schiff's stain technique was used. After deparaffinization and dehydration, the sections were stained with Alcian blue for 10 min. After the sections were washed with distilled water, the slides were sequentially stained with periodic acid and Schiff's stain. Finally, they were counterstained with hematoxylin and observed under a light microscope. AB-PAS-positive cells were quantified along the mucosa of the unilateral nasal septum at $\times 400$ magnification.

Statistical analysis

The results were expressed as mean $\pm$ SD. The statistical analysis was performed using the Statistical Package for the Social Sciences (Version 18.0; SPSS Inc., Chicago, IL, USA). The Student's *t*-test was used to analyze the expression of HMGB1. A value of $p < 0.05$ was considered to be statistically significant.

Figure 2 Effect of EP on nasal symptoms, Th-2 cytokines in NLF, and IgE in sera. Numbers of sneeze and rubbing were markedly increased after OVA challenge while the numbers were decreased with EP administration (a). IL-4, IL-13 levels in NLF and IgE level in sera were significantly elevated in AR group compared to control group. The increased cytokines levels were reduced significantly by administration of EP in a dose-dependent manner (b, c). IgE level only was decreased in 100 mg EP group (d). *compare to control group, $p < 0.05$; #compare to AR group, $p < 0.05$; \$ compare between 50 and 100 mg group, $p < 0.05$

Results

Effects of EP on nasal symptoms in a mouse AR model

The effect of EP on nasal symptoms is shown in Figure 2. Treatment with either 50 or 100 mg/kg EP significantly suppressed the number of sneezes and nose rubbings in the EP group compared to the AR group (Figure 2(a), $p < 0.05$). Moreover, the incidence of sneezes but not rubbings after the final OVA challenge was reduced more in the 100 mg/kg EP group compared to that in the 50 mg/kg EP group (Figure 2(a), $p < 0.05$).

Effects of EP on cytokine levels in NLF and total IgE in sera

To determine the effects of EP on Th-2 inflammation in AR, we measured levels of IL-4 and IL-13 in NLF. The levels of IL-4 and IL-13 in NLF were significantly elevated in the AR group compared to the control group (Figure 2(b) and (c), $p < 0.05$). The increased IL-4 and IL-13 levels were reduced by the administration of EP in a dose-dependent manner, but only significantly in the 100 mg/kg EP group (Figure 2(b) and (c)). Like the Th-2 cytokines, the total IgE

in sera was only significantly reduced in the 100 mg/kg EP group (Figure 2(d), $p < 0.05$).

Effect of EP on serum OVA-specific antibodies

The OVA-specific IgE, IgG1, and IgG2a levels were significantly elevated in OVA-challenged mice compared to the control animals. The 100 mg/kg EP group had significantly reduced the levels of OVA-specific IgE whereas the 50 mg/kg EP group did not. However, EP had no effect on OVA-specific IgG1 or IgG2a (Figure 3).

Effect of EP on pathological changes in AR

The histological analysis revealed evident infiltration of inflammatory cells, including eosinophils, and notable epithelial proliferation in the OVA-sensitized/challenged mice (Figure 4(a) to (e)). The EP displayed an extensive capacity to alleviate airway inflammation in nasal mucosa. The number of AB-PAS positive cells in the airway epithelia of OVA challenged mice was obviously more than that in the control group (Figure 4(f) and (g), $p < 0.05$). Treatment with EP at a dose of 100 mg/kg body weight was more effective

Figure 3 Effect of EP on serum OVA-specific antibodies. The OVA-specific IgE, IgG1, and IgG2a levels were significantly increased in AR and EP treatment groups compared to control group. Treatment with EP at dose of 100 mg/kg significantly reduced the levels of OVA-specific IgE. In contrast, EP had no effect on OVA-specific IgG1 or IgG2a (Figure 3). The levels of OVA-specific antibodies were expressed as arbitrary unit (AU). *compare to control group, $p < 0.05$; #compare to AR group, $p < 0.05$

at reducing goblet cell hyperplasia than treatment with a dose of 50 mg/kg (Figure 4(h) to (j), $p < 0.05$).

Effects of EP on HMGB1 expression in OVA-challenged mice

To explore the role of HMGB1 in OVA-induced airway response, we observed the localization and expression of HMGB1 using immunohistochemistry and the western blot technique. Immunohistochemical analysis of snout sections revealed that HMGB1 was highly expressed in both the epithelium and lamina propria of the nasal mucosa of the control group (Figure 5(a)). The expression of HMGB1 was up-regulated in the AR group (Figure 5(b)). Moreover, the transport of HMGB1 was observed in the AR group. Generally, HMGB1 was predominantly expressed in the nuclei (Figure 5(a), red arrow), although a bit of cytoplasmic staining was observed. However, much more cytoplasmic staining was found in the AR group (Figure 5(b), black arrow). The amount and release of HMGB1 were reduced by the 100 mg/kg EP treatment but not by the 50 mg/kg treatment (Figure 5(c) and (d)). Similar results were

obtained with the western blot with the nasal mucosa samples (Figure 5(e)). Compared to the control group, the HMGB1 protein level in the AR, 50 mg/kg EP, and 100 mg/kg EP groups were 1.78 ± 0.25 fold, 1.39 ± 0.12 fold, and 1.21 ± 0.05 fold, respectively.

Discussion

OVA-induced AR, characterized by Th2-dominated airway inflammation, produces clinical symptoms such as sneezing and nasal itch, IgE production, eosinophil recruitment, and Th-2 cytokines expression.^{32,33} In our study, allergic inflammation in the OVA-challenge groups was characterized by airway eosinophilic infiltration and goblet cell hyperplasia, along with increased NLF cytokines including IL-4 and IL-13. The number of nasal rubbings and sneezes increased in the AR group. Serum total IgE and OVA-specific antibodies were significantly elevated. The characteristics of AR were verified in our experiment, and we established a mouse AR model successfully. In addition, significantly increased HMGB1 was found in the AR group, where cytoplasmic transfer of the protein was also observed.

Figure 4 Effect of EP on pathological change. Evident eosinophilic infiltration and goblet cell hyperplasia were observed in the nasal mucosa from AR mice (b, g), while no obvious inflammatory cells were found in control mice (a, f). Treatment with EP alleviated inflammatory cell infiltration and goblet cell hyperplasia in a dose-dependent manner (c, d, e, h, i, j). *compare to control group, $p < 0.05$; #compare to AR group, $p < 0.05$; \$ compare between 50 and 100 mg group, $p < 0.05$. (A color version of this figure is available in the online journal.)

Our results demonstrated that the abundant expression of HMGB1 in the nasal mucosa of normal mice is restricted to the nuclei. The expression of HMGB1 was remarkably increased after an OVA challenge. Furthermore, HMGB1 translocation was promoted by allergen exposure, and released HMGB1 was observed in the epithelium and lamina propria cells. Previous research has shown that HMGB1 has different functions based on its compartment-specific expression.³⁴ HMGB1 is ubiquitously expressed in almost all cell types and is common in the nucleus, where it regulates gene transcription and chromatin structure.^{15,35,36} Extracellular HMGB1 is an important cytokine in a variety of immune responses.³⁷ It can be passively released from the dead cells³⁸ and actively secreted from inflammatory cells such as macrophages, monocytes, dendritic cells, and natural killer cells.¹⁹ As an important mediator of inflammation, HMGB1 can increase its inflammatory activity by binding with pro-inflammatory cytokines such as TNF α , IL-6, IL-8,²⁴ which were elevated in AR.²³ Moreover, a recent study demonstrated that HMGB1 mediates the release of Th-2 cytokines by the receptor for

advanced glycation end products and thus amplifies allergic inflammation.²² This suggests that the increased expression of HMGB1 might lead to the up-regulation of downstream inflammatory cytokines expression that is involved in the pathogenesis of AR, and thus mediate the allergic inflammation of nasal mucosa.

EP, a derivative of pyruvic acid, strongly suppresses inflammation.^{26,30} Its positive effect on inflammation has been demonstrated in a wide variety of experimental inflammatory disease models.^{13,17,27,28} However, the effect of EP on AR has not been previously reported. In our study, EP significantly inhibited a number of features of the allergic inflammatory response, including nasal symptoms, airway eosinophilia, Th2-cytokines expression, and total and OVA-specific IgE and goblet cell hyperplasia. Many different mechanisms may participate in the anti-inflammatory effects of EP.³⁰ Among them, EP can effectively inhibit the release of the pro-inflammatory DNA-binding protein HMGB1. Our results showed that the expression and translocation of HMGB1 were significantly inhibited by treatment with EP in a dose-independent manner (Figure 4).

Figure 5 Respective expression of HMGB1 in the nasal mucosa of mice from each group. (a) HMGB1 is abundantly expressed in the epithelium and lamina propria, and mainly located at the nucleus in mucosa from control mouse (black arrow). (b) shows that expression of HMGB1 was significantly increased in the nasal mucosa of AR mice and the migration of HMGB1 from the nucleus to the cytoplasm was clearly observed (red arrow). (c, d) Secretion of HMGB1 was inhibited by treatment with 100 mg EP rather than 50 mg. Similar result was confirmed by western blot (e). (A color version of this figure is available in the online journal.)

However, the exact signal pathway through which EP inhibits HMGB1 expression needs to be established. Previous studies have speculated about the exact mechanism. Wang *et al.* provided evidence that HMGB1 release was mediated by the activation of NF- κ B.³⁹ Furthermore, Song *et al.* and Han *et al.* proved that EP can decrease NF- κ B-dependent signaling.^{40,41} We speculate that EP may suppress the expression and translocation of HMGB1 by regulating NF- κ B.

Another important mechanism contributing to the protective effect of EP on AR may be the suppression of NF- κ B pathway activation.^{42–44} NF- κ B may participate in the pathogenesis of AR, as NF- κ B-mediated signaling regulates numerous relevant products. NF- κ B is pertinent to the regulation of the expression of pro-inflammatory cytokines, such as IL-1, IL-6, and TNF α ; the expression of adhesion molecules, such as E-selectin, intercellular cell adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1); and the expression of chemokines, such as eotaxin, regulated on activation, normal T cell expressed and secreted (RANTES), and monocyte chemoattractant protein-1 (MCP-1).⁴⁵ An EP-induced decrease of NF- κ B signals might affect the aforementioned inflammatory mediators. For instance, IL-6, an important Th-2

pro-inflammatory cytokine, has been shown to be elevated in patients with AR and is known to provoke an increase in nasal secretions.⁴⁶ It can stimulate T lymphocytes, promote the differentiation of B lymphocytes, and co-promote with IL-4 and IL-13 to generate IgE.⁴⁵ EP may decrease IL-6 production by regulating NF- κ B and thus attenuating IgE synthesis and secretion.⁴⁷ EP is also thought to act as an antioxidant,⁴⁸ and oxidative stress plays a role in the pathogenesis of AR. Increased oxidative stress exacerbates AR, nasal secretions, and eosinophil infiltration.⁴⁹ EP exerts an anti-inflammatory effect by scavenging reactive oxygen species,⁵⁰ and it may inhibit the production of reactive oxygen species and thus prevent AR exacerbation.

Eosinophilic infiltration is a characteristic of AR.^{4,51} Eosinophil is recruited to nasal mucosa by chemoattractants produced by T cells, mast cells, and epithelium.^{51,52} Recent studies have proved that eosinophil can be recognized and activated by a damage-associated molecular pattern molecule released from necrotic epithelium.⁵³ Moreover, HMGB1 can be a chemokine to eosinophil and can enhance eosinophil survival.⁵² Eosinophil transmigration into nasal mucosa is also coordinated by specific chemokines eotaxin and RANTES in combination with adhesion molecules such as ICAM-1, VCAM-1, and E-selectin.⁵⁴ Both the

chemokines⁵⁵ and adhesion molecules⁵⁶ mentioned earlier were elevated in AR and played roles in the pathogenesis of AR. Treatment with EP significantly reduced the expression of HMGB1 and eosinophil aggregation, supporting the argument that EP can prevent HMGB1 secretion by restraining eosinophilia in AR. We also found that the effects mentioned earlier were dose dependent. These data suggest that HMGB1 may play an important role in the allergic response and eosinophilic infiltration in AR. Furthermore, EP can also inhibit NF- κ B signaling to prevent adhesion molecules and chemokines up-regulating, therefore decreasing eosinophil recruitment. AR was attenuated by treatment with EP, which suppressed the expression and release of HMGB1.

The Th1 immune response is characterized by the enhanced production of IgG2a and IgG2b in mice whereas the Th2 response is characterized by the production of IgG1.⁵⁷ In our study, EP had no effect on OVA-specific IgG1 or IgG2a (Figure 3). IgE mediates the inflammatory response in allergic diseases such as asthma, AR, and food allergies.⁵⁸ In our study, the level of total and specific IgE in sera of AR mice was reduced after treatment with EP in a dose-dependent manner. Tang *et al.* obtained similar results with a murine asthma model.¹³ The reduced IgE production may be modulated by the following mechanism. First, IgE synthesis can be simulated by the Th2 cytokines IL-4 and IL-13.⁵⁹ Treatment with EP would significantly decrease the levels of IL-4 and IL-13, therefore IgE synthesis may be down-regulated. Many studies of asthma, which is an allergic disease in the lower respiratory region, have proven that the blockage of HMGB1 activity with antibodies or agents such as EP will significantly decrease either total IgE or allergen-specific IgE levels,^{13,22,60} indicating that EP administration may decrease total IgE levels by inhibiting HMGB1 expression. In addition, NF- κ B is involved in the synthesis of IgE, and EP has been proven to be an effective agent for inhibiting NF- κ B activity.³⁰ Therefore, EP may reduce IgE production by suppressing NF- κ B activity.

Although we demonstrated that EP administration could significantly attenuate AR, we recognize that EP is a non-selective HMGB1 antagonist, and further research should be conducted using an anti-HMGB1 neutralized antibody. In summary, we demonstrated that EP might decrease airway eosinophil inflammation partly by inhibiting HMGB1 in a murine AR model. Thus, HMGB1 may be a novel therapeutic target for AR.

Authors' contributions: WK and YW designed the study; SC, GG, and YN conducted the experiments; WK and JC analyzed the data; SC and YW wrote the paper. WK supervised the project. SC and YW contributed equally to this work.

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