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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 μL/ear). On days 15, 18, and 21 the mice underwent an oropharyngeal aspiration (20 μL) 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 $CaCl_2$ (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 μm) (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 ×g, 4 °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 ×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 ×g, 10 min). The supernatant was frozen (−80 °C) until further analysis. For differential cell counts, a cytospin 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 μm) 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× 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.

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