



Original Article

Effect of diosmetin on airway remodeling in a murine model of chronic asthma

Ai Ge[†], Yanan Liu[†], Xiaoning Zeng[†], Hui Kong, Yuan Ma, Jiaxiang Zhang, Fangfang Bai, and Mao Huang*

Department of Respiratory Medicine, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China

[†]These authors contributed equally to this work.

*Correspondence address. Tel: +86-25-68136269; Fax: +86-25-83673567; E-mail: huangmao6114@126.com

Received 31 January 2015; Accepted 17 April 2015

Abstract

Bronchial asthma, one of the most common allergic diseases, is characterized by airway hyperresponsiveness (AHR), inflammation, and remodeling. The anti-oxidant flavone aglycone diosmetin ameliorates the inflammation in pancreatitis, but little is known about its impact on asthma. In this study, the effects of diosmetin on chronic asthma were investigated with an emphasis on the modulation of airway remodeling in BALB/c mice challenged with ovalbumin (OVA). It was found that diosmetin significantly relieved inflammatory cell infiltration, goblet cell hyperplasia, and collagen deposition in the lungs of asthmatic mice and notably reduced AHR in these animals. The OVA-induced increases in total cell and eosinophil counts in bronchoalveolar lavage fluid were reversed, and the level of OVA-specific immunoglobulin E in serum was attenuated by diosmetin administration, implying an anti-Th2 activity of diosmetin. Furthermore, diosmetin remarkably suppressed the expression of smooth muscle actin alpha chain, indicating a potent anti-proliferative effect of diosmetin on airway smooth muscle cells (ASMCs). Matrix metalloproteinase-9, transforming growth factor- β 1, and vascular endothelial growth factor levels were also alleviated by diosmetin, suggesting that the remission of airway remodeling might be attributed to the decline of these proteins. Taken together, our findings provided a novel profile of diosmetin with anti-remodeling therapeutic benefits, highlighting a new potential of diosmetin in remitting the ASMC proliferation in chronic asthma.

Key words: airway remodeling, diosmetin, airway smooth muscle cells (ASMCs), TGF- β 1, chronic asthma

Introduction

Asthma, a disorder encompassing features such as airway hyperresponsiveness (AHR), chronic inflammation, and remodeling [1], is a serious public problem with increasing morbidity and mortality across the globe. Many strategies have been developed for the management of asthma, but few are particularly effective. The primary therapeutic option for asthma is to address the imbalance between Th1 and Th2 cytokines such as interleukin (IL)-4, IL-5, and IL-13 [2,3]. Although the severity of this disease is definitively dependent on the extent of airway remodeling [4], including subepithelial fibrosis, mucous hypersecretion, extracellular matrix (ECM) deposition, increased airway smooth muscle (ASM) mass, and vascular alterations [5,6], little

attention has been paid to anti-remodeling as a cure for asthma. Glucocorticoids are the first-line agents for asthma patients, owing to their therapeutic effects against airway inflammation and remodeling. However, some patients are insensitive to glucocorticoids and so benefit little from glucocorticoids [4,7,8]. A range of adverse effects such as hyperglycemia, hyperlipidemia, hypertension, osteoporosis, and susceptibility to pathogens also occur during treatment [9,10]. So, there is an urgent need to develop new therapeutic agents for asthma with low toxicity.

Diosmetin (3',5,7-trihydroxy-4'-methoxyflavone) (Fig. 1A) is a flavone aglycone found in citrus species [11] and olive leaves [12]. It was reported that diosmetin could elicit chemopreventive and

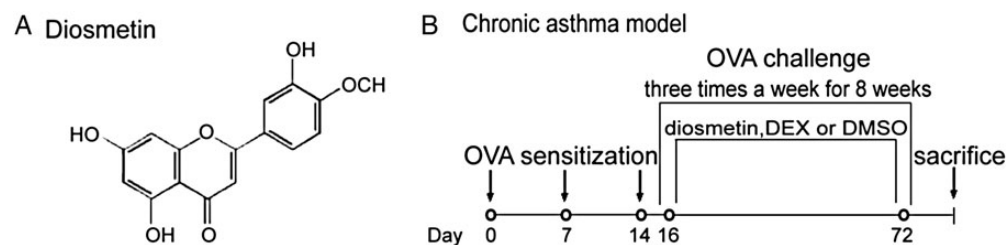


Figure 1. Chemical structure of diosmetin and experimental protocols for OVA-induced remodeling (A) Chemical structure of diosmetin. (B) BALB/c mice were sensitized with OVA by three intraperitoneal injections on days 0, 7, and 14, and then received an aerosolized OVA challenge three times a week from days 16 to 72. Diosmetin, dexamethasone (DEX), or DMSO was given 30 min before each OVA challenge.

anti-proliferative effects in various tumors [13–16]. Other properties of diosmetin have also been documented, such as anti-microbial activity [17,18] and anti-inflammatory activity [16,19,20]. Recently, emerging evidence has suggested that diosmetin possesses strong anti-oxidant activity through scavenging intracellular reactive oxygen species (ROS), which causes pathophysiologic events implicated in asthma by contracting ASM, altering protein structure, initiating mucus hypersecretion, and exacerbating microvascular permeability [21,22]. The airway inflammation and remodeling are critical determinants of asthma, but the advantages of diosmetin in allergen-induced asthma have not been well determined. In this study, an ovalbumin (OVA)-induced chronic model of asthma was established to elucidate the potential benefits of diosmetin against lung inflammation and remodeling, which opens a new perspective of diosmetin as a therapeutic agent for asthma.

Materials and Methods

Animals

Specific pathogen-free female BALB/c mice (6–8 weeks of age and 18–22 g of weight) were obtained from Vital River Laboratories (Beijing, China). The mice were housed at room temperature under a 12-h light/dark cycle and allowed free access to standard chow and water *ad libitum*. The average lifespan of the animals was ~1 year in the Animal Facility of Nanjing Medical University. All experiments involving animals and tissue samples were conducted according to the Guidelines of the National Institutes of Health. The research procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

OVA sensitization and challenge

Female BALB/c mice were intraperitoneally immunized on days 0, 7, and 14 with 0.5 mg/kg OVA (Grade V; Sigma, St Louis, USA) emulsified in 2 mg aluminum hydroxide gel (InvivoGen, San Diego, USA) in a total volume of 200 μ l. The OVA-sensitized mice were exposed to aerosolized 5% OVA in 51 $\times$ 31 $\times$ 21 cm chambers connected to an ultrasonic nebulizer (NE-U11B; OmronCorp, Tokyo, Japan) for 8 weeks: from days 16 to 72, three times a week, 30 min for each time. And, the OVA-sensitized mice were given 0.05, 0.1, 0.5, or 1 mg/kg diosmetin (Sigma) intraperitoneally 30 min before each inhalation challenge (Groups D0.05, D0.1, D0.5, and D1, respectively). An initial stock concentration of diosmetin was made at 100 mM in dimethylsulfoxide (DMSO) and then diluted with saline. Dexamethasone (DEX, 1 mg/kg, positive control) and DMSO (0.4 μ l in a total volume of 200 μ l with saline, solvent control) were also given intraperitoneally 30 min before nebulization. The control group was subject to the same protocol with saline only. All mice were euthanized 24 h after

the last challenge, and the bronchoalveolar lavage fluid (BALF), serum, and lung tissue were collected for analyses. The procedures for the experiment are summarized in Fig. 1B.

Measurement of AHR

AHR was measured using whole-body plethysmography (Buxco Electronics, Troy, USA), which has been recently validated for measuring lung function in murine asthma models [23]. The mice were anesthetized by pentobarbital sodium (70 mg/kg), and the trachea was surgically exposed to connect with a computer-controlled ventilator via an intratracheal tube. Mice were nebulized with phosphate-buffered saline (PBS) and then with increasing concentrations of methacholine from 3.125 to 50 mg/ml by the nebulizer. The results were recorded as enhanced pause (Penh) values.

BALF collection and differential cell counts

BALF was collected by lavaging three times with 1.2 ml saline, and 85%–90% of the total input volume was recovered. BALF obtained from each mouse was centrifuged at 100 g for 15 min at 4°C to extract the supernatant for transforming growth factor (TGF)- β 1 detection. Total cells in BALF were counted with a hemocytometer. The cell differentials were then enumerated based on morphological criteria by Wright's staining. The counting was performed by two independent observers in a single-blind study, in which at least 200 cells from three different random locations were analyzed using a microscope.

Enzyme-linked immunosorbent assay (ELISA)

The level of TGF- β 1 in BALF was detected using a mouse TGF- β 1 ELISA kit (R&D Systems, Abingdon, UK) following the instructions provided by the manufacturer. The level of OVA-specific immunoglobulin E (IgE) in serum was determined using a mouse OVA-specific IgE ELISA kit (Shibayagi, Gunma, Japan). All samples were analyzed in triplicate.

Lung histology

The lower right lungs were fixed in 4% paraformaldehyde for 18–24 h and then embedded in paraffin. Serial 5- μ m tissue sections were obtained and stained with hematoxylin–eosin (H&E), periodic acid Schiff (PAS), and Masson's trichrome to detect inflammatory cell infiltration, mucus production, and collagen deposition around the airway, respectively. Immunohistochemistry was used to evaluate the expression of smooth muscle actin alpha chain (α -SMA) and matrix metalloproteinase (MMP)-9. Quantitative analyses of inflammatory cells and goblet cells in lung tissues were performed in blind fashion as previously described [24]. Briefly, an inflammation score was used to assess the severity of infiltration, based on a five-point scoring

system: 0, normal; 1, a few cells; 2, a ring of cells 1-cell-layer deep; 3, a ring of cells 2- to 4-cell deep; and 4, a ring of cells of >4-cell deep. A mucus score was used to evaluate the extent of mucus secretion using a five-point grading system: 0, no goblet cells; 1, <25% goblet cells; 2, 25%–50% goblet cells; 3, 50%–75% goblet cells; and 4, >75% goblet cells. The scores were statistically calculated from at least three different fields for each lung section, and a mean score was obtained from three animals.

Western blot analysis

Lung tissues were excised on ice and homogenized 1:10 (w/v) in RIPA lysis buffer (Thermo Scientific, Rockford, USA) supplemented with a protease inhibitor cocktail (Roche, Indianapolis, USA). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, USA) by the wet transfer method. Nonspecific sites were blocked with 5% nonfat milk in TBS Tween 20 (TBST) (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature. Then, the blots were incubated with anti-SMA (1:100; Abcam, Cambridge, UK), anti-MMP-9 (1:1000; Abcam), anti-vascular endothelial growth factor (VEGF, 1:1000; Abcam), and anti-glyceraldehyde-3 phosphate dehydrogenase (GAPDH, 1:5000; Bioworld, Nanjing, China) antibody overnight at 4°C. The horseradish peroxidase-labeled secondary antibodies (1:10,000; Cell Signaling Technology, Inc., Beverly, USA) were then added and

incubated at room temperature for 1 h. After the membranes were treated with enhanced chemiluminescence western blot detection reagents (Thermo), the binding of specific antibodies was visualized using the Bio-Rad Gel Doc/Chemi Doc Imaging System and analyzed using Quantity One software.

Statistical analysis

The data are presented as the mean $\pm$ SEM. All statistical analyses were performed using one-way ANOVA, followed by a Dunnett's post hoc test, employing Prism 6.00 software (GraphPad Software, San Diego, USA) and SPSS version 20 (SPSS, Inc., Chicago, USA). $P < 0.05$ was considered statistically significant.

Results

Effect of diosmetin on AHR and serum level of OVA-specific IgE

The *in vivo* airway reactivity to aerosolized methacholine was assessed to determine whether diosmetin treatment improves the pulmonary function of allergic mice after chronic challenge with OVA. Results showed a significant decrease in Penh values in diosmetin-treated groups compared with OVA group induced by 12.5 mg/ml methacholine (D0.1, 2.19 ± 0.05 , $P < 0.05$; D0.5, 2.15 ± 0.04 , $P < 0.05$; D1, 2.28 ± 0.10 , $P < 0.05$ vs. OVA, 2.80 ± 0.08) (Fig. 2A). Similar results were also obtained in OVA-sensitized mice induced by 50 mg/ml methacholine (D0.1, 3.48 ± 0.19 , $P < 0.05$; D0.5, 2.99 ± 0.07 , $P < 0.05$; D1,

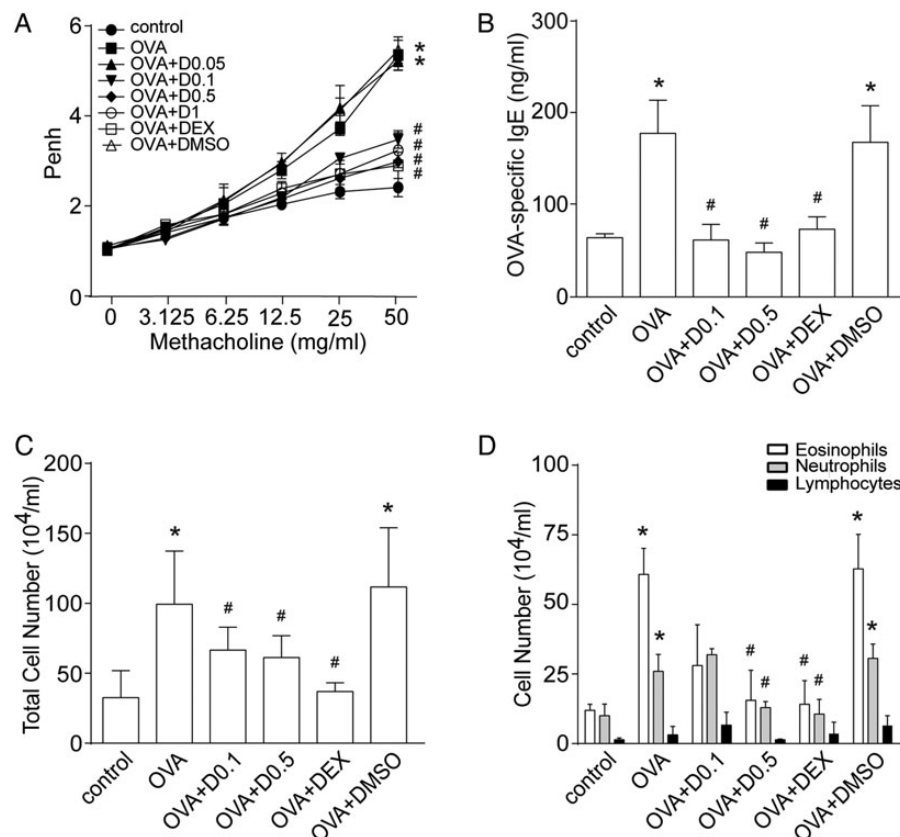


Figure 2. Treatment with diosmetin inhibits AHR, OVA-specific IgE in serum, and inflammatory cell infiltration in OVA-sensitized mice (A) AHR was determined in the response to methacholine on 24 h after the last OVA challenge. Mice inhaled increasing doses of methacholine (3.125–50 mg/ml), and AHR was measured and shown as Penh value. (B) OVA-specific IgE serum level was measured by ELISA. (C and D) The total cell counts (C) and the differential cell counts (D) in BALF were examined. The data are expressed as the mean $\pm$ SEM ($n = 6$). * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the OVA group.

3.24 ± 0.39 , $P < 0.05$ vs. OVA, 5.52 ± 0.37). For all doses of methacholine, no prominent effect was observed in D0.05 group compared with OVA group. Due to a poor solubility resulted from the high concentration of diosmetin (1 mg/kg), there was no significant difference between D0.5 and D1 groups, so we chose 0.1 and 0.5 mg/kg diosmetin as optimal doses for the subsequent experiments. To confirm that treatment with the OVA antigen results in sensitization, the OVA-specific IgE level was measured by ELISA. The serum IgE level was notably increased in the OVA group (178.6 ± 36.2 ng/ml) compared with that in the control group. However, high and low doses of diosmetin (0.5 and 0.1 mg/kg) could greatly attenuate the level of serum IgE to 48.5 ± 10.2 and 62.1 ± 16.9 ng/ml, respectively (Fig. 2B).

Effect of diosmetin on airway inflammation

The amount of total and differential cell counts in BALF were assessed 24 h after the last challenge. In OVA group, the numbers of total cells, eosinophils, and neutrophils were significantly higher in the BALF compared with the control group ($P < 0.05$), but there was no significant difference in lymphocytes between the two groups (Fig. 2C,D). The high dose of diosmetin (0.5 mg/kg) significantly decreased the number of total cells, eosinophils, and neutrophils to 61.6%, 25.5%, and 49.9% ($P < 0.05$), respectively, whereas the low dose of diosmetin (0.1 mg/kg)

had only a slight effect. Lung sections were stained with H&E to evaluate airway inflammation. As expected, the OVA-challenged mice displayed extensive inflammatory infiltration around the peribronchial and peri-vascular areas compared with that of the control group. Diosmetin (0.5 mg/kg) and DEX (1 mg/kg) treatments dramatically alleviated this infiltration (Fig. 3A,B).

Effect of diosmetin on goblet cell hyperplasia

To evaluate the degree of goblet cell hyperplasia and mucus hypersecretion, lung sections were stained with PAS and the percentage of PAS-positive cells in the airway epithelia was determined. Few goblet cells were observed in the lungs of the control group, whereas the amount of positively stained cells was increased significantly in the OVA-challenged mice (Fig. 3A,C). Diosmetin administration (0.5 mg/kg) prominently suppressed goblet cell hyperplasia in the airway similar to the treatment with DEX, implying an equivalent effect between them.

Effect of diosmetin on lung collagen deposition

ECM deposition is an important manifestation of lung remodeling. Dense collagen deposition/fibrosis in the peribronchial region was revealed by Masson's trichrome staining in the OVA group. As

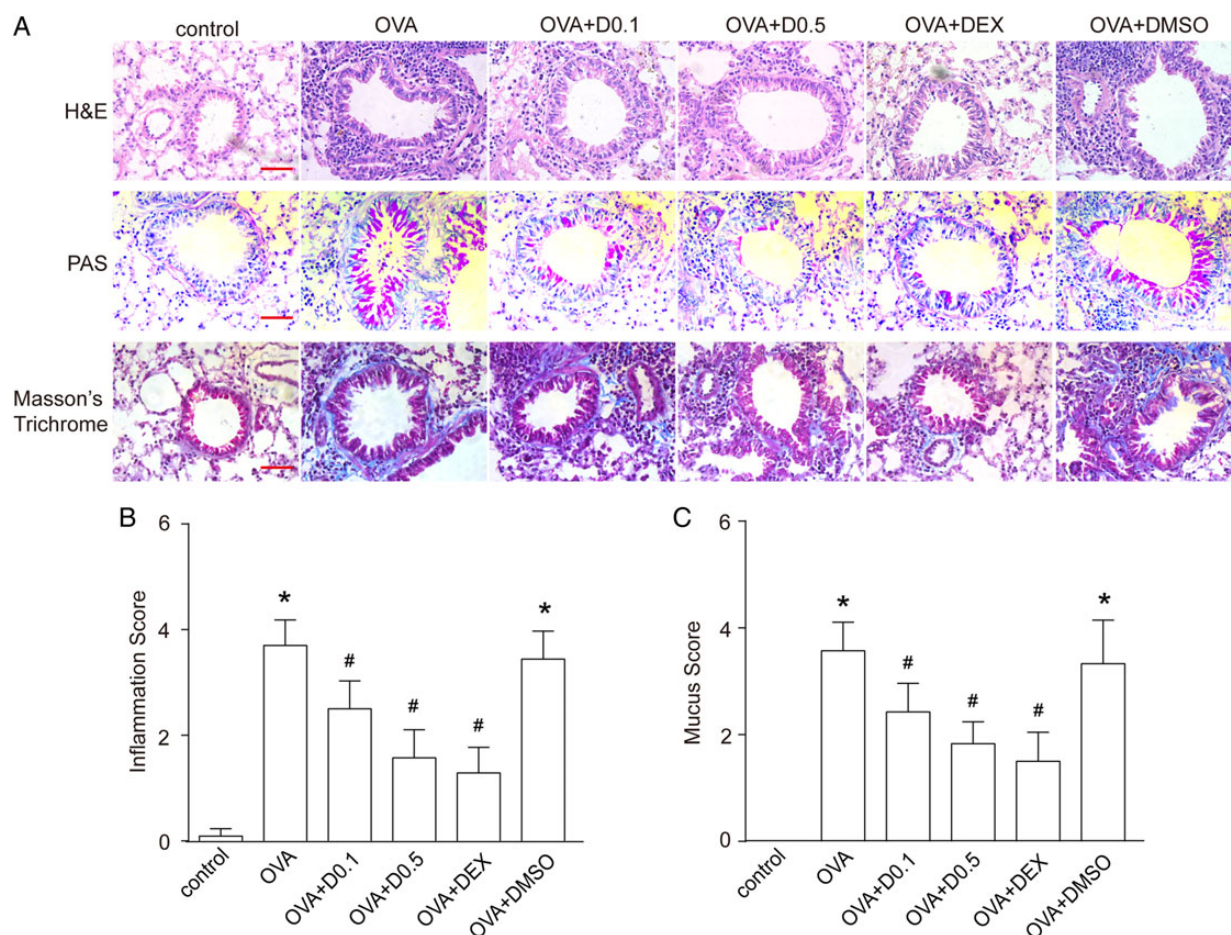


Figure 3. Treatment with diosmetin decreases inflammatory cell infiltration, goblet cell hyperplasia, and collagen deposition in the asthmatic airway (A) Lung sections were subject to H&E, PAS, and Masson's trichrome staining to assess inflammation cells, mucus production, and collagen deposition, respectively. Magnification, 400x. Bars indicate 50 μ m. Large amounts of inflammatory cells, mucus, and collagen were identified in OVA and OVA+DMSO groups while reduced in diosmetin- and DEX-treated group. (B and C) Graphs represent the inflammation score and mucus score expressed as the mean $\pm$ SEM ($n = 6$). * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the OVA group.

shown in Fig. 3A, the high dose of diosmetin (0.5 mg/kg) and DEX could drastically attenuate collagen deposition, whereas a modest effect was observed with the low dose of diosmetin (0.1 mg/kg).

Effect of diosmetin on the expression of α -SMA and MMP-9

As shown in Fig. 4A, the area of α -SMA staining in the OVA-challenged mice was profoundly enhanced compared with that of the control group. The administration of diosmetin and DEX remarkably decreased the expression of α -SMA. Additionally, the staining density of MMP-9 around the bronchioles and the infiltrated inflammatory cells in the OVA-challenged mice was much higher than that in the control mice (Fig. 4A). Consistent with these observations, western blot analysis also showed a striking increase in α -SMA and MMP-9 expressions in the OVA group compared with the control group ($P < 0.05$). This increase was significantly attenuated by the high dose of diosmetin (0.5 mg/kg). The level of MMP-9 was dramatically lowered in the DEX group, whereas α -SMA was marginally reversed by DEX (Fig. 4B), indicating that their underlying anti-remodeling mechanisms are different.

Effect of diosmetin on the expression of VEGF and the level of TGF- β 1

To further clarify the mechanisms involved in the anti-remodeling effects of diosmetin in asthma, we investigated the TGF- β 1 level in BALF and

the VEGF expression in the lungs. ELISA analysis demonstrated that the level of TGF- β 1 in BALF was much higher (804.5 ± 196.9 pg/ml) in the OVA group; the high dose of diosmetin (0.5 mg/kg) could decrease its level to 388.9 ± 69.3 pg/ml, and the low dose of diosmetin (0.1 mg/kg) could decrease its level to 480.9 ± 127.5 pg/ml (Fig. 5A). OVA-challenged mice also showed remarkable overexpression of VEGF in comparison with the control; this overexpression was reversed by both diosmetin and DEX treatments (Fig. 5B).

Discussion

In traditional Chinese medicine and dietary supplements, some types of single agents, pure compounds, or complex herbal formulas present specific properties. Various studies have confirmed an anti-inflammatory role of diosmetin by inhibiting the generation of pro-inflammatory mediators such as IL-1 β , IL-6, and tumor necrosis factor alpha (TNF- α) [20]; preventing LPS-induced NO production [25]; and alleviating c-Kit signal that suppresses stem cell factor/Ultraviolet B-induced melanogenesis [26]. Diosmetin also contains anti-proliferative potential by inducing G2/M arrest of the cell cycle in oncotherapy [15]. However, little is known about its effects on asthma, the key events of which are inflammation and airway smooth muscle cell (ASMC) proliferation.

In this study, a well-characterized murine model of allergic asthma was established to evaluate the effects of diosmetin. Repeated

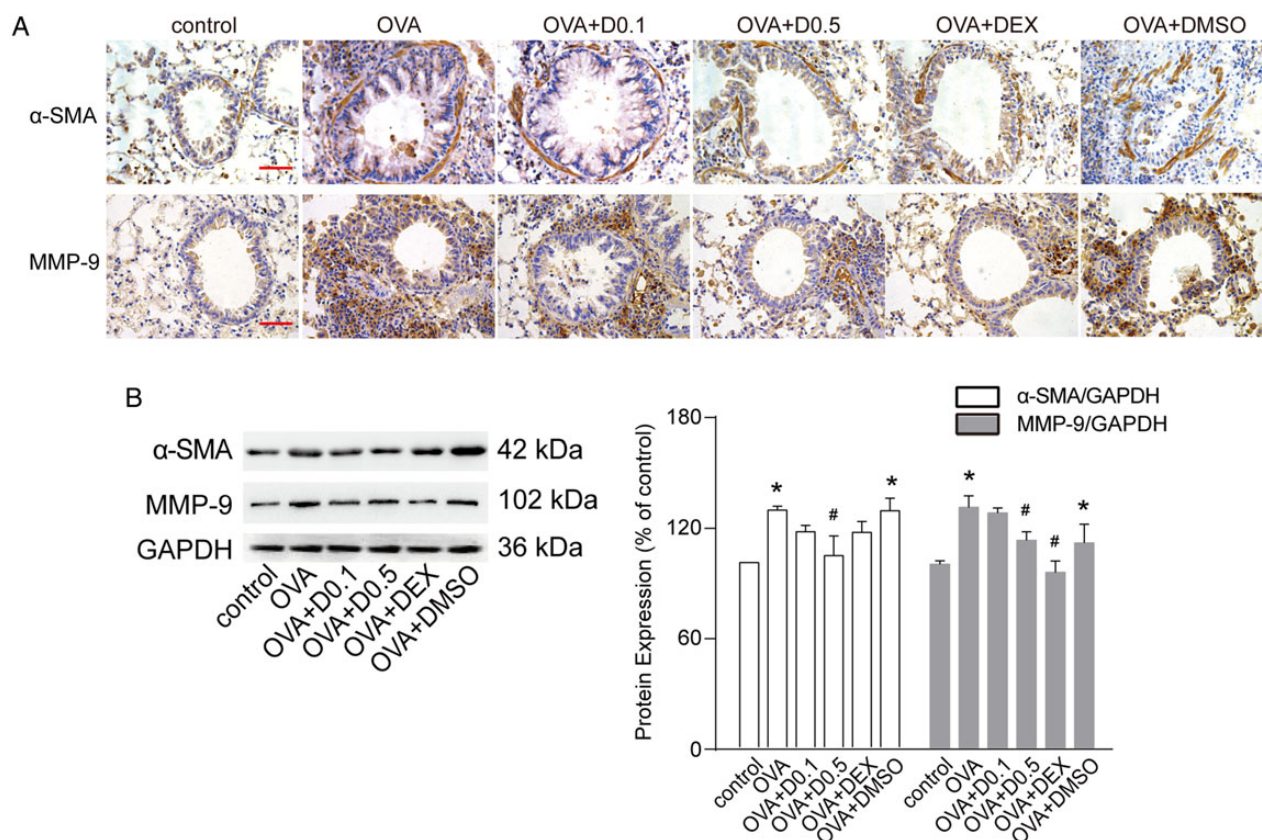


Figure 4. Treatment with diosmetin attenuates the expressions of α -SMA and MMP-9 in OVA-sensitized mice (A) Immunohistochemistry was employed to identify α -SMA around the airway and MMP-9 in the peri-vascular and peri-bronchiolar regions. α -SMA- and MMP-9-immunostained regions were remarkably reduced in OVA+D 0.5 and OVA+DEX groups compared with OVA group. Magnification, 400x. Bars indicate 50 μ m. (B) Lung tissue proteins were extracted 24 h after the final OVA challenge and subject to western blot analysis of α -SMA and MMP-9. GAPDH was utilized as the standard control. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the OVA group.

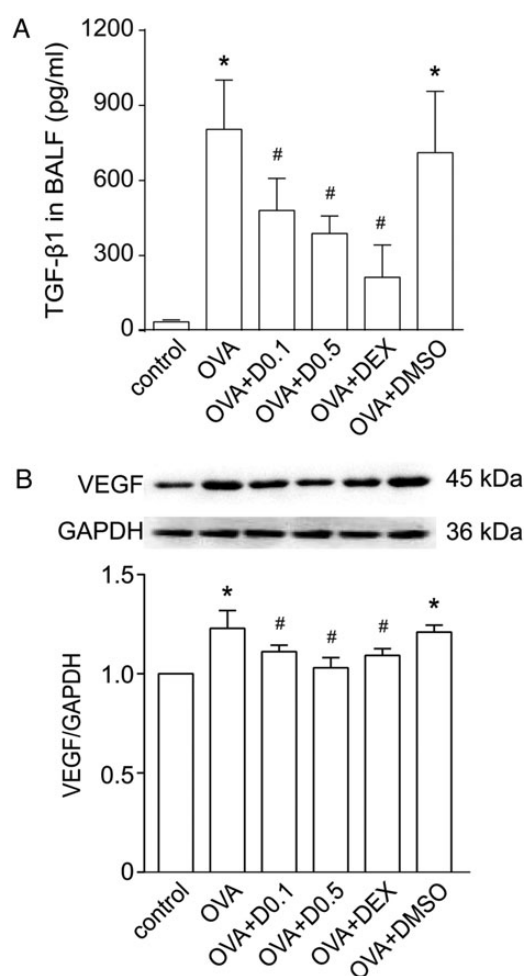


Figure 5. Treatment with diosmetin reduces the level of TGF-β1 in BALF and the VEGF expression (A) TGF-β1 in BALF was examined by ELISA. The data were expressed as the mean ± SEM ($n=6$). (B) Western blot analysis of VEGF. Results were expressed as the ratio to GAPDH. * $P<0.05$ compared with the control group; # $P<0.05$ compared with the OVA group.

exposures to OVA were associated with an antigen-specific IgE response accompanied by AHR and eosinophil accumulation [27,28]. Our findings revealed that diosmetin significantly reduced antigen-specific IgE release in serum, partially accounting for the suppression of AHR. Moreover, diosmetin showed anti-inflammatory activity as evidenced by the reduction in total cells and the percent of eosinophils. Furthermore, H&E staining demonstrated that inflammation in the diosmetin group was remarkably relieved. Collectively, these data indicated that diosmetin might represent a prominent anti-allergic and anti-inflammatory agent for the management of allergic airway diseases.

As mentioned previously, airway remodeling encompasses mucus gland hyperplasia, ASMC proliferation, and ECM protein alterations. Owing to the goblet cell hyperplasia and submucosal gland hypertrophy, mucus accumulation results in exaggerated airflow obstruction, contributing to the morbidity and mortality of asthma [29,30]. To gain insight into the properties of diosmetin, we examined the airways of OVA-exposed mice by PAS staining in the presence or absence of diosmetin. The results revealed that the airways of diosmetin-treated mice contained much fewer mucin-filled goblet cells than the airways exposed to OVA alone, implying a suppression of mucus hypersecretion by diosmetin.

As an important type of structural cell, ASMC hypertrophy and hyperplasia tend to cause airway narrowing and obstruction, accelerating the progression of airway remodeling [31]. α -SMA, a marker for ASMCs, is pivotal for the contraction, migration, and proliferation of ASMCs and ECM [32]. It has been shown that α -SMA levels are higher in patients with severe asthma than in control subjects. Therefore, it is urgent to develop safe and effective therapeutic approaches for these patients [31]. In this study, the results of immunohistochemical staining and western blot analysis demonstrated that diosmetin remarkably decreased the expression of α -SMA, whereas DEX-treated mice showed a slight reduction in α -SMA expression, suggesting that diosmetin may offer some therapeutic advantages over DEX in improving ASM hypertrophy and hyperplasia, providing a potential option for the treatment of severe asthma. Furthermore, ASMCs may produce and release ECM proteins [33]. Pathological studies in patients with chronic asthma revealed that ECM components, particularly collagen deposition, account for subepithelial fibroblasts [34]. In our animal model, diosmetin pretreatment during chronic allergen challenge protected against extensive ECM deposition, implying a suppressive action for diosmetin on asthmatic airway remodeling.

To clarify the mechanisms implicated in the effects of diosmetin on airway remodeling, we investigated mediators, such as TGF-β1, VEGF, and MMP-9, that are involved in the development of asthmatic remodeling. TGF-β1, which is expressed in the asthmatic remodeled airway, can induce subepithelial fibrosis mediated by the activation of fibroblasts to produce ECM proteins such as collagen [35]. TGF-β1 also plays an essential role in the alternation of ASMC function by prompting their hypertrophy and proliferation [36]. In addition, a high level of TGF-β1 has been observed in the airways of patients with fatal asthma [37], and recent evidence has revealed that diosmetin reduces the generation of TGF-β1 in mouse macrophages [19]. Our study showed that diosmetin significantly reduced the level of TGF-β1 compared with that of OVA-challenged mice, suggesting that TGF-β1 might be a key point by which diosmetin inhibits airway remodeling. TGF-β1 has also been reported to augment VEGF release by ASMCs through the PI3K pathway [38]. VEGF, an endothelial cell-specific mitogenic peptide, stimulates angiogenesis, epithelial cell proliferation, and endothelial cell survival [39]. Evidence has suggested that VEGF transgenic mice have an asthma-like phenotype, showing vascular remodeling, inflammation, parenchymal remodeling, and myocyte hyperplasia [40]. Thus, VEGF is a mediator of vascular and extravascular remodeling and inflammation that enhances antigen sensitization, and the inhibition of VEGF may be a promising therapeutic strategy. These results showed that diosmetin administration significantly reduced the level of VEGF and indicated a specific contribution of diosmetin to anti-angiogenic remodeling.

MMPs are a large family of zinc-dependent neutral endopeptidases that degrade most components of ECM proteolytic enzymes [41]. MMPs are known to participate in physiologic and pathologic processes, including ECM turnover, tissue degradation, tissue repair, cell migration, and inflammation. Among this family, MMP-9 is one of the major proteinases implicated in chronic airway inflammation and remodeling of asthma. Accumulating evidence has verified that MMP-9 levels are higher in the sputum of patients, accompanied by elevated ECM [42]. Significantly, reductions in collagen deposition and peribronchial fibrosis were also found in MMP-9-deficient mice [42,43]. Therefore, we explored whether MMP-9 is involved in the improvement of airway remodeling by diosmetin. The results indicated that diosmetin significantly alleviated MMP-9 expression, which might be responsible for the lessened ECM.

Recently, airway remodeling has gained sufficient attention for its key role in asthma, and inflammation and remodeling are considered to interact with each other via multiple pro-inflammatory cytokines [44]. By far, it has been suggested that excessive ROS could lead to tissue injury and promote inflammatory responses [22]. It was reported that diosmetin can reduce the generation of ROS [21]. So, it was suggested that the anti-remodeling effect of diosmetin might be attributed to its anti-inflammatory and anti-oxidant activities.

In conclusion, this study demonstrated that diosmetin could inhibit inflammatory cell infiltration, goblet cell hyperplasia, collagen deposition, and ASMC proliferation in mice with asthma induced by chronic exposure to aerosolized allergen. The reduced levels of TGF- β 1, MMP-9, and VEGF might be attributed to the anti-remodeling activity of diosmetin. Our findings revealed that diosmetin might be a novel therapeutic approach for pulmonary remodeling, highlighting the potential of diosmetin in blocking ASMC proliferation involved in chronic asthma.

Funding

This work was supported by the grants from the National Major Scientific and Technological Special Project for 'Significant New Drugs Development' (No. 2011ZX09302-003-02), the 'Six Talent Peak' Project of Jiangsu Province (No. 2013-WSN-059), the Jiangsu Province Major Scientific and Technological Special Project (No. BM2011017), the Jiangsu Province Special Program of Medical Science (No. BL2012012), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

References

- Al-Muhsen S, Johnson JR, Hamid Q. Remodeling in asthma. *J Allergy Clin Immunol* 2011, 128: 451–462.
- Olin JT, Wechsler ME. Asthma: pathogenesis and novel drugs for treatment. *BMJ* 2014, 349: g5517.
- Larché M. Immunoregulation by targeting T cells in the treatment of allergy and asthma. *Curr Opin Immunol* 2006, 18: 745–750.
- Ammit AJ. Glucocorticoid insensitivity as a source of drug targets for respiratory disease. *Curr Opin Pharmacol* 2013, 13: 370–376.
- McMillan SJ, Lloyd CM. Prolonged allergen challenge in mice leads to persistent airway remodelling. *Clin Exp Allergy* 2004, 34: 497.
- Zhang WX, Li CC. Airway remodeling: a potential therapeutic target in asthma. *World J Pediatr* 2011, 7: 124–128.
- Vazquez-Tello A, Halwani R, Hamid Q, Al-Muhsen S. Glucocorticoid receptor-beta up-regulation and steroid resistance induction by IL-17 and IL-23 cytokine stimulation in peripheral mononuclear cell. *J Clin Immunol* 2013, 33: 466–478.
- Xiong Y, Wang J, Yu H, Zhang X, Miao C, Ma S. The effects of nodakenin on airway inflammation, hyper-responsiveness and remodeling in a murine model of allergic asthma. *Immunopharmacol Immunotoxicol* 2014, 4: 1–8.
- Cates CJ, Jaeschke R, Schmidt S, Ferrer M. Regular treatment with salmeterol and inhaled steroids for chronic asthma: serious adverse events. *Cochrane Database Syst Rev* 2013, 3: 6922.
- Cates CJ, Lasserson TJ. Regular treatment with formoterol and an inhaled corticosteroid versus regular treatment with salmeterol and an inhaled corticosteroid for chronic asthma: serious adverse events. *Cochrane Database Syst Rev* 2010, 1: 7694.
- Roowi S, Crozier A. Flavonoids in tropical citrus species. *J Agric Food Chem* 2011, 59: 12217–12225.
- Meirinhos J, Silva BM, Valentão P, Seabra RM, Pereira JA, Dias A, Andrade PB, et al. Analysis and quantification of flavonoidic compounds from Portuguese olive (*Olea europaea* L.) leaf cultivars. *Nat Prod Res* 2005, 19: 189–195.
- Androutsopoulos VP, Mahale S, Arroo RR, Potter G. Anticancer effects of the flavonoid diosmetin on cell cycle progression and proliferation of MDA-MB 468 breast cancer cells due to CYP1 activation. *Oncol Rep* 2009, 6: 1525–1528.
- Androutsopoulos V, Wilsher N, Arroo RR, Potter GA. Bioactivation of the phytoestrogen diosmetin by CYP1 cytochromes P450. *Cancer Lett* 2009, 274: 54–60.
- Androutsopoulos VP, Spandidos DA. The flavonoids diosmetin and luteolin exert synergistic cytostatic effects in human hepatoma HepG2 cells via CYP1A-catalyzed metabolism, activation of JNK and ERK and P53/P21 up-regulation. *J Nutr Biochem* 2013, 24: 496–504.
- Zhao R, Chen Z, Jia G, Li J, Cai Y, Shao X. Protective effects of diosmetin extracted from *Galium verum* L. on the thymus of U14-bearing mice. *Can J Physiol Pharmacol* 2011, 89: 665–673.
- Bae EA, Han MJ, Kim DH. *In vitro* anti-helicobacter pylori activity of some flavonoids and their metabolites. *Planta Med* 1999, 65: 442–443.
- Chan BC, Ip M, Gong H, Lui SL, See RH, Jolivald C, Fung KP. Synergistic effects of diosmetin with erythromycin against ABC transporter over-expressed methicillin-resistant *Staphylococcus aureus* (MRSA) RN4220/pUL5054 and inhibition of MRSA pyruvate kinase. *Phytomedicine* 2013, 20: 611–614.
- Comalada M, Ballester I, Bailón E, Sierra S, Xaus J, Gálvez J, de Medina FS, et al. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: analysis of the structure-activity relationship. *Biochem Pharmacol* 2006, 72: 1010–1021.
- Yu G, Wan R, Yin G, Xiong J, Hu Y, Xing M, Cang X, et al. Diosmetin ameliorates the severity of cerulein-induced acute pancreatitis in mice by inhibiting the activation of the nuclear factor- κ B. *Int J Clin Exp Pathol* 2014, 7: 2133–2142.
- Liao W, Ning Z, Chen L, Wei Q, Yuan E, Yang J, Ren J, et al. Intracellular antioxidant detoxifying effects of diosmetin on 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced oxidative stress through inhibition of reactive oxygen species generation. *J Agric Food Chem* 2014, 62: 8648–8654.
- Park SJ, Lee KS, Lee SJ, Kim SR, Park SY, Jeon MS, Lee HB, et al. L-2-Oxothiazolidine-4-carboxylic acid or α -lipoic acid attenuates airway remodeling: involvement of nuclear factor- κ B (NF- κ B), nuclear factor erythroid 2p45-related factor-2 (Nrf2), and hypoxia-inducible factor (HIF). *Int J Mol Sci* 2012, 13: 7915–7937.
- Castro SM, Guerrero-Plata A, Suarez-Real G, Adegbuyoga PA, Colasurdo GN, Khan AM, Garofalo RP, et al. Antioxidant treatment ameliorates respiratory syncytial virus-induced disease and lung inflammation. *Am J Respir Crit Care Med* 2006, 174: 1361–1369.
- Bao Z, Guan S, Cheng C, Wu S, Wong SH, Kemeny DM, Leung BPA. Novel antiinflammatory role for andrographolide in asthma via inhibition of the nuclear factor- κ B pathway. *Am J Respir Crit Care Med* 2009, 179: 657–665.
- Shanmugam K, Holmquist L, Steele M, Stuchbury G, Berbaum K, Schulz O, Benavente Garcia O, et al. Plant-derived polyphenols attenuate lipopolysaccharide-induced nitric oxide and tumour necrosis factor production in murine microglia and macrophages. *Mol Nutr Food Res* 2008, 52: 427–438.
- Lee SJ, Jung TH, Kim H, Jeong D, Choi G, Park WK, Kong JY, et al. Inhibition of c-Kit signaling by diosmetin isolated from *Chrysanthemum morifolium*. *Arch Pharm Res* 2014, 37: 175–185.
- Renz H, Smith HR, Henson JE, Ray BS, Irvin CG, Gelfand EW. Aerosolized antigen exposure without adjuvant causes increased IgE production and increased airway responsiveness in the mouse. *J Allergy Clin Immunol* 1992, 89: 1127–1138.
- Oshiba A, Hamelmann E, Takeda K, Bradley KL, Loader JE, Larsen GL, Gelfand EW. Passive transfer of immediate hypersensitivity and airway hyperresponsiveness by allergen-specific immunoglobulin (Ig) E and IgG1 in mice. *J Clin Invest* 1996, 97: 1398–1408.
- Curran DR, Cohn L. Advances in mucous cell metaplasia: a plug for mucus as a therapeutic focus in chronic airway disease. *Am J Respir Cell Mol Biol* 2010, 42: 268–275.

30. Lai H, Rogers DF. New pharmacotherapy for airway mucus hypersecretion in asthma and COPD: targeting intracellular signaling pathways. *J Aerosol Med Pulm Drug Deliv* 2010, 23: 24.
31. Bentley JK, Hershenson MB. Airway smooth muscle growth in asthma: proliferation, hypertrophy, and migration. *Proc Am Thorac Soc* 2008, 5: 89–96.
32. Rao KB, Malathi N, Narashiman S, Rajan ST. Evaluation of myofibroblasts by expression of alpha smooth muscle actin: a marker in fibrosis, dysplasia and carcinoma. *J Clin Diagn Res* 2014, 8: 14–17.
33. James AL, Maxwell PS, Pearce-Pinto G, Elliot JG, Carroll NG. The relationship of reticular basement membrane thickness to airway wall remodeling in asthma. *Am J Respir Crit Care Med* 2002, 166: 1590–1595.
34. Eap R, Jacques E, Semlali A, Plante S, Semlali CJ. Cysteinyl leukotrienes regulate TGF- β 1 and collagen production by bronchial fibroblasts obtained from asthmatic subjects. *Prostaglandins Leukot Essent Fatty Acids* 2012, 86: 127–133.
35. Makinde T, Murphy RF, Agrawal DK. The regulatory role of TGF- β 1 in airway remodeling in asthma. *Immunol Cell Biol* 2007, 85: 348–356.
36. Sturrock A, Huecksteadt TP, Norman K, Sanders K, Murphy TM, Chitano P, Wilson K, *et al.* Nox4 mediates TGF β 1-induced retinoblastoma protein phosphorylation, proliferation and hypertrophy in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2007, 292: 1543–1555.
37. Tillie-Leblond I, Pugin J, Marquette CH, Lamblin C, Saulnier F, Brichet A, Wallaert B, *et al.* Balance between proinflammatory cytokines and their inhibitors in bronchial lavage from patients with status asthmaticus. *Am J Respir Crit Care Med* 1999, 159: 487–494.
38. Shin JH, Shim JW, Kim DS, Shim JY. TGF-beta effects on airway smooth muscle cell proliferation, VEGF release and signal transduction pathways. *Respirology* 2009, 14: 347–353.
39. Clifford RL, John AE, Brightling CE, Knox AJ. Abnormal histone methylation is responsible for increased VEGF165a secretion from airway smooth muscle cells in asthma. *J Immunol* 2012, 189: 819–831.
40. Lee CG, Link H, Baluk P, Homer RJ, Chapoval S, Bhandari V, Kang MJ, *et al.* Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung. *Nat Med* 2004, 10: 1095–1103.
41. Chaudhuri R, McSharry C, Brady J, Grierson C, Messow CM, Spears M, Miele G, *et al.* Low sputum MMP-9/TIMP ratio is associated with airway narrowing in smokers with asthma. *Eur Respir J* 2014, 44: 895–904.
42. Lim DH, Cho JY, Miller M, McElwain K, McElwain S, Broide DH. Reduced peribronchial fibrosis in allergen-challenged MMP-9-deficient mice. *Am J Physiol Lung Cell Mol Physiol* 2006, 291: 265–271.
43. Vignola AM, Riccobono L, Mirabella A, Profita M, Chanez P, Bellia V, Mautino G, *et al.* Sputum metalloproteinase-9/ tissue inhibitor of metalloproteinase-1 ratio correlates with airflow obstruction in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 1998, 158: 1945–1950.
44. Capra V, Rovati GE. Rosuvastatin inhibits human airway smooth muscle cells mitogenic response to eicosanoid contractile agents. *Pulm Pharmacol Ther* 2013, 27: 10–16.