

Original Article

Effect of diammonium glycyrrhizinate on airway inflammation and Nrf2 Expression in obese mice with asthma

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Abstract: Obese asthma is a distinct and novel phenotype of asthma, which is associated with glucocorticoid resistance. Oxidative stress may contribute to the pathophysiological process. Nrf2 exerts protective effects of ameliorating oxidative stress. We investigated whether Diammonium glycyrrhizinate (DG) can attenuate airway inflammation, reduce oxidative stress and activating Nrf2 in mouse model of obese asthma. Mice were divided into four groups: control, asthma, obese asthma, and obese asthma with DG treatment. We developed mouse models of asthma by OVA and obesity by high-fat diet, and then performed a pulmonary function test to assess airway responsiveness. We also investigated lung histology and measured cell counts in bronchoalveolar lavage fluid (BALF). MDA and GSH in lung were measured, multiplex luminex assay for inflammatory cytokines in BALF were conducted. The protein expression of Nrf2 was determined with Western blot. Mice in both obese asthma group and asthma group developed obvious airway inflammation and AHR to methacholine. Obese asthma mice treated with DG exhibited significantly declined AHR, attenuated airway inflammation and lower pro-inflammatory mediator levels. And MDA was decreased, GSH was increased in treatment group. Also, treated with DG significantly enhanced Nrf2 expression in lung tissue compared to mice in the obese asthma group. The results suggest DG has an anti-inflammatory activity in obese asthma through activating Nrf2 and restoring the oxidant-antioxidant balance. DG may be an acceptable treatment of obese asthma.

Keywords: Asthma, obesity, diammonium glycyrrhizinate, Nrf2, oxidative stress

Introduction

Asthma is a common chronic airway disease affecting about 300 million people worldwide and is characterized by airway inflammation, airway remodeling and airway hyper-responsiveness (AHR). To date, inhaled corticosteroids (ICS) are accepted as preferred anti-inflammatory treatment for asthma which are effective enough in the most conditions. However, asthma is a heterogeneous chronic inflammatory disorder with complex pathophysiology and different subphenotypes [1]. There still are some patients show lower responsiveness or even resistance to corticosteroids. These patients often suffer severe refractory asthma. On the other hand, long-term administration of corticosteroids is often to cause undesirable ad-

verse effects, such as candidiasis, osteoporosis, growth retardation and pharyngitis etc.

Obese asthma is a distinct and novel phenotype of asthma, which is associated with an altered response to asthma controller therapy, glucocorticoid resistance, and poor asthma control [2]. Epidemiological evidence suggests that the incidence of both obesity and asthma has increased in the past decades. They exert a great impact on public health. And obesity affects the incidence and severity of asthma. As obese asthma is difficult to control, a novel strategy is urgently needed to prevent or treat obese asthma.

Diammonium glycyrrhizinate (DG), an active compound extracted from liquorices root, ex-

erts multiple biochemical and pharmacological activities including antioxidant and anti-inflammatory properties in numerous studies. Ram A et al. [3] have reported Glycyrrhizin has therapeutic efficacy in asthma for it alleviates asthmatic features. Diammonium glycyrrhizinate can inhibit airway smooth muscle proliferation possibly through up-regulation of PPAR γ in a murine model of chronic asthma [4]. In many observation studies, the protective effects of DG were associated with reducing generation of reactive oxygen species (ROS) and restoring oxidant-antioxidant balance.

It has been demonstrated that obesity can induce systemic oxidative stress [5]. Oxidative stress is the imbalance between the production of oxidants and endogenous antioxidant defenses in cells. Increased ROS lead to oxidant-antioxidant imbalance which contributes to the pathological features of asthma. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key regulator of the cellular antioxidant defenses. Nrf2 regulates the expression of about 1% of human genes, which encode many cytoprotective proteins that ensure cellular tolerance to multiple stressors by participating in biotransformation, antioxidant reactions, and inflammation, and by modifying the cellular metabolic program [6].

We hypothesised that Diammonium glycyrrhizinate can protect obese asthma through antioxidant effect and enhancing Nrf2 activation. Herein, we investigated whether DG can attenuate airway inflammation; inhibit ROS production and activating Nrf2 in mouse model of obese asthma. We will provide experimental results to highlight DG as a new therapeutic potential for prevention and treatment of obese asthma.

Materials and methods

Animals

Specific pathogen-free male BALB/C mice, 3-4 weeks old, weight 18-22 g, were supplied by Laboratory Animal Center of Guangxi Medical University (Nanning, Guangxi, China). Mice were fed for 8 weeks with either a standard chow diet (70% carbohydrate; 20% protein, 10% fat) or a high-fat diet that induces obesity (29% carbohydrate, 16% protein, 55% fat), with food and water ad libitum, in conditions of tempera-

ture (20°C) and relative humidity (40-60%) with a 12:12-h light-dark cycle. All animal care and experimental protocols followed the Guide for the Care and Use of Laboratory Animals. All of the experimental protocols used in this study were approved by the Ethical Principles in Animal Research adopted by the Guangxi Medical University for Animal Experimentation.

Experimental protocols and OVA sensitization and challenge

A total of twenty-four mice were randomly divided into four experimental groups (n=6/group): the control group (group A), the asthma group (group B), obese asthma group (group C) and obese asthma with DG treatment group (group D). The mice of group A and B were fed standard mouse chow, and group C and D were fed with a high-fat diet to prepare a diet-induced obesity (DIO) model according to the method described in our previous study [7]. During 12 weeks of fed, the body weight and length of all mice were measured weekly on a certain day (at 16.00). In the last 4 weeks, sensitization and challenging animals to Ovalbumin (OVA) was performed using the method described previously [8]. In brief, mice were IP injected 25 μ g OVA (grade V; Sigma) complexed with 1 mg Al(OH)₃ (Sigma) on day 1, day 7 and 14, and then mice were challenged with aerosol OVA (2% in saline) for 20 min daily for 7 consecutive days in a closed chamber. The mice in the control group were challenged with aerosolized saline. The mice of group D were treated with 50 mg/kg DG by intraperitoneal injection once daily before each time challenge.

Airway responsiveness measurement

Airway responsiveness was assessed in anesthetized and tracheotomized mice that were mechanically ventilated in response to increasing dose of methacholine inhalation, using a computerized small animal ventilator (Finepoint, Buxco Electronics, Wilmington, NC). The test procedures were performed as previously described [9]. Methacholine is used to diagnose asthma by inducing bronchoconstriction. Lung resistance (RL, cmH₂O.s/ml) were measured to determine the airway responsiveness of each mice, recording the dose responsiveness to methacholine (6.25, 12.5, 25 and 50 mg/ml). Mice were allowed to stabilize on the ventilator for 5 min prior to measurements.

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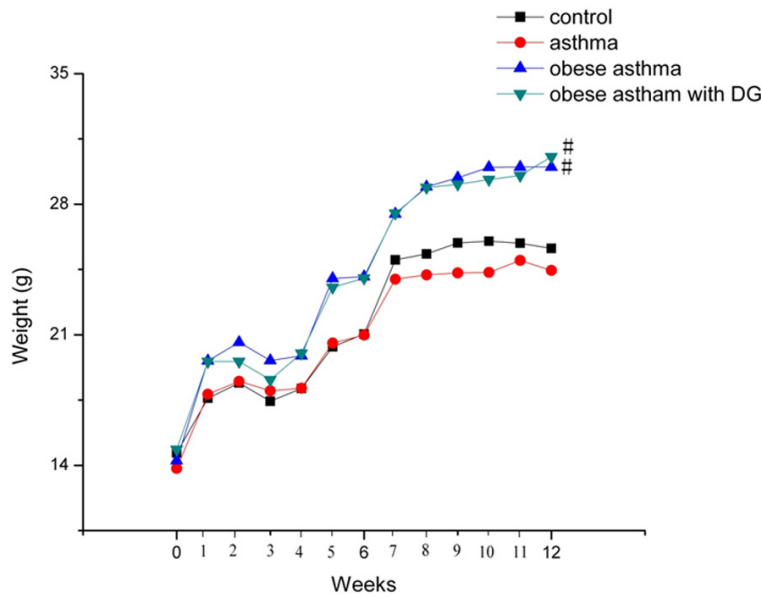


Figure 1. Measurement of body weight. Body weight of the mice with a high-fat diet increased more rapidly compared to mice with a normal diet. The final body weight of mice with high-fat diet was significantly higher than animals with normal-diet ($p < 0.05$).

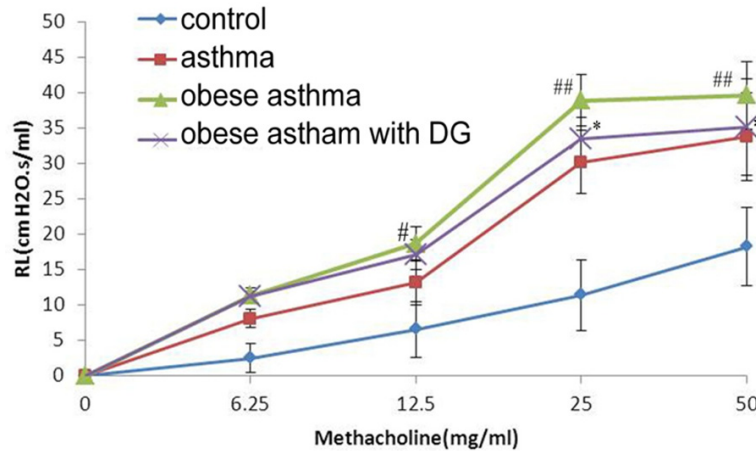


Figure 2. Airway hyperresponsiveness test of all groups. AHR was assessed using a computerized small animal ventilator. Lung resistance (RL, cmH₂O.s/ml) was recorded to determine the airway responsiveness. DG treatment suppressed AHR in obese asthmatic mice. # $p < 0.05$, ## $p < 0.01$, significant difference compared with the normal control group; * $p < 0.05$, significant difference compared with the obese asthma group. $n = 6$ per group.

Sample collection

Mice were sacrificed 24 hours after the last aerosol challenge with an overdose of pentobarbital sodium (100 mg/kg). The lungs were lavaged using 500 μ L iced PBS three times, and the bronchoalveolar lavage fluid (BALF) was collected. Lung tissues were harvested. The supernatant of BALF was stored at -20° until next assay for cytokine determination. The

right upper lobes were fixed in formalin for histology; the right lower lobes were stored for testing bio-markers of oxidative stress; the left lobes were stored at -80° for Western blotting analysis.

Inflammatory cell count in BALF

The supernatant of BALF suspension was used for cytokine quantification. The remaining cell pellet was resuspended by 200 μ L PBS. Fifty microliters of cell suspension was determined by a hemocytometer. Differential cell counts were assessed. Another 50 μ L suspension was subjected to cytopspin at 450 rpm for 5 min, followed by Diff-Quick staining (Sysmex Corporation) to detect inflammatory cells. A total of 300 cells were counted under microscopic examination. Types of inflammatory cells were determined in randomly selected fields of the slide with a differential cell counter (Hwashin Tech) based on morphologic criteria and staining characteristics. Inflammatory cells were classified as eosinophils, neutrophils, macrophages, or lymphocytes.

Lung histology

The lung was fixed in 4% paraformaldehyde in PBS and paraffin-embedded. The lung sections of 4 μ m were stained with hematoxylin and eosin (HE) and Periodic acid-Schiff (PAS) was used for morphological evaluation of the lung tissue. Lung inflammation was assessed by the degree of peribronchiolar and perivascular inflammation. Goblet cell hyperplasia in the epithelium and submucosal gland hypertrophy were assessed. Quantitative analysis was performed by two independent pathological examiners in a blind fashion. A grading scoring system to elucidate the extent of inflammation and goblet cell hyperplasia was adopt-

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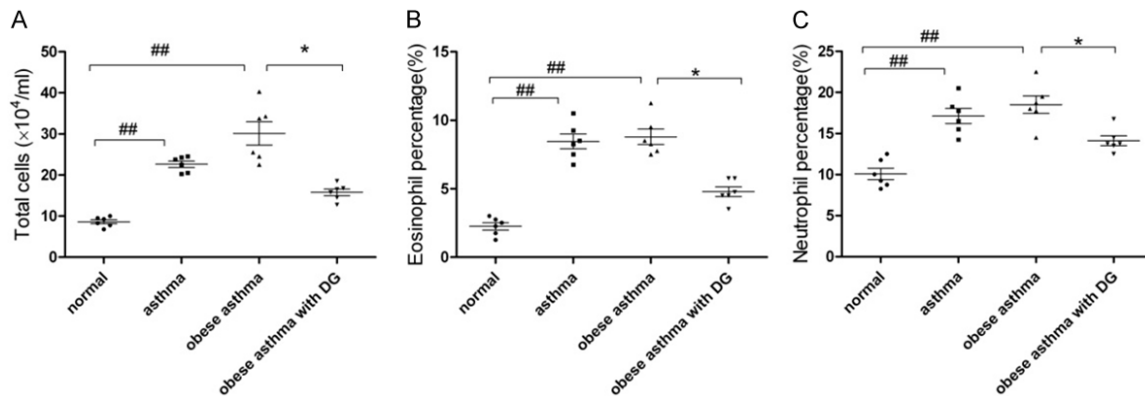


Figure 3. Cell counts in BALF. Cell fractionation and differential cell count in BALF were performed. Data expressed as mean \pm SEM (n=6/group). A. Total inflammatory cell count. B. The mean percentage of eosinophils. C. The mean percentage of neutrophils. # p <0.05, ## p <0.01, significant difference compared with the normal control group; * p <0.05, significant difference compared with the obese asthma model group.

ed, making a semi-quantitative analysis as described previously [8]. Scoring of inflammatory cells and goblet cells was performed in at least three different fields for each lung section; the average value was considered as the final result.

Measurement of bio-markers of oxidative stress

The right lower lobe was crushed in PBS, and lung homogenate was made, centrifuged for 2 min at 4°C (1500 rpm). Then supernatants were collected and total protein level was measured using BCA assay. After lung homogenate preparation, the Malondialdehyde (MDA) and Glutathione (GSH) contents in lung tissue were determined using GSH and MDA test kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to manufacturer's instructions. The concentration of MDA and GSH was showed in μ mol/g-protein.

Cytokine and chemokine analysis in BALF

The amounts of cytokine/chemokine of interleukin (IL)-17A, IL-4, IL-6, tumor necrosis factor α (TNF- α), Monocyte chemotactic protein 1 (MCP-1), keratinocyte-derived chemokine (KC) and RANTES in BALF were analyzed by a multiplex Luminex assay using a Milliplex Mouse Cytokine/Chemokine Immunoassay (Millipore Corporation, Billerica, MA), following the manufacturer's instructions strictly.

Western blot analysis of Nrf2

Western blot analysis for Nrf2 was conducted in lung tissues. Lung tissue was homogenized

in the frozen state in ice-cold lysis buffer. RIPA buffer (Thermo, Rockford, IL) was used to extract protein from crushing lung tissues, following the manufacturer's protocol. Protein content was determined by the Bradford method using Bio-Rad protein assay (Bio-Rad, Hercules, CA). 20 μ g were used for Western blot analysis to determine Nrf2 protein expression with Nrf2 antibody. (Bio-Rad, Hercules, CA). Equal amounts of proteins were separated by 10% SDS-PAGE. The proteins were then transferred electronically onto a polyvinylidene difluoride membrane (PVDF) membrane (0.45 μ m; Millipore Corp., Billerica, MA, USA). The PVDF membranes were incubated with a blocking buffer (5% nonfat milk in 20 mM Tris-HCl pH 7.5), 137 mM NaCl, and 0.1% Tween 20 for 1 h at room temperature. Then the membranes were probed with primary anti-Nrf2 antibodies (Abcam, Cambridge, MA, UK) overnight at 4°C, washed 3 times (20 mM Tris-HCl pH 7.5, 137 mM NaCl, and 0.1% Tween 20), incubated with HRP-conjugated secondary antibodies (1:5000 dilution) for 1 h at room temperature, washed thrice, and then detected with ECL (Amersham Pharmacia Biotech). Densitometry was performed using ImageJ Software.

Statistical analysis

Data analyses were performed with SPSS version 17.0 statistic software. Multiple groups were compared to determine differences using a one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test of means. All data were shown as means \pm SEM (standard error of the mean). Histological sco-

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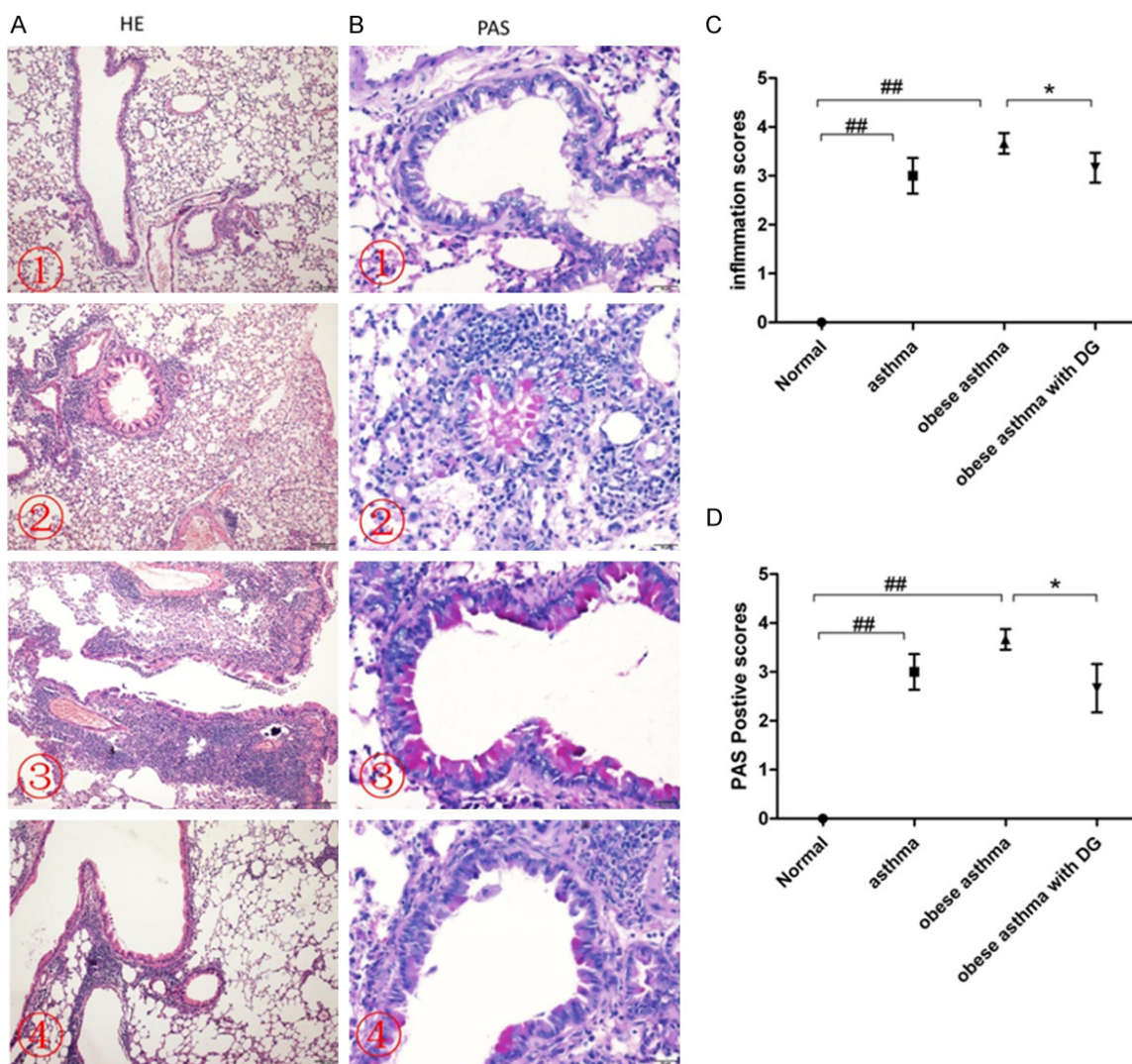


Figure 4. Histological study of lung tissues by HE and AB-PAS staining. Lung inflammation and mucus production were assessed, as described in Materials and Methods. A. HE staining; B. AB-PAS staining; C. The Inflammation scores; D. PAS positive scores. Original magnification: $\times 400$. Data expressed as mean \pm SEM ($n=6$ /group). 1 control group; 2 asthma group; 3 obese asthma group; 4 obese asthma with DG group. Asthma group and obese asthma group mice exhibited an obvious inflammatory cell infiltration shown in HE staining and Goblet cell hyperplasia, mucus hypersecretion in PAS staining. DG treatment reduced the changes. # $p<0.05$, ## $p<0.01$, significant difference compared with the normal control group; * $p<0.05$, significant difference compared with obese asthma model group.

res were compared by the Kruskal-Wallis test. Statistical significance of differences was set at $P<0.05$.

Results

Physiological outcomes and body weights

And results showed the body weights gained in mice on a high fat diet are more rapidly than in mice on a normal diet. The total body mass of high fat diet mice was substantially greater than that of normal diet mice. The mice induced

by OVA exhibited the expected physiological outcomes, including sneezing, nasal itching, catching the ear, urinary and fecal incontinence, and asthma attacks. Obese asthma mice demonstrated the above outcomes more seriously. However, in obese asthma mice with DG, these symptoms were alleviated. (**Figure 1**).

Airway hyperresponsiveness of mice

The mice in both asthma group and obese asthma group, as immunized by OVA, showed a

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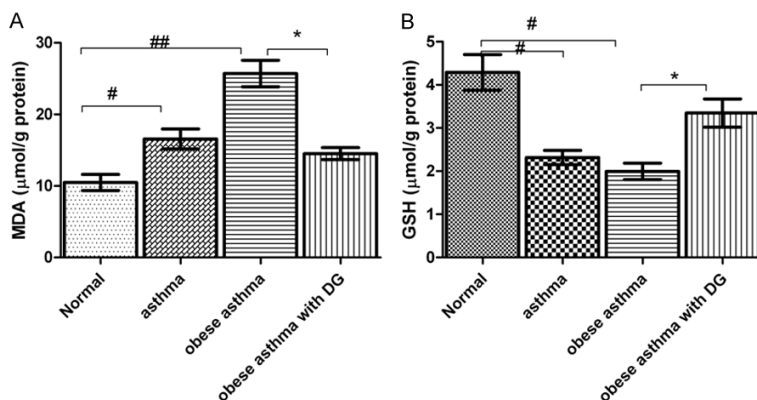


Figure 5. Measurement of bio-markers of oxidative stress in lung. MDA, GSH contents in lung tissue were examined. Data expressed as mean \pm SEM (n=6/group). # p <0.05, ## p <0.01, significant difference compared with the normal control group; * p <0.05, significant difference compared with obese asthma model group.

gradually increase of airway responsiveness after methacholine inhalation compared with the control group. The RL growths in mice of obese asthma group were significantly greater at each concentration of methacholine compared to normal group. In addition, at concentration of 25 g/L, 50 g/L methacholine, the RL growth of obese asthma mice showed a significant increase compared to asthma group mice, which indicating manifest airway hyperreactivity in obese asthma (p <0.01). However, the obese asthma mice treated with DG resulted in lower AHR at high concentrations of methacholine (25 g/L and 50 g/L) (Figure 2).

Effect of DG treatment on cell counts in BALF and lung histological changes

Compared with normal control group, the mice of asthma group, obese asthma group showed a markedly rise in the number of total inflammatory cells in the BALF (p <0.01). The mean percentage of eosinophils and neutrophils in the BALF of the two groups was also markedly increased (p <0.01). In obese asthma with DG treatment group, the total number of cells in the BALF was significantly reduced compared with the obesity group and asthma group. The mean percentage of eosinophils and neutrophils in the BALF of DG treatment group were significantly lower than in the obese asthma group (p <0.05) (Figure 3).

In normal control group mice, lung histological examination showed normal tissue, without

infiltration of inflammatory cells. Asthma group and obese asthma group mice exhibited an obvious inflammatory cell infiltration, which around airways and blood vessels. Goblet cell hyperplasia was observed in asthma group and obese asthma group, and in obese asthma mice goblet cell hyperplasia and mucus hypersecretion was more obvious than mice of asthma group. The treatment of DG reduced the infiltration of inflammatory cells in lung compared with the obese asthma model mice (Figure 4A and 4B). The Inflammation scores

and mucus scores in all groups were shown in Figure 4C and 4D.

Detection of bio-markers of oxidative stress

The levels of MDA in the lung tissue of model groups (asthma and obese asthma group) were significantly higher than those of control group, but the obese asthma was more severe. Moreover, GSH was lower in the both model groups than control group. DG treatment significantly reduced levels of lung MDA and increased the level of GSH in lung tissue of obese asthmatic mice (p <0.05). (Figure 5).

Cytokine concentrations

Asthma group mice and obese asthma group mice showed a increase in cytokine of IL-17A, IL-4, IL-6, TNF, MCP-1, KC and RANTES in the BAL fluid as compared with the normal control mice. In obese asthma group, these pro-inflammatory cytokines were increased the most obviously. The obese asthma with DG treatment group exhibited a significantly reduce of those pro-inflammatory mediator concentrations. (Table 1).

Effect of DG treatment on expression of Nrf2 protein

Nrf2 protein expression was up-regulated in both asthma group and obese asthma group, reflecting a balance for the production of ROS. Treatment of DG significantly promoted the expression of total Nrf2 protein even more.

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Table 1. Cytokine/Chemokine concentrations in BALF

Group	n	IL-4	IL-6	IL-17	MCP-1	KC	TNF- α	RANTS
A	6	11.64 \pm 1.61	4.32 \pm 1.47	4.308 \pm 0.53	7.66 \pm 1.04	48.05 \pm 6.37	12.84 \pm 2.80	3.33 \pm 0.61
B	6	29.56 \pm 2.86 [#]	35.73 \pm 7.17 [#]	25.57 \pm 3.11 [#]	32.73 \pm 3.59 [#]	276.92 \pm 45.18 [#]	87.23 \pm 7.13 [#]	8.73 \pm 1.26 [#]
C	6	75.59 \pm 6.31 [#]	53.26 \pm 12.61 ^{##}	33.64 \pm 7.13 ^{##}	61.13 \pm 10.74 ^{##}	513.80 \pm 96.04 ^{##}	137.60 \pm 12.49 ^{##}	9.51 \pm 1.99 [#]
D	6	53.36 \pm 4.90 [*]	13.12 \pm 1.99 [*]	10.01 \pm 2.41 [*]	35.79 \pm 3.89 [*]	124.20 \pm 23.22 [*]	64.34 \pm 11.45 [*]	3.70 \pm 0.66 [*]

Data expressed as mean \pm SEM (n=6/group). [#]p<0.05, ^{##}p<0.01, significant difference compared with the normal control group; ^{*}p<0.05, significant difference compared with the obese asthma model group.

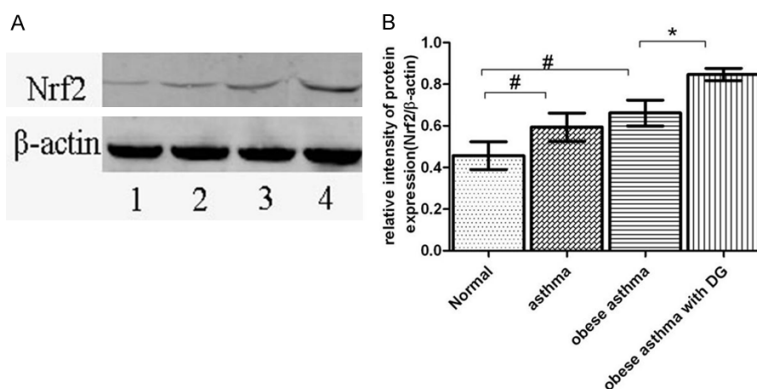


Figure 6. Nrf2 protein expression in lung tissue. Nrf2 expression levels were analyzed by western blot using β -actin as an internal control. A. Western blot images of Nrf2 protein expression. 1, Control group; 2, asthma group; 3, obese asthma group; 4, obese asthma with DG treatment group. B. Relative expression of Nrf2 protein. Relative units are expressed as the relative ratio of Nrf2 to β -actin. Data expressed as mean \pm SEM. (n=6/group). [#]p<0.05, significant difference compared with the normal control group; ^{*}p<0.05, significant difference compared with obese asthma model group.

(**Figure 6A**). Relative expressions of Nrf2 protein to β -actin are shown in **Figure 6B**.

Discussion

We studied the potential of DG treatment for obese asthma using a murine model of obesity with asthma. We investigated the effect of DG on airway inflammation and AHR. Our findings indicate that DG has an obvious efficacy in suppressing the development of airway inflammation and inhibiting AHR. The effect of DG on airway inflammation and AHR involves up-regulating Nrf2 protein expression and reducing oxidative stress in lung tissue of mice.

Bronchial asthma is a common airway disease and inflammation is the hallmark of asthma. Obesity is a low-grade, chronic systemic inflammation state which may affect the lungs to exacerbate asthma. To investigate airway inflammation, we assessed the total cell and differential counts in the BALF and made histological examination of murine lung tissue. In

our present study, we found inflammatory cell numbers were increased in asthma mice and obese asthma mice, and the increase of inflammatory cell in obese asthma mice was more significant. Inflammatory cell numbers in BALF were significantly reduced after treatment of DG. Together, in obese asthma mice, the percentage of eosinophils and neutrophils were increased significantly compared to the control group. DG treatment decreased neutrophil and eosinophil percentage significantly compared with the obese asthma model group. As shown by HE staining and AB-PAS staining, there was a reduction of inflammatory cell infiltrate, goblet cell hyperplasia and mucus hypersecretion in lung tissue in obese asthmatic mice treated with DG.

Another feature of asthma is the relatively high AHR to methacholine. Increased AHR correlates with disease severity and can serve as a monitoring indicator of asthma. Johnston R.A. et al. [10] reported that after OVA sensitization and challenge, obese mice exhibit enhanced airway responsiveness to methacholine. In the current study we found obese mice showed a significant rise of AHR. Our results show the treatment of DG lead to lower AHR at high concentrations of methacholine.

The pathogenesis of asthma involves multiple cells and a network of cytokines and chemokines, adhesion molecules generated by these cells. It has been suggested that T helper 2 (Th2) cells and Th2 cytokines play crucial roles in the development of allergic asthma. However, IL-17 has been largely reported to associated

with severe asthma and steroid-nonresponsive asthma, including obese asthma. Therefore, in the current study we observed the treatment of DG in influencing cytokines and chemokine generation (IL-17A, IL-4, IL-6, TNF- α , MCP-1, KC and RANTES). We observed that, in DG treatment group, these inflammatory mediators production was decreased compared with the obese asthma model group. This result shows that anti-inflammatory effect of DG may associated with suppressing inflammatory mediator secretion.

As previously mentioned, obesity induces systemic oxidative stress. It has been well established that oxidative stress contributes to asthma development. ROS results from inflammation and in turn triggers inflammation [11]. The enhanced oxidative stress can promote the NF- κ B, which upgrade the expression of many genes of pro-inflammatory cytokines and chemokines, including IL-4, IL-5, IL-10, IL-13, IL-8, RANTES, TNF- α and ICAM-1 etc. Besides, oxidative stress is an important factor in corticosteroid insensitivity [12]. MDA level represents the lipid peroxidation in tissues. GSH is an important endogenous antioxidant. We detected MDA and GSH concentration in the pulmonary tissue to access the state of oxidative stress. In the obese asthma model group, the concentration of MDA was higher than those in the normal control group and the concentration of GSH was lower than those in the normal control group. DG treatment reduced the MDA concentration and increased GSH levels. Therefore, our results illustrate that DG can restore the oxidant-antioxidant balance in the obese asthma. We used Western blotting to determine the contribution of the Nrf2 to the balance of oxidant-antioxidant system. Nrf2 expression was significantly increased in lungs from obese asthma mice with DG treatment, relative to the obese asthma model group. Nrf2 transcription factor has been known as a regulator of antioxidant gene. So, we demonstrate DG has an anti-inflammatory activity in obese asthma through activating Nrf2 and restoring the oxidant-antioxidant balance.

Overall, the findings highlight that DG may be used for treatment of obese asthma and may provide a potential strategy for patients with steroids-resistant asthma. Further studies are needed to confirm its clinical usefulness in obese asthma of human beings.

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Disclosure of conflict of interest

None.

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