



Preliminary report

Ethyl pyruvate decreases airway neutrophil infiltration partly through a high mobility group box 1-dependent mechanism in a chemical-induced murine asthma model



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ABSTRACT

Background: Diisocyanates are one of the leading causes of occupational asthma, which is dominated by granulocytic inflammation in the airway. In this study, we intended to explore the role of ethyl pyruvate (EP) on neutrophil infiltration in a toluene-2,4-diisocyanate (TDI)-induced murine asthma model.

Methods: The experimental mice were first dermally sensitized and then challenged with TDI via oropharyngeal aspiration. The mice were treated intraperitoneally with 100, 50 or 10 mg/kg EP 1 h before each challenge. One day after the last challenge, airway reactivity to methacholine was measured by a barometric plethysmographic chamber. Total and differential cell counts, along with levels of macrophage inflammatory protein-2 (MIP-2), TNF- α in bronchoalveolar lavage (BAL) fluid and mRNA expression of CXCR2 in the lung were assessed. To depict neutrophils, a naphthol AS-D chloroacetate esterase kit was used. High mobility group box 1 (HMGB1) was determined by western blot and immunohistochemistry.

Results: Treatment with EP dramatically decreased airway hyperresponsiveness in TDI-challenged mice, as well as numbers of neutrophils in BAL fluid and peribronchovascular regions. Both the TDI-induced raised protein level and abnormal distribution of HMGB1 were significantly recovered by EP in a dose-dependent manner. The concentration of MIP-2 in TDI-induced asthma mice was significantly higher than that of the control ones, while EP had few effects on MIP-2. The mRNA expression of CXCR2 didn't change significantly, and TNF- α was not detected in BAL fluids.

Conclusion: EP reduces airway neutrophil infiltration partly through downregulating HMGB1 in a chemical-induced murine asthma model.

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1. Introduction

Polymorphonuclear neutrophils (PMN) are key components of the first line of defense against microbial pathogens, being rapidly recruited to inflammatory sites in response to a variety of stimuli. Their role in the inflammatory process was once thought to be restricted to phagocytosis and the release of enzymes and other cytotoxic agents, but it is now known that these cells can release diverse mediators that have profound effects on the airways of asthmatic individuals [1]. Although eosinophilic airway inflammation is recognized as an important feature of most patients with chronic, stable asthma, evidence indicates a

critical role for neutrophils in asthma. At the same time, increased neutrophil levels have been found in patients with acute severe asthma [2]. Furthermore, it has become apparent that certain phenotypes of asthma are characterized by an influx of neutrophils in the airways, as assessed by analysis of induced sputum or BAL fluid [3]. This is especially the case with occupational asthma (OA). Previous investigation of bronchial mucosa using immunohistochemistry revealed that neutrophil count is significantly higher in subjects with TDI-induced asthma than those of allergic asthma [4]. Several other studies also showed neutrophil accumulation in patients with isocyanate-induced OA, as well as in experimental animal models [5–7]. Recently, De Vooght et al. demonstrated an important role for neutrophils in TDI-induced OA [8]. Blocking neutrophils by antibodies can prevent airway hyperresponsiveness (AHR) and lung epithelial injury and dramatically reduce airway inflammation, a direct proof supporting that neutrophils are involved in the pathogenesis of TDI-induced asthma.

Ethyl pyruvate (EP) is derived from the endogenous metabolite pyruvic acid and supposed to be a potent antioxidant and free-

Abbreviations: TDI, toluene-2,4-diisocyanate; EP, ethyl pyruvate; HMGB1, high mobility group box 1; MIP-2, macrophage inflammatory protein-2; BAL, bronchoalveolar lavage; TNF, tumor necrosis factor; IL, interleukin.

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radical scavenger. The pharmacological effects of EP include amelioration of redox-mediated damage to cells and tissues, inhibition of pro-inflammatory cytokine secretion, and suppression of apoptosis [9–11]. Treatment with EP significantly inhibits the recruitment of neutrophils into the bronchoalveolar space after lipopolysaccharide (LPS) administration [12,13], implying a therapeutic role for EP in pulmonary neutrophil infiltration of TDI-induced asthma.

We have successfully developed a murine asthma model characterized by neutrophil-dominated airway inflammation using TDI [14], one of the most common causes of OA [15–18]. In this study, we hypothesized that EP may attenuate airway neutrophil infiltration in a TDI-induced asthma model. Therefore we tested the hypothesis with our asthma model and explored the mechanisms involved.

2. Methods

2.1. Animals and materials

Six-week old specific-pathogen-free male BALB/c mice (20–22 g) were purchased from Southern Medical University. The mice were housed in a SPF house with 12-h dark/light cycles. All studies were conducted in accordance with the committee of Southern Medical University on the use and care of animals. The protocols were approved by the Animal Subjects Committee of Nanfang Hospital (application number: NFYY-2012-055). Toluene-2,4-diisocyanate, methacholine, EP and acetone were obtained from Sigma-Aldrich (Shanghai, China). Pentobarbital was obtained from Weijia Biotechnology Company (Guangzhou, China). The vehicle (AOO) used to dissolve TDI consists of a mixture of 2 volumes of acetone and 3 volumes of olive oil (Selection de Almazara, Carbonell, Madrid, Spain) for the dermal sensitization, and 1 volume of acetone and 4 volumes of olive oil for the challenge. IL-4, IFN- γ , MIP-2 and TNF- α ELISA kit were purchased from BoShiDe biological engineering company (Wuhan, China); IgE ELISA kit from American Laboratory Products Company; and HMGB1 antibody from Abcam (Hong Kong, China).

2.2. TDI-induced asthma and EP administration

TDI-induced asthma model was prepared by a modification of Hoet's method [5]. All mice were randomized to the following five groups (20 mice/group): (1) AOO-sensitized, AOO-challenged, and PBS-treated mice (AOO group); (2) TDI-sensitized, TDI-challenged, and PBS-treated mice (TDI group); (3) TDI-sensitized, TDI-challenged, and 10 mg/kg EP-treated mice (EP10 mg/kg group); (4) TDI-sensitized, TDI-challenged, and 50 mg/kg EP-treated mice (EP50 mg/kg group); and (5) TDI-sensitized, TDI-challenged, and 100 mg/kg EP-treated mice (EP100 mg/kg group). Briefly, on days 1 and 8, the mice were dermally treated with 0.3% TDI on the dorsum of both ears (20 μ L/ear). On days 15, 18, and 21 the mice underwent an oropharyngeal aspiration (20 μ L) of 0.01% TDI under light pentobarbital anesthesia (50 mg/kg body weight). As a control, the AOO group was sensitized and challenged with a mixture of acetone and olive oil. Ethyl pyruvate working solution was prepared as 28 mM EP, 130 mM NaCl, 4 mM KCl, and 2.7 mM CaCl₂ (pH = 7.0). 10, 50 and 100 mg/kg EP were administered via intraperitoneal injection (i.p.) respectively 1 h before each challenge. By comparison, the control group received the same dose of vehicle.

2.3. Airway hyperresponsiveness (AHR)

AHR to methacholine was assessed 24 h after the third challenge. Briefly, mice were placed in a barometric plethysmographic chamber (Buxco Electronics, Troy, NY). Aerosolized methacholine in increasing concentrations (0–25 mg/mL) was nebulized through an inlet of the main chamber for 3 min. Readings were taken and averaged for 3 min after each nebulization. The bronchopulmonary resistance was expressed as enhanced pause (Penh).

2.4. Preparing lymph node cells

Cervical lymph nodes were dissected from the mice and processed for each mouse separately, then kept on ice in RPMI-1640 medium and cell suspensions were obtained by pressing the lymph nodes through a cell strainer (40 μ m) (BD Bioscience, Erembodegem, Belgium) and rinsing with 10 mL tissue culture medium (RPMI-1640). Cells were counted using a Bürker hemocytometer. Lymphocytes were then washed three times and suspended (10^7 cells/mL) in complete tissue culture medium (RPMI-1640 supplemented with 10% heat-inactivated FBS). Cells were seeded into 48-well culture plates at a density of 10^6 cells/mL and incubated in complete RPMI-1640 medium for 43 h with 5 mg/mL of concanavaline A (Sigma-Aldrich, Shanghai, China), then centrifuged (1000 $\times$ g, 4 $^{\circ}$ C, 10 min) and the supernatants were stored at -80° C for further measurement of IL-4 and IFN- γ .

2.5. Total serum IgE

The mice were sacrificed with pentobarbital (100 mg/kg, i.p.) on day 23. Blood samples were taken from the retro-orbital plexus/sinus, rest for 1 h at room temperature, then centrifuged (3000 $\times$ g, 20 min) and supernatants were harvested and stored at -80° C. Total serum IgE was measured by ELISA according to the manufacturer's instructions.

2.6. Bronchoalveolar lavage (BAL) fluid

After cervical lymph nodes were obtained, the lungs were lavaged in situ, three times with 0.8 mL sterile saline (0.9% NaCl), and the recovered fluid was pooled. Total cells in the BAL fluid were counted, and the BAL fluid was centrifuged (1000 $\times$ g, 10 min). The supernatant was frozen (-80° C) until further analysis. For differential cell counts, a cytopspin sample (Shandon Scientific, Runcorn, UK) was prepared and stained using hematoxylin and eosin (H&E) (Nanjing Jiancheng, China). For each sample, a total of 200 cells were counted for the number of macrophages, eosinophils, neutrophils and lymphocytes.

2.7. Lung histology for H&E, PAS, specific esterase staining

Twenty-four hours after the last airway challenge, mice were euthanized. The left lungs were infused with 4% neutral buffered formalin to fully inflate all lobes (judged visually), then fixed, and embedded in paraffin. Lung sections (5 μ m) were cut with a Leica microtome 2030 (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Lung tissues were stained with hematoxylin and eosin for blinded histopathologic assessment. Lung inflammation was quantified by a modification of K. Tournoy's method [19]. Briefly, two criteria were scored to document the pulmonary inflammation: peribronchial inflammation and perivascular inflammation. A value of 0 was adjudged when no inflammation was detectable, a value of 1 for occasional cuffing with inflammatory cells, a value of 2 for most bronchi or vessels surrounded by thin layer (one to ten cells) of inflammatory cells and a value of 3 when most bronchi or vessels were surrounded by a thick layer (more than ten cells) of inflammatory cells. As 8–10 tissue sections per mouse were scored, inflammation scores could be expressed as a mean value and could be compared between groups.

To visualize mucin production, periodic acid-Schiff base (PAS) staining was utilized and semiquantitative scoring was used to compare groups. PAS-stained tissues were examined under light microscopy for PAS-positive staining in the upper airway epithelium in five fields at 200 $\times$ magnification. PAS-positive epithelial cells of the total epithelial cells were counted to obtain a percentage and compare groups. To depict neutrophils, other sections were stained with naphthol AS-D chloroacetate esterase (Sigma-Aldrich, Shanghai, China), with counterstaining by hematoxylin.

2.8. Immunohistochemistry and western blot

For immunohistochemistry of HMGB1, sections were deparaffinized with xylene and rehydrated with graded ethanol to distilled water, then submerged in citrate buffer (pH 6.0) and subjected to antigen retrieval. To block the endogenous peroxidase, samples were treated with 3% H₂O₂ for 15 min, and then incubated overnight at 4 °C in a 1:100 dilution of polyclonal rabbit anti-HMGB1 primary antibody. After washing with PBS, slices were incubated with anti-rabbit secondary antibody for 30 min at room temperature. Signals were visualized with a DAB peroxidase substrate kit.

For western blot analysis, lung tissue (60 mg) from animals was homogenized in 3 mL of ice-cold homogenization buffer (containing protease inhibitor, calcineurin inhibitors and PMSF). Homogenates were then centrifuged at 4 °C, 9000 ×g for 30 min. Supernatants were collected and centrifuged at 4 °C, 14,000 ×g for 30 min before mixing with loading buffer and boiled for 10 min, then separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After being blocked with 5% skim milk at room temperature for 2 h, the membranes were incubated with polyclonal rabbit anti-HMGB1 at 4 °C overnight. The membranes were washed three times in TBST. The samples were then incubated for 1 h at room temperature with a HRP-conjugated secondary antibody. The membranes were exposed to Odyssey® CLx Imager and image capture or data analysis was done with Odyssey Software.

2.9. Real-time RT-PCR

The expression of a major IL-8 receptor CXCR2 was detected by the SYBR green-based real-time RT-PCR technique. Total RNAs were isolated from lung tissues, using an RNA isolation kit (Trizol reagent, Invitrogen, GuangZhou, China). RNA samples were then reverse transcribed into cDNA. Oligonucleotide primers were designed on the basis of the published sequences for mouse CXCR2. The primers for amplifying CXCR2 cDNA were 5'-CAG CTG CCT TAA CCC CAT CA-3' (forward) and 5'-CTT GAG AAG TCC ATG GCG AAA-3' (reverse). The primers for amplifying GAPDH were 5'-TGA AGC AGG CAT CTGA GG G-3' (forward) and 5'-CGA AGG TGG AAG AGT GGG AG-3' (reverse).

Real-time RT-PCR was performed using the Stratagene Mx3005P, and the cycling conditions used were 95 °C for 15 s, 60 °C for 1 min, for 40 cycles, followed by a melting point determination or dissociation curves. The gene expression level is indicated by the cycle number needed for the cDNA to be amplified to reach a threshold. The amount of DNA is calculated from the cycle numbers by using standard curves, and the results are normalized to the housekeeping gene GAPDH from the same sample.

2.10. Lung myeloperoxidase activity

The lung tissue was homogenized immediately on ice in 5 vol of normal saline. The myeloperoxidase (MPO) activity was measured using an MPO assay kit (Nanjing Jiancheng, China) following the manufacturer's recommendations. One unit of MPO activity is defined as degrading 1 μmol of hydrogen peroxide at 37 °C, and MPO activity of tissue was expressed as unit per gram (U/g).

2.11. Statistical analysis

Statistical analysis was performed using SPSS version 17.0. Data were expressed as mean ± standard error (SE) and comparisons among groups were analyzed by one-way analysis of variance (ANOVA) accompanied by Bonferroni post hoc test for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of EP on airway reactivity and airway inflammation in a TDI-induced murine asthma model

Effects of EP on AHR, IL-4, IFN-γ and total IgE are shown in Fig. 1. The differences of Penh values between TDI group and AOO group were significant when stimulated by Ach (3.12, 6.25, 12.5 and 25.0 mg/mL) ($p < 0.05$). While AHR was reduced in mice treated with EP (10, 50, 100 mg/kg) compared with the TDI group when stimulated by Ach (6.25, 12.5 mg/mL) ($p < 0.05$), but only 100 mg/kg can reduce AHR when stimulated by Ach (25.0 mg/mL) ($p < 0.05$). Fig. 1 also shows the levels of IL-4 and IFN-γ in supernatants of cultured cervical lymph node cells. The release of IL-4 and IFN-γ was significantly increased in the TDI group compared with that from the AOO group (272.66 ± 4.79 pg/mL vs 20.48 ± 2.18 pg/mL; 167.92 ± 7.56 pg/mL vs 37.47 ± 5.27 pg/mL) ($p < 0.05$). Both 50 mg/kg and 100 mg/kg EP could markedly decrease IL-4 (185.42 ± 4.93 pg/mL vs 272.66 ± 4.79 pg/mL; 170.63 ± 4.05 pg/mL vs 272.66 ± 4.79 pg/mL), IFN-γ (126.55 ± 5.71 pg/mL vs 167.92 ± 7.56 pg/mL; 92.11 ± 4.91 pg/mL vs 167.92 ± 7.56 pg/mL) ($p < 0.05$), as well as total serum IgE (348.11 ± 5.97 pg/mL vs 431.78 ± 16.21 pg/mL; 345.67 ± 2.21 vs 431.78 ± 16.21 pg/mL) ($p < 0.05$).

3.2. Effect of EP on pathological changes in TDI-induced asthma

On histological examination, TDI-sensitized/challenged mice had heavy infiltration of inflammatory cells and notable epithelial proliferation while EP with 50 mg/kg and 100 mg/kg displayed extensive capacity of suppressing inflammation in the peribronchial and perivascular regions compared with TDI group (Fig. 2A, C and 2D). The percentage of cells stained with PAS in airway epithelia of TDI-inhaled mice was obviously greater than that of the AOO group, which was reduced after administration of 100 mg/kg EP rather than 10 mg/kg and 50 mg/kg (Fig. 2B and E).

3.3. Effect of EP on neutrophil infiltration

Total cell counts, as well as numbers of macrophages, neutrophils, eosinophils and lymphocytes were assessed in the BAL fluid (Fig. 3A). In agreement with total cell counts, higher amount of neutrophils was found after sensitization and challenge with TDI ($p < 0.05$), which was significantly decreased by EP in a dose-dependent manner. In addition, both MPO assay and specific esterase staining showed increased neutrophils in TDI group compared with AOO group. And administration of EP reduced MPO activity as well as number of neutrophil-specific esterase-positive staining cells in lung tissues (Figs. 3B and 4).

3.4. Effect of EP on HMGB1, MIP-2 and CXCR2

To evaluate the role of neutrophil chemotactic factors in TDI-induced airway response, we measured levels of HMGB1 and CXCR2 in lung tissue and MIP-2 and TNF-α in BAL fluid. Compared with AOO group, both the location and amount of HMGB1 changed after TDI challenge. In AOO group, HMGB1 was observed almost only in the nuclei. After TDI challenge, we found that bronchial epithelium, alveolar epithelium, airway fibroblasts and infiltrating inflammatory cells all expressed HMGB1 in the cytoplasm (Fig. 5). Similar results were obtained by western blot. The protein levels of HMGB1 were significantly different between AOO group and TDI group, while EP administration effectively downregulated HMGB1 in a dose-dependent manner (Fig. 6A and B). The concentration of MIP-2 in BAL fluid 24 h after the last challenge was obviously increased in TDI group ($p < 0.05$), but no significant reduction was seen in EP treated groups (Fig. 6C). Besides, we measured the mRNA level of CXCR2, an important receptor of neutrophil chemoattractants, in the lung tissue. As we can see from Fig. 6D, there's a mild decrease

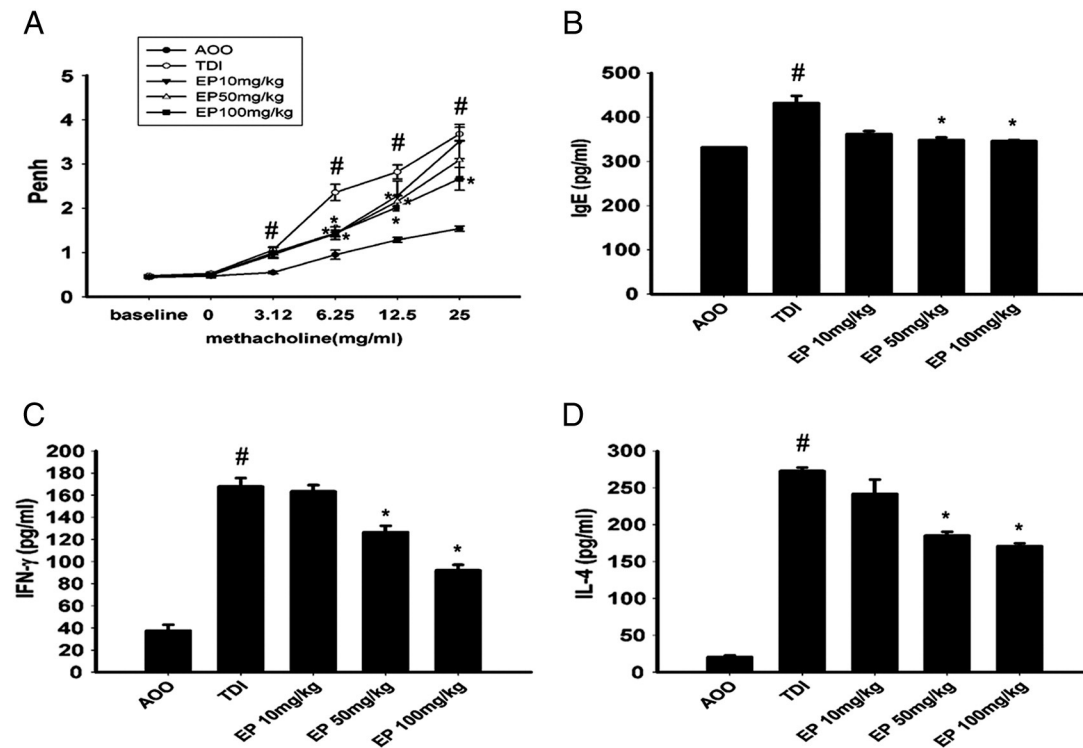


Fig. 1. EP reduced TDI-induced AHR, Th1/Th2 inflammation and total serum IgE. (A) 24 h after the last challenge, AHR to methacholine was assessed. (B) On the following day, mice were sacrificed. Blood samples were first taken from the retro-orbital plexus/sinus, and serum total IgE was measured using ELISA. The cervical lymph nodes were processed for each mice separately, the obtained lymph node cells were cultured with concanavaline A (5 mg/mL) for 43 h. (C) IFN- γ and (D) IL-4 were measured via ELISA in the supernatant of cultured lymphocytes. Data are presented as mean $\pm$ SE (n = 6–8 per group). # = $p < 0.05$ compared with AOO group; * = $p < 0.05$ compared with TDI group.

of relative mRNA expression of CXCR2 after TDI challenge, which was raised when pretreated with EP, but the differences were not significant. Unfortunately, TNF- α was not detected in the BAL fluids (data not shown).

4. Discussion

To our knowledge, this is the first report to demonstrate that EP could suppress neutrophil infiltration in a TDI-induced murine asthma model.

TDI-induced asthma is characterized by Th2-dominated airway inflammation [20], which was verified in our study. Inflammation was characterized by airway neutrophilia and eosinophilia, along with elevated BAL cytokines, raised levels of IL-4 and IFN- γ in supernatant of cultured lymphocytes, and increased total serum IgE, accompanied by airway epithelial goblet cell hyperplasia.

EP has been shown to be an effective anti-inflammatory agent in a wide variety of in vivo and in vitro models of inflammation-mediated cellular or tissue injury, including severe sepsis, hemorrhagic shock, ischemia/reperfusion-induced intestinal mucosal injury and ileus induced by bowel manipulation in mice [21–26]. However, EP used for treatment in TDI-induced asthma has not been studied yet. Neutrophils are the major inflammatory cells involved in the process of ALI [27], as well as asthma [28]. They can release diverse mediators that have profound effects on the airways of asthmatic individuals, such as IL-8, MMP-9 and elastase [1]. Studies have revealed that neutrophils are involved in the process of TDI-induced asthma [8,29]. In our study, more neutrophils were found in the peribronchial and perivascular regions and BAL in TDI group compared with AOO group. Administration of EP can inhibit neutrophil infiltration in a dose-dependent manner, suggesting a therapeutic role for ethyl pyruvate in TDI-induced asthma. But the mechanisms involved remains unclear.

Prevention of inflammatory mediator release (for example tumor necrosis factor and HMGB1) emerged as a key mechanism underlying

the anti-inflammatory function of EP [21,30,31]. HMGB1 is found in all mammalian tissues and is highly conserved among species. It can be largely secreted into the extracellular space after activation of macrophages, natural killer cells and mature dendritic cells [32,33]. Extracellular HMGB1 may interact with Toll-like receptor (TLR) 2 and TLR4, and receptor for advanced glycation end products (RAGE) [34,35]. As a danger signal, HMGB1 would be expected to trigger inflammation. It stimulates the release of TNF- α , IL-1 β and other inflammatory cytokines from macrophage, and mediates ALI and lethality [36]. In addition to acting as an inflammatory mediator, HMGB1 also serves as a chemoattractant to neutrophils and prevents neutrophil apoptosis [37]. Studies have clarified that HMGB1 can mediate neutrophil recruitment [38,39]. Blockade of HMGB1 by administration of anti-HMGB antibodies inhibits accumulation of neutrophils in the lung [40,41]. Recent studies performed in asthmatics suggested a significant correlation between HMGB1 and neutrophilic inflammation found in airways [42–44]. In comparison with asthma induced by ovalbumin, where eosinophil is the characteristic cell type present in the BAL fluid, asthma induced by TDI is associated with an influx of mainly neutrophils [18,45], which are implicated in the pathogenesis of TDI-induced asthma. Intriguingly, in this study, we found that the protein level of HMGB1 was notably increased in TDI group, accompanied by its abnormal distribution in the lung tissue. Thus, it is reasonable to hypothesize that HMGB1 is associated with neutrophilic airway inflammation found in asthma induced by TDI. However, TNF- α was not detected in TDI group (data not shown).

To examine whether HMGB1 contributes to airway neutrophil recruitment in TDI-induced asthma, EP, a nonselective HMGB1 antagonist [46], that was commonly used for evaluating the function of HMGB1 in vivo [47,48], was administered at a dose of 10 mg/kg, 50 mg/kg and 100 mg/kg respectively via intraperitoneal injection (i.p.) 1 h before TDI challenge. As a result, treatment with EP, especially with the concentration of 100 mg/kg, significantly reduced the expression of HMGB1 as well as neutrophil aggregation, supporting that EP can prevent HMGB1 release to decrease airway neutrophilia in TDI-induced

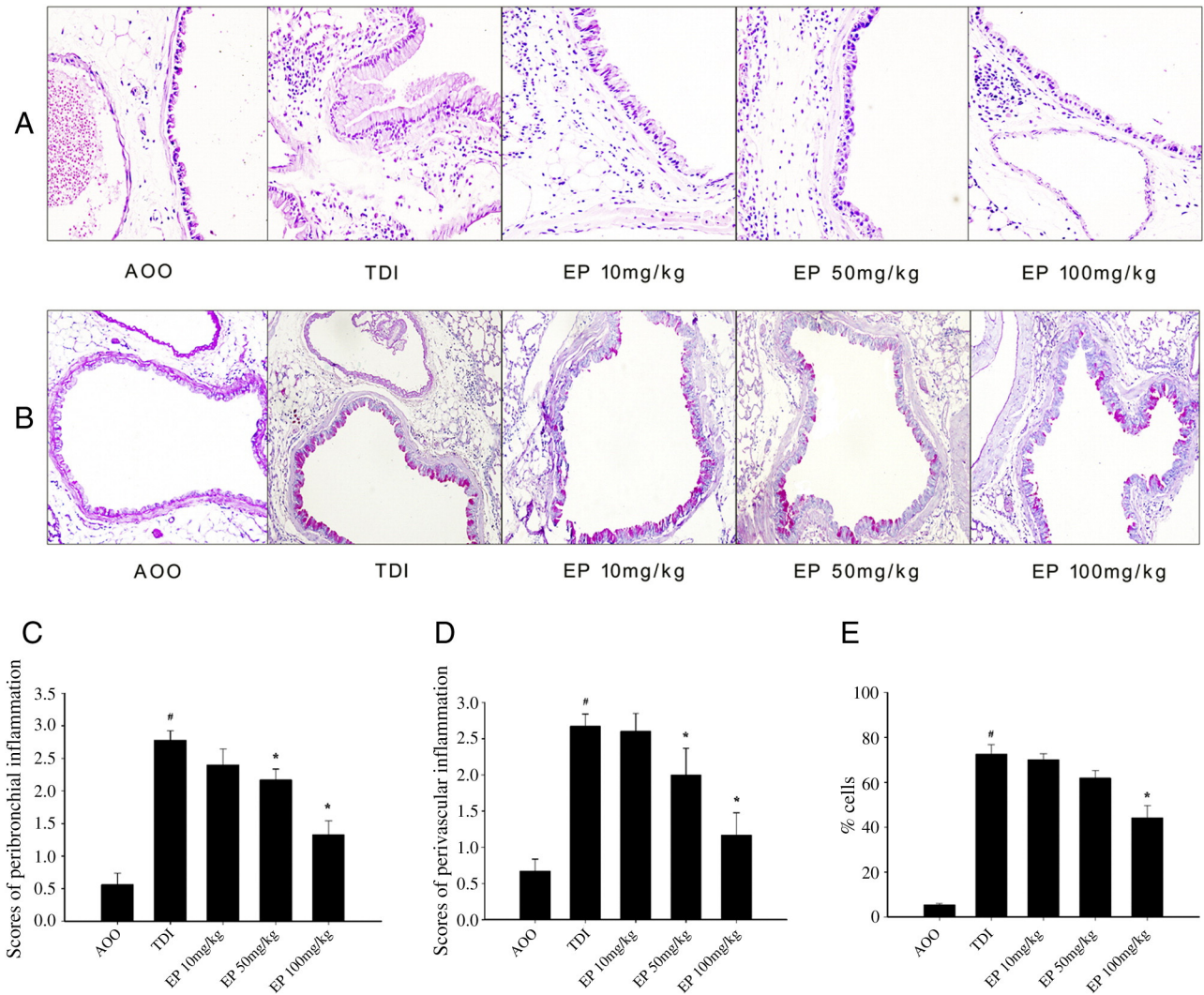


Fig. 2. Effects of EP on pathologic changes in lung tissues 48 h after oropharyngeal aspiration. (A) H&E staining was performed on lung slices ($n = 6-8$) per group. Images were at $400\times$ original magnification. (B) Representative PAS-stained sections of the lungs ($n = 6-8$ per group). Magnification for each image was $200\times$. (C) Results of histological scoring for peribronchial inflammatory changes of lung sections from different groups. (D) Histological scoring for perivascular inflammatory changes of lung sections. (E) Quantification of PAS-positive staining was determined by counting the number of PAS-positive epithelial cells. [#] $p < 0.05$ compared with the AOO group; ^{*} $p < 0.05$ compared with the TDI group.

asthma. However, the precise pathway by which EP inhibits HMGB1 expression is still unclear. Though Su's study provided evidence that EP could downregulate the expression of HMGB1 via selective inhibition of TLR4 and NF- κ B [11], future studies are still needed to confirm

that. Moreover, as bronchial epithelium, alveolar epithelium, airway fibroblasts and infiltrating inflammatory cells were all found to have higher expression of HMGB1 in the cytoplasm after TDI challenge, the main contributor to airway neutrophilia remains inconclusive. We also

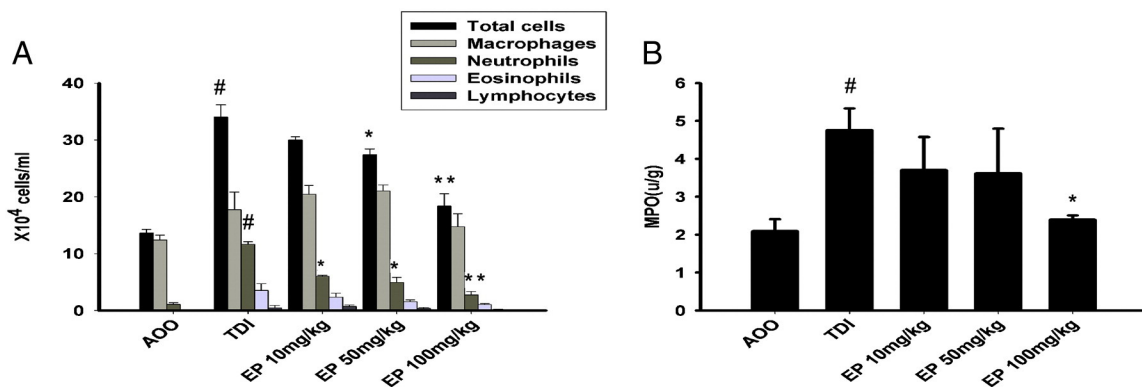


Fig. 3. Effects of EP on neutrophils infiltration. (A) The numbers of total cell counts, macrophages, neutrophils, eosinophils and lymphocytes were determined in the BALF. (B) MPO activity was significantly higher in the lungs of TDI-treated mice than those of the control ones. While treatment of EP had little effect on MPO activity. Data are presented as mean $\pm$ SE ($n = 6-8$ per group). [#] $p < 0.05$ compared with the AOO group; ^{*} $p < 0.05$, ^{**} $p < 0.001$ compared with the TDI group.

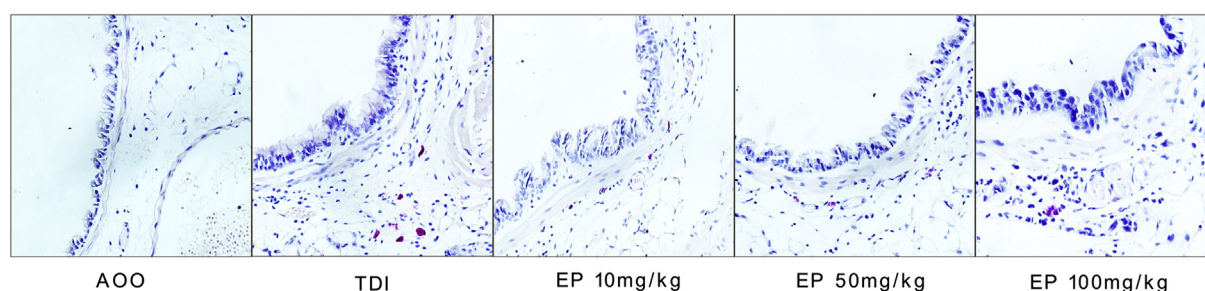


Fig. 4. Histological examination of neutrophils around the peribronchovascular regions. Neutrophils (red-stained cells) were detected with chloroacetate esterase substrate. Original magnification was 400 $\times$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

realize that EP is not a specific antagonist, future work should seek to provide more definite evidence using siRNA to knockdown HMGB1 or anti-HMGB1 neutralizing antibody to reduce the levels of active protein.

As an important chemoattractant of neutrophils, IL-8 takes part in a variety of neutrophilic inflammatory diseases. Elevated IL-8 quantified by ELISA was found in the sputum of TDI-induced asthmatics as well as in vitro models [7,49]. Mice lack the IL-8 gene, but they do express homologs of human IL-8, such as keratinocyte-derived cytokine (KC) and MIP2, which exert neutrophil chemoattractant function through interaction with their major receptor, CXCR2 [50], so we measured CXCR2 instead. However, in the present study we found no significant differences of CXCR2 gene among all enrolled groups. Yet as a bioactive receptor of various ligands, its gene or protein expression need not to be altered in a disease state [51]. The exact mechanisms still need to be clarified in future researches.

In addition to HMGB1, CXCR2 and TNF- α , MIP-2 is also involved in the recruitment of neutrophils. MIP-2 has been shown to elicit a localized inflammatory response when injected subcutaneously into the footpads of C3H/HeJ mice, has a potent chemotactic activity for human PMN, and possesses marked capacity for inducing lysosomal degranulation of PMN [52]. Additionally, MIP-2 has been shown to apparently reduce mycobacterial-induced recruitment of neutrophils [53]. Previous studies have shown that MIP-2 rose in TDI-induced asthma [54,55]. Also, an influx of neutrophils, accompanied by a marked increase in MIP-2 level, was found in BAL of TDI group in the present study. But administration of EP cannot suppress the increase of MIP-2, which is in consistent with previous report [56]. These suggest that MIP-2 may also facilitate migration of neutrophils in TDI-induced asthma, while EP may not attenuate neutrophil infiltration through MIP-2-mediated pathway.

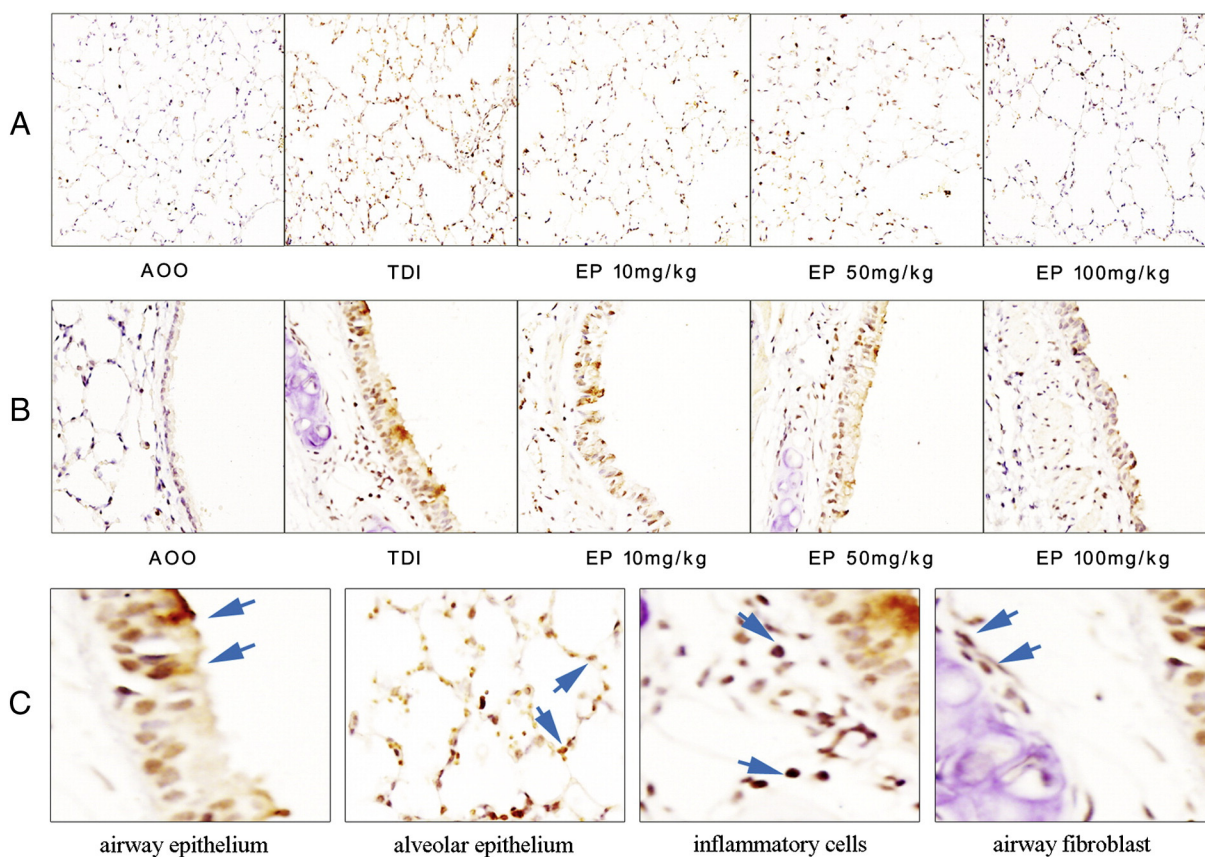


Fig. 5. Changes in lung HMGB1 location after treatment with EP. (A) Immunohistochemistry of HMGB1 in lung alveolar cells. (B) Immunohistochemistry of HMGB1 in lung epithelial cells. (C) High-resolution view of cells expressing HMGB1 in the cytoplasm in lungs of TDI group. The blue arrow indicates cells expressing HMGB1 in the cytoplasm. Original magnification of each image was 400 $\times$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

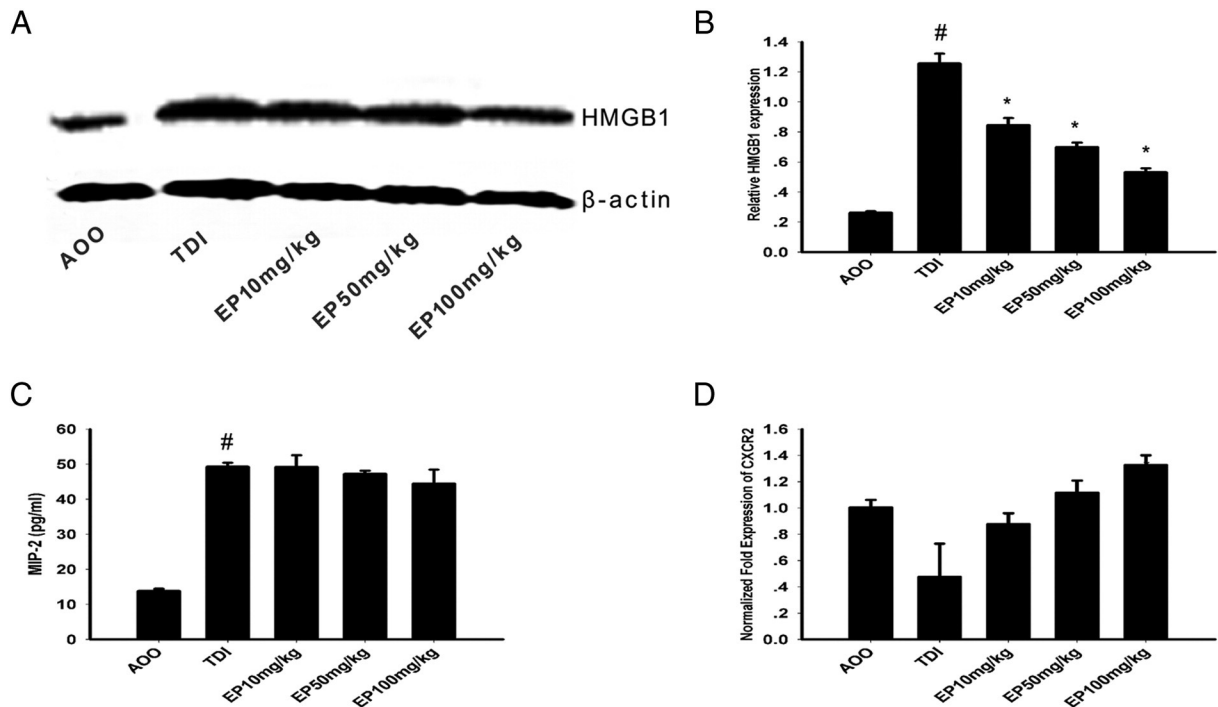


Fig. 6. Effects of EP on HMGB1 and MIP-2 expression. (A) Western blotting to assess HMGB1 levels in all enrolled groups. (B) Western blotting showed the relative expression of HMGB1 was elevated in TDI group; when pretreated with EP, it decreased in a dose dependent manner compared with TDI group. (C) MIP-2 levels in BAL 24 h after oropharyngeal aspiration of vehicle or TDI in BALB/c mice ($n = 6-8$ per group). (D) Relative lung mRNA expression of CXCR2 in all groups. $\#p < 0.05$ compared with the AOO group, $*p < 0.001$ compared with the TDI group.

5. Conclusions

In conclusion, we demonstrated that EP may decrease airway neutrophil inflammation partly through downregulating HMGB1 in a TDI-induced murine asthma model. Our data suggest novel therapeutic targets for the treatment of TDI-induced asthma.

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