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Original Article

Grape seed proanthocyanidin extract influences susceptibility to steroid resistance via regulating Nrf2-iNOS-HDAC2 axis in murine models of asthma

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Abstract: Most patients diagnosed with asthma maintain control reasonably well and do not experience asthma exacerbations; however, 30% of patients achieve suboptimal control, have severe or difficult-to-treat asthma, and are relatively nonresponsive to the same medications that achieve and maintain asthma control for most patients. Steroid resistance causes most severe or difficult-to-treat asthma, but the underlying mechanism still remains unclear. Our study confirmed that the low expression of Nrf2 up-regulates iNOS and down-regulates GSH in murine models of difficult-to-treat asthma. The down-regulation of GSH causes disorder of HDAC2 function and activity and ultimately results in steroid resistance. Overall, our findings demonstrated the disorder of Nrf2-iNOS-HDAC2 axis promotes the steroid resistance. Additionally, we found Grape seed proanthocyanidin extract (GSPE) relieves the steroid resistance via regulating Nrf2-iNOS-HDAC2 axis in mice.

Keywords: Asthma, glucocorticoid resistance, grape seed proanthocyanidin extract (GSPE)

Introduction

Asthma is a chronic inflammatory disorder of the airways associated with airway hyper responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing [1]. Asthma exacerbations are characterized by decreased expiratory airflow that can be quantified by measure of lung function (peak expiratory flow or forced expiratory volume in 1 second) [2]; they are often treated with bursts of oral corticosteroids and are the source of significant asthma morbidity [3]. Exacerbations, especially in difficult-to-treat asthma, represent asthma treatment failure and account for a substantial portion of the total costs of asthma [4]. According to the 2014 Global Initiative for Asthma report, between 30 and 50% of all asthma medical expenditures are the result of exacerbations [5, 6].

Previous studies have demonstrated that steroid resistance might be one of the most important etiologies of difficult-to-treat asthma, but the underlying mechanism still remains unknown [7]. Illustrating the pathogenesis of steroid resistance and seeking out the corresponding drug have vital implications for the therapy of difficult-to-treat asthma [8]. It has been reported that the function defect of HDAC2 caused by oxidation/nitration imbalance is a vital etiology of steroid resistance [9-11]. In addition, inducible nitric oxide synthase (iNOS) and NF-E2-related factor (Nrf2) have been confirmed to exert a role in the regulation of HDAC2 [12-14]. We previously found Grape seed proanthocyanidin extract (GSPE) decreases the progression of airway inflammation and hyperresponsiveness by downregulating the iNOS expression, promising to be a potential in the treatment of allergic asthma [15].

In our study, we confirmed that the low expression of Nrf2 up-regulates iNOS and down-regulates GSH in murine models of refractory asthma. The down-regulation of GSH causes disorder of HDAC2 function and activity and ultimately results in steroid resistance. Overall, our findings demonstrated the disorder of Nrf2-

iNOS-HDAC2 axis promotes the steroid resistance. Additionally, we found Grape seed proanthocyanidin extract (GSPE) relieves the steroid resistance via regulating Nrf2-iNOS-HDAC2 axis in mice.

Materials and methods

Materials

Unless otherwise stated, all biochemical reagents used in this study were purchased from Sigma (St. Louis, MO). GSPE was purchased from Jianfeng, Inc. (Tianjin, China). HDAC2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). LPS exposure studies in mice were performed as described previously. Briefly, age-matched WT, Nrf2 and HDAC2 were exposed to aerosolized Escherichia coli LPS (1 mg/ml) for 8 minutes. Aerosolized saline-exposed mice were used as controls, and animals were sacrificed at 24 h post last exposure.

Multi-allergen challenge stimulates steroid-resistant airway inflammation [16], steroid-resistant murine models of asthma are established according to the literature [17]. Corticosteroid treatment Budesonide in dry-powered form was dissolved in 70% ethanol and then diluted with saline prior to administration. Twenty-five microliters of budesonide solution, corresponding to 1 or 3 mg/kg body weight, was administered via an intranasal route to each mouse for 3 days followed by LPS exposure at 1 h after last budesonide treatment.

Bronchoalveolar lavage

Mice were anaesthetized by pentobarbital (Abbott Laboratories, Abbott Park, IL) intraperitoneal injection (100 mg/kg body weight) before sacrifice. Lungs were then removed and lavaged three times with 0.6 ml of 0.9% sodium chloride with cannula inserted into the trachea. Total lavage fluid for each mouse was combined and then centrifuged. Supernatants were frozen at -80°C until required for further analysis and cell pellets resuspended in 1 ml of saline, and total number of cells determined using a haemocytometer. Differential counts (minimum 500 per slide) were determined using Diff-Quik (Dade Behring, Newark, DE)-stained cytospin slides.

RNA preparation, reverse transcription and quantitative real-time PCR

Total RNA from frozen samples and cell lines was extracted by Invitrogen (Invitrogen, USA) according to the manufacturer's protocol. cDNAs from all samples were synthesized from 1 μg of total RNA by PrimeScript RT Master Mix kit (Takara, Dalian, China). The expression of MEG3 and miR-770 was analyzed by qRT-PCR using Quantifast SYBR Green PCR Kit (Qiagen, German) at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Data analyses for the gene expression were performed using the 2-ΔΔCt method.

Lung tissue protein extraction

Cytoplasmic and nuclear proteins were extracted from frozen lung tissue samples as described. Whole cell lysate was extracted from lung tissue after homogenization in RIPA buffer.

Protein extraction and Western blotting

Total protein was extracted using a Qproteome Mammalian Protein Prep Kit (Qiagen, German) in accordance with the manufacturer's instruction. Typically, 20 μg of the protein was loaded per lane. Protein samples were resolved using SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk for one hour at room temperature and incubated at 4°C overnight with the antibodies. Sequentially, the secondary antibodies were conjugated to horseradish peroxidase, and the proteins were visualized via chemiluminescence (Beyotime, China). Actin (Abcam, UK) was used to normalize the quantity of the protein.

Histochemistry

The lungs were inflated with 0.6 ml of 10% buffered formalin, fixed for 24 h at 4°C, before histochemical processing. The whole lung was embedded in paraffin, sectioned at a 5-μm thickness, and stained with H&E (n=6) for routine histopathology. Tissue sections were also stained with PAS for the identification of stored mucosubstances within the mucus goblet cells lining the main axial airways (proximal), as previously described. The number of PAS-positive cells was counted on longitudinal lung sections

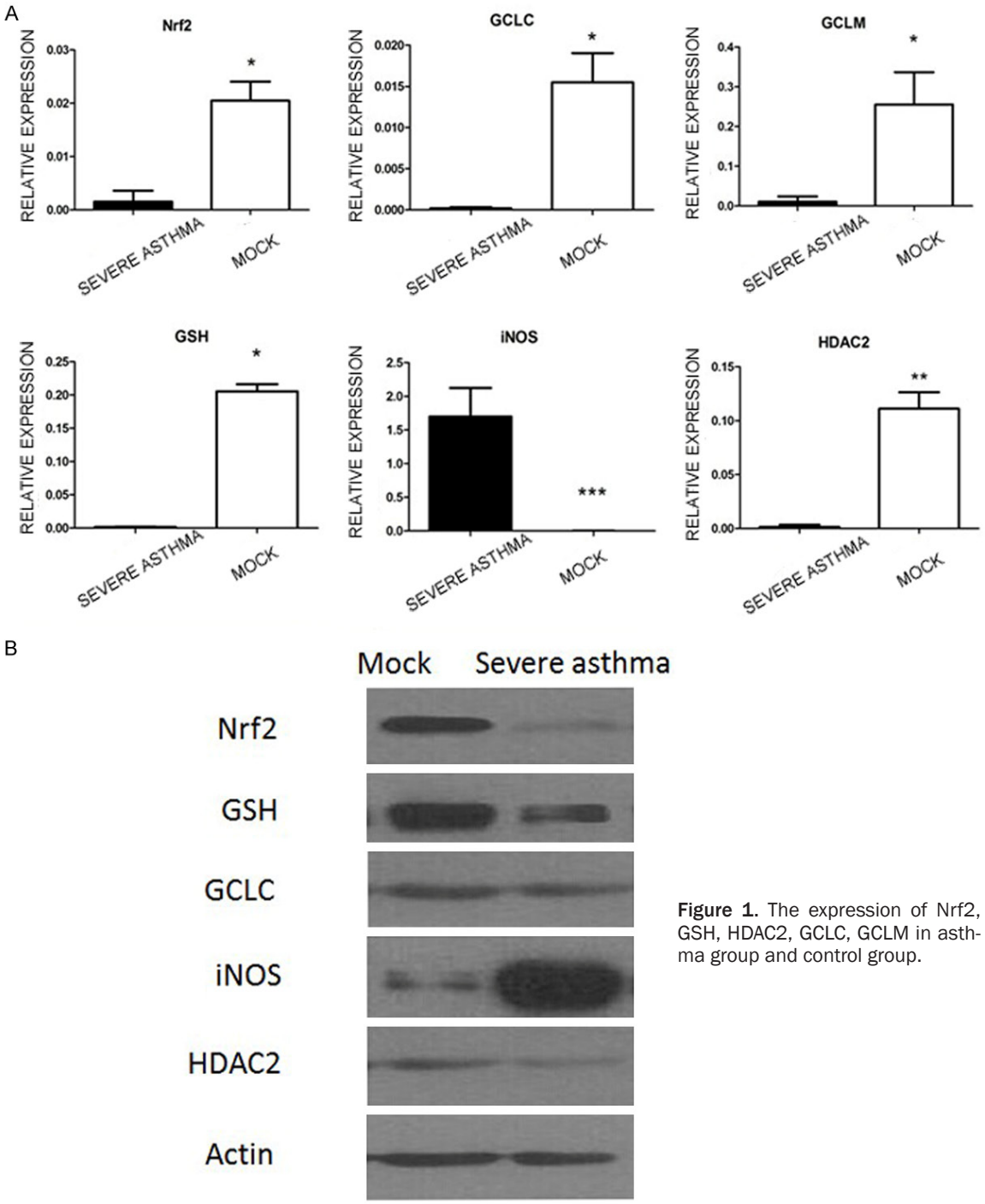

Figure 1. The expression of Nrf2, GSH, HDAC2, GCLC, GCLM in asthma group and control group.

of the proximal airways. The percentage of PAS-positive cells was determined by counting the mucus-positive cells and unstained epithelial cells in the proximal airways under the microscope with a grid at 100×. Six animals were used for each treatment. The sum of the values of five fields per slide for five slides is provided for each animal. The data are expressed as means ± SEM.

Enzyme-linked immunosorbent assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) for the measurement of serum of mice lung tissues was developed. Microtiter plates were coated with a mixture of two monoclonal antibodies and bound t-IL-8/MCP-1 was quantitated with a third monoclonal antibody linked to peroxidase. The lower limit of sensitivity of the

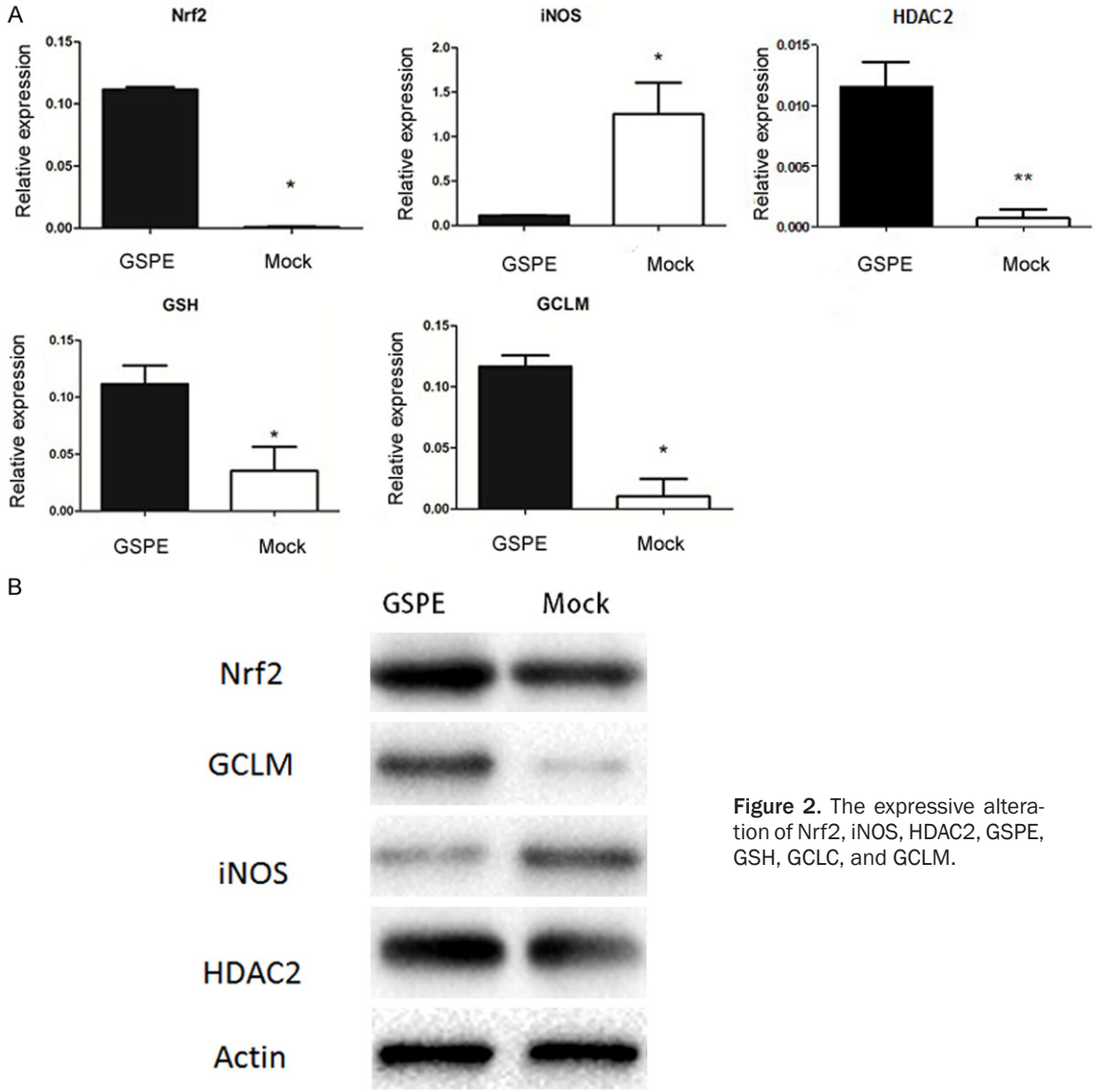

Figure 2. The expressive alteration of Nrf2, iNOS, HDAC2, GSPE, GSH, GCLC, and GCLM.

assay was 0.2 ng of t-IL-18/MCP-1 per ml. The assay had a good reproducibility with values of 3.8, 6.5 and 4.9 percent respectively for the intra-, inter-assay and inter-dilution variation coefficients. The results of the ELISA assay on serum samples from lung tissues of mice , over a wide concentration range, with those obtained with a previously described two-site immuno-radiometric assay (r=0.96). This ELISA with monoclonal antibodies constitutes a stable and reproducible set of reagents for the measurement of t-IL-8/PMCP-1 antigen in biological fluids, avoiding the disadvantages of the use of radioisotopes and of polyclonal antibodies.

HDAC2 activity assay

HDAC2 was immunoprecipitated from lung homogenates (500 μg protein) by incubating overnight with anti-HDAC2 antibody (2 μg). Beads were washed and incubated with Color de Lys substrate (Biomol) for 80 minutes with rocking at 37°C. 30 μL aliquots from each sample were placed in 96-well plates and HDAC specific buffer added. Color de Lys developer was then added and incubated for a further 20 minutes with rocking at 37°C.

Color development was monitored at 405 nm. HDAC2 activity was expressed relative to stan-

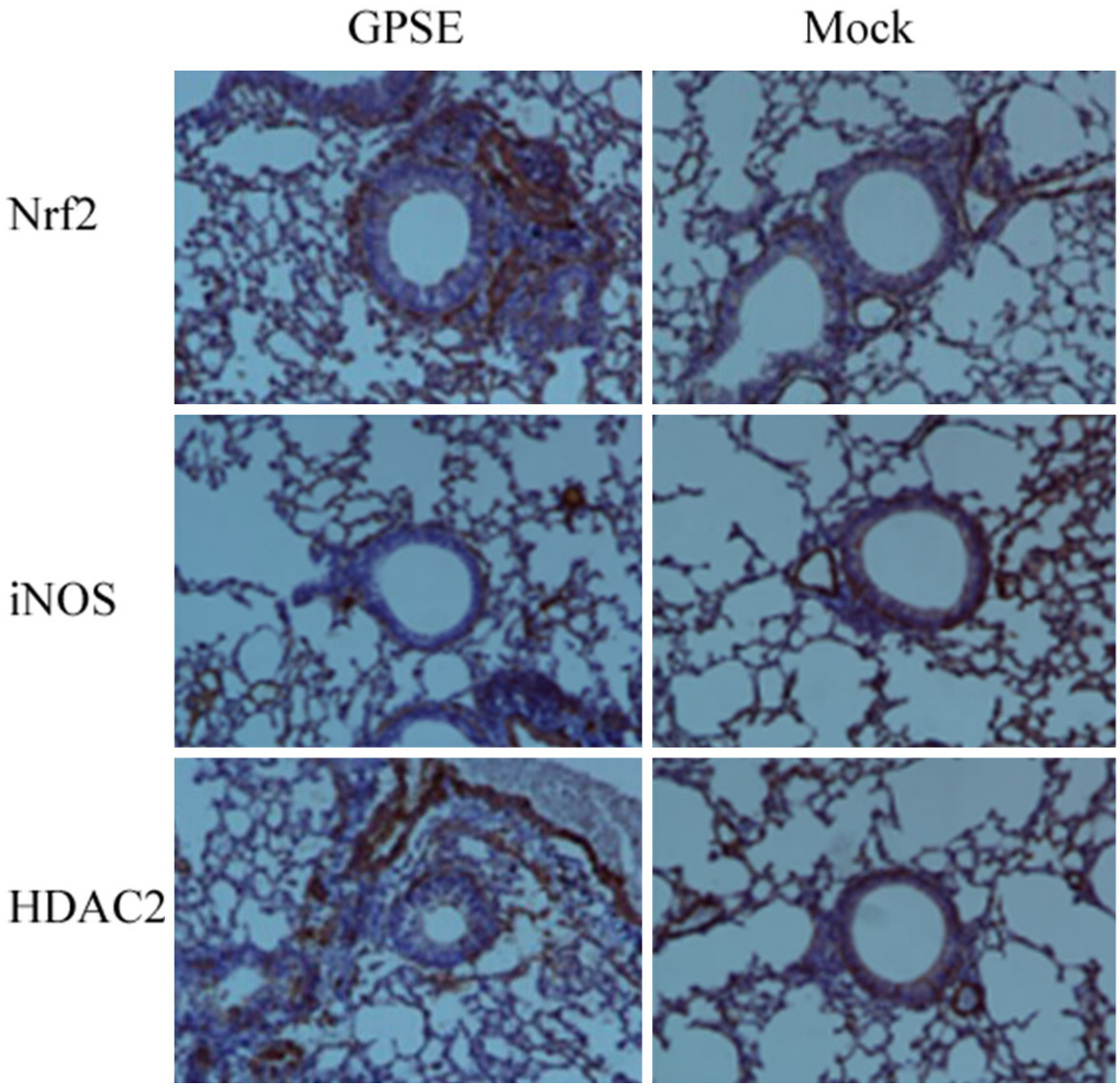

Figure 3. The immunohistochemistry results showed the expression of Nrf2, HDAC2 in GSPE group was up-regulated compared with control group. While iNOS is down-regulated in GSPE group.

dard curve generated from 0-500 μM Color de Lys deacetylated standard.

Statistical analysis

Data expressed as mean ± SEM. Statistical significance was calculated using one-way Analysis of Variance (ANOVA) with STATVIEW software. NIH Image J software was used for densitometry analysis. $P<0.05$ as significant compared to relative controls.

Results

Expression of Nrf2, iNOS, HDAC2, GCLM, GCLC in murine models of asthma

Previous study has verified that steroid resistance might be one of the most important etiologies of difficult-to-treat asthma, but the exact mechanism still remains unclear. Illustrating the pathogenesis of steroid resistance and seeking out the corresponding drug have important implications for the therapy of difficult-to-treat asthma. It has been reported that the function defect of HDAC2 caused by oxidation/nitration imbalance is a vital etiology of steroid resistance. In addition, iNOS and Nrf2 have been confirmed to exert a role in the regulation of HDAC2. Thus, we built murine models of asthma and control models, and sacrificed the mice to gather the lung tissues in 10 weeks. Then we utilized the real-time-PCR and western blot to detect to expression of Nrf2, iNOS, HDAC2, GCLM, GCLC in the asthma model and control groups. As shown in **Figure**

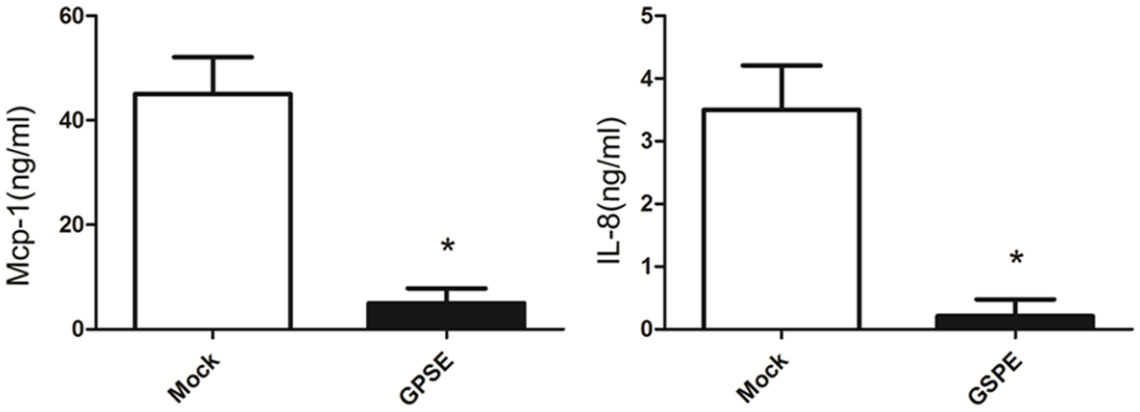

Figure 4. ELISA detection of IL-8 and MCP-1 in the serum of serve asthma model and control groups.

1A the expression of Nrf2, GSH, HDAC2, GCLC, GCLM in serve asthma group was remarkably down-regulated compared with control group. On the contrary, the expression level of iNOS in serve asthma group was obviously up-regulated. Furthermore, western blot (**Figure 1B**) has achieved the same results.

GSPE has a positive application for the therapy of serve asthma via regulating Nrf2-iNOS-HDAC2 axis

Our results have confirmed that the low expression of Nrf2 might up-regulate iNOS and down-regulates GSH in murine models of asthma. The down-regulation of GSH caused disorder of HDAC2 function and activity and ultimately results in steroid resistance. In general, our findings demonstrated the disorder of Nrf2-iNOS-HDAC2 axis promotes the steroid resistance. Moreover, we found that GSPE which was applicable to the clinical measurement of serve asthma could influence the expression of Nrf2, iNOS, HDAC2. GSPE could promote the Nrf2, HDAC2, GSH, GCLC, GCLM expression but suppressed the iNOS (**Figure 2A**, **2B**). The effect on Nrf2-iNOS-HDAC2 might mean that GSPE exert a positive role in the therapy of serve asthma via regulating Nrf2-iNOS-HDAC2 axis. The immunohistochemistry has confirmed the results of western blot and real time PCR (**Figure 3**).

GSPE exerts a role in the therapy of serve asthma through regulating the expression of IL-8 and MCP-1

We have demonstrated that GSPE exert a positive role in the therapy of serve asthma via regulating Nrf2-iNOS-HDAC2 axis. However, the distinct mechanism still remains unclear. In our study, we preformed enzyme-linked immunosorbent assay (ELISA) to test IL-8 and MCP-1 in the serum of serve asthma model and control groups and found that IL-8 and MCP-1 expressions were suppressed by GSPE (**Figure 4**). Thus, we illustrated that the pharmacological mechanisms of the GSPE in the therapy of serve asthma.

Discussion

Asthma is a chronic inflammatory disorder of the airways associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing. Asthma exacerbations are characterized by decreased expiratory airflow that can be quantified by measure of lung function; they are often treated with bursts of oral corticosteroids and are the source of significant asthma morbidity. Exacerbations, especially in difficult-to-treat asthma, represent asthma treatment failure and account for a substantial portion of the total costs of asthma. According to the 2014 Global Initiative for Asthma report, between 30 and 50% of all asthma medical expenditures are the result of exacerbations. It has been reported that steroid resistance might be one of the most important etiologies of difficult-to-treat asthma, but the underlying mechanism still remains unclear. Illustrating the pathogenesis of steroid resistance and seeking out the corresponding drug have vital implications for the therapy of difficult-to-treat asthma. It has been verified that the function HDAC2 defect caused by oxidation/nitration imbalance is a vital etiology of steroid resis-

tance. Additionally, inducible nitric oxide iNOS and Nrf2 have been confirmed to exert a role in the regulation of HDAC2.

Gene expression of iNOS greatly increased [18] in Nrf2 gene knockout mice, activation of Nrf2 can inhibit the expression of iNOS [18]; therefore, Nrf2 is negative regulation of expression of iNOS. Recently, Adenuga [19] reported lung inflammation induced by lipopolysaccharide (LPS) of Nrf2 wild type mice can be effectively reduced by steroid, but in Nrf2 knockout mice steroid shows no reducing effect. Meanwhile, HDAC2 gene expression is decreased in lung tissues of Nrf2 gene knockout mice, this demonstration that the Nrf2-HDAC2 axis plays an important role in the mechanism of hormone resistance.

Illustrating the pathogenesis of steroid resistance and seeking out the corresponding drug have vital implications for the therapy of difficult-to-treat asthma. It has been reported that the function defect of HDAC2 caused by oxidation/nitration imbalance is a vital etiology of steroid resistance. In addition, inducible nitric oxide synthase (iNOS) and NF-E2-related factor (Nrf2) have been confirmed to exert a role in the regulation of HDAC2. GSPE is a mixture of polyphenols, which are mainly dimers, trimers and highly polymerized oligomers of monomeric catechins [20]. In recent years, mounting evidence indicates that GSPE can counter oxidative stress, protect circulation, and has anti-inflammatory and anti-carcinogenic activity. Reportedly, GSPE is also involved in the immunoregulatory activity in vitro and in vivo [21].

In our present research, we confirmed that the low expression of Nrf2 up-regulates iNOS and down-regulates GSH in murine models of asthma. The down-regulation of GSH causes disorder of HDAC2 function and activity and ultimately results in steroid resistance. Overall, our findings demonstrated the disorder of Nrf2-iNOS-HDAC2 axis promotes the steroid resistance. Additionally, we found GSPE relieves the steroid resistance via regulating Nrf2-iNOS-HDAC2 axis in mice. Conclusively, our study illustrated the pathogenesis of steroid resistance and sought out the corresponding drug have vital implications for the therapy of difficult-to-treat or serve asthma.

Taken together, this study is the first to demonstrate that GSPE modulates inflammatory response in the steroid resistant asthma by regulating Nrf2-iNOS-HDAC2 axis in mice. With anti-inflammatory and immunomodulatory role, GSPE may be useful as an adjuvant therapy for asthmatic patient especially refractory asthma phenotype in the future.

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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