

Original Article

Emodin inhibited ozone stress-induced airway hyperresponsiveness by regulating epithelial protection mechanisms against injury

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Abstract: Background: Airway hyperresponsiveness (AHR) is the common clinical features of respiratory diseases such as asthma, chronic bronchitis and chronic obstructive pulmonary disease (COPD). Emodin is one of the main active components of a Himalayan rhubarb, which has demonstrated many beneficial effects against inflammation; however, the mechanism remains unclear. The present study was designed to detect the effects and related mechanisms of Emodin on AHR in both vivo and in vitro experiments. Methods: The Airway resistance (AR) was assayed by Buxco Resistance and Compliance (RC) system, lung histological pathology, inflammatory cell enumeration and inflammatory media in bronchoalveolar lavage fluid (BALF) were analyzed on an ozone-stressed animal model by direct microscopic observation. In vitro experiments, cell viability was determined by suphorhodamine B (SRB) assays. TGF- β and PGE2 was assayed by ELISA. Cellular protection against injury was assayed by ³H-Udr and LDH secretion and catalase activity. Results: Emodin inhibited AR, inflammatory cell infiltration, Th1 and Th17 production. On bronchial epithelial cells (BECs), Emodin displayed a obvious cytotoxicity over 20 μ g/mL. Emodin inhibited TGF- β production and promoted PGE2 production under ozone stress. Emodin also inhibited ³H-Udr and LDH secretion and promoted catalase activity. Conclusion: Our data suggest that Emodin has strong anti-AHR effects, which was related with improvement of epithelial protection against injury.

Keywords: Airway hyperresponsiveness, Emodin, Ozone, TGF- β , PGE2

Introduction

Airway hyperresponsiveness (AHR) is the common characteristic features of many respiratory diseases such as asthma, chronic bronchitis, chronic obstructive pulmonary disease (COPD) etc, which are characterized by over responsiveness to general strength of stimuli or some endogenous active substances, leading to cause repeated airway inflammation, mucus secretion, airway remodeling and increased airway resistance. The repeated attacks of these diseases can make the progressive damage to the lung function and irreversible complications. AHR diseases have become an important global public health problem, consuming large amounts of medical and economic resources. AHR diseases are often associated with airway

chronic inflammation, increased airway reactivity and airway remodeling [1]. Nowadays, the drugs which are commonly used for treatment for AHR include anti-inflammatory drugs and bronchodilators. Inhaled corticosteroids have become first-line drugs for the treatment of asthma. Aminophylline and dyphylline are commonly used bronchodilators. However, although these drugs can relieve symptoms but can't correct airway remodeling and alleviate the trend of re-occurrence and disease progression. And some cases are insensitive to glucocorticoid [2]. In addition, clinicians often face a dilemma in the medication for these drugs because of their different degrees of side effects to human body, leading to increased tolerance year by year. Therefore, new drugs with less side effects and better treatment effects are urgent.

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Looking for drugs with better effects from natural herbs becomes a new research hot spot.

Emodin is one of the main active components of rhubarb, which has effects in preventing viral infections [3], tumors [4], atherosclerosis [5], fungal infections [6] and allergies. The recent study showed that Emodin also had a strong anti-inflammatory effect on airway [7]. This study was designed to detect the anti-inflammatory effects of inhaled emodin on an ozone-stressed AHR animal model. By detecting AR, lung pathology, inflammatory cell infiltration and epithelial protection against injury, we observed that after administration of emodin, airway resistance and pulmonary inflammation decreased significantly, lung pathological structure changed, and epithelial function improved, which have the same exact anti-inflammatory effects with corticosteroids.

Material and methods

Ozone-stressed animal models

Balb/c mice (20 g) were obtained from the experimental animal center of Quzhou People Hospital. All mice were housed under pathogen-free conditions with random access to food and water. Animal care and handling were in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals". The mice were kept in a tightly sealed chamber with whole-body exposure to ozone at a concentrations of 1, 5 ppm for 30 min/day for consecutive 5 days (n=6/group), while they were awake and breathing spontaneously in the chamber. Drugs (50 mg/kg, i.p.) were administered by aerosol inhalation 4 hour later after ozone stress.

Airway function assay

The invasive airway resistance (AR) test system was from Buxco, USA. After treatments, the mice were anesthetized with pentobarbital sodium (60 mg/kg, i.p.). Then a tracheostomy was made, the trachea was cannulated and one end of cannula was connected with buxco. In this way, the intrapulmonary pressure and airflow signal could be recorded synchronically. AHR was assessed by measuring changes in AR in response to increasing concentrations of inhaled methacholine (Mch). A stable baseline airway pressure (<5% variation over 2.5 min-

utes) was reached, Phosphate buffered saline (PBS) and increasing concentrations of Mch (0.78, 1.56, 3.12 and 6.25 mg/ml) in succession were administered via a jet nebulizer. AR were determined and expressed as percent change from the baseline value.

Cell enumeration of bronchoalveolar lavage fluid (BALF)

Immediately following assessment of AR, mice were sacrificed with an i.p. injection of sodium pentobarbitone (100 mg/kg). The trachea was cannulated and BALF was obtained by washing the airway lumina. Briefly, cells in the lungs were recovered by flushing 1 mL of PBS into the lungs. BALF was centrifuged and the cell pellet was suspended in 200 µl of PBS and counted using a hemocytometer. Then, the cell suspensions were then centrifuged onto glass slides using a cytospin centrifuge at 1000 g for 5 min at room temperature. Cytocentrifuged cells were air dried and stained with a Wright-Giemsa staining which allows differential counting of various cells. At least 300 cells per sample were counted by direct microscopic observation.

ELISA assay

The supernatant of BALF and cultured BECs was used for determination of cytokine levels. Interleukin IFN γ , IL-4 and IL-17 (R&D Systems, USA) production in BALF and interleukin TGF- β and PGE2 (R&D Systems, USA) production from BECs were measured by ELISA according to the manufacturer's instructions.

Lung histological analysis

After the left lung had been lavaged, the right lung was excised from each mouse, fixed in 4% formalin, and embedded in paraffin. Sections 3-4 µm thin from blocks were stained with H&E. Histopathological assessment (light microscopy) was performed blind on randomized sections. HE-stained slides were scored as follows. Each sample was assigned between 0 and 4 scores based on the epithelial damage, inflammatory infiltrate, edema of the alveoli and mesenchyme, alveolar hemorrhage

Cell culture

Human bronchial epithelial cells (BECs) line (16HBE14o-) were incubated in DMEM:F12

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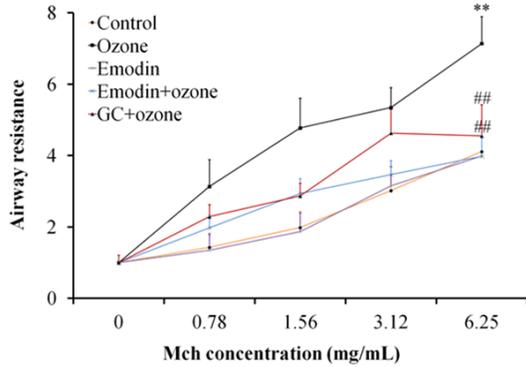


Figure 1. AR was assayed by Buxco system (n=6). Ozone resulted in increased AR to methacholine (Mch) and Emodin (50 mg/kg) abolished the increased AR induced by ozone (** $P < 0.01$ versus control; ## $P < 0.01$ versus Ozone).

(1:1) containing 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 10% heat inactivated fetal bovine serum at 37°C in an atmosphere containing 5% CO₂.

Cytotoxicity assays of Emodin

Emodin (molecular weight: 270.24) was purchased from ACOS organics, Belgium. The drug was dissolved in 1 mL of ethanol to a stock concentration of 100 mg/mL according to the instruction and diluted sequentially in medium to a final concentration of 1, 10, 20 and 50 μ g/mL. Cells were plated in 96-well plates to 80% confluence and treated with different concentrations of Emodin (1, 10, 20 and 50 μ g/mL) for 72 hours, followed by fixing the cells in 30% of trichloroacetic acid for 2 h at 0°C. After 3 times of washing, cells were exposed to 0.5% suphrodamine B (SRB) solution for 15 min in dark place and subsequently washed with 1% acetic acid. After drying overnight, Tris-HCl was used to dissolve the SRB-stained cells and color intensity was measured at 540 nm. Data are represented as a percentage of control cells.

Determination of protection against injury

BECs was labeled by ³H-Udr (1 μ i/ml) overnight, and then washed three times with PBS. BECs were divided into control group (the cells were incubated for 1.5 h), Emodin group (the cells were incubated for 30 min, treated by Emodin for 30 min, and then incubated for 30 min), O₃ stress group (the cells were stressed by O₃ for 30 min then incubated 1 h), O₃ + Emodin group

(the cells were stressed by O₃ for 30 min, treated by Emodin for 30 min, then incubated for 30 min), and GC group (the cells were stressed by O₃ for 30 min, treated by GC for 30, then incubated for 30 min). For determination of cell damage, then biggest release was obtained by dissolving the cell in 0.1% Triton X-100 and collecting centrifugal supernatant. 0.1 ml supernatant fluid was added to 3 ml liquid scintillation and dpm values were determined. Each samples for determination of triple tube. ³H-Udr release rate was calculated by dividing the biggest release. For determination of LDH release activity, supernatants of each group were collected and assayed by using LHD kit. The standard curve was prepared according to the instruction and the OD440 nm of each group was assayed. The unit was U/10⁶ cells. For determination of Catalase activity, the supernatant was abandoned, and 100 ul cold saline was added to each well. The cells were lysed via ultrasonic grinding cells, the centrifugal supernatant was obtained after 3000 r/min 15 min. The standard curve was prepared according to the instruction and the OD240 nm of each group was assayed. The unit was mU/10⁶ cells.

Statistical analysis

Experimental data was analyzed with the SPSS15.0 package. The results were presented as mean \pm standard deviation (SD). If the data consisted of a normal distribution and homoscedasticity, we did one-way analysis of variance (ONE-WAY ANOVA). If the data were not consistent with a normal distribution, we did a rank test whose statistical significance was set at $P < 0.05$.

Results

Assessment of airway resistance

As shown in **Figure 1**, after the animals were challenged by different concentrations of methacholine (Mch) (0.78, 1.56, 3.12 and 6.25 mg/mL), the AR of animals increased in a dose-dependent manner. The AR of ozone-stressed animals increased significantly compared with those of the control animals; while the AR of ozone-stressed animals significantly decreased after treatment with Emodin (50 mg/kg) (** $P < 0.01$, **Figure 1**). And the effects were the

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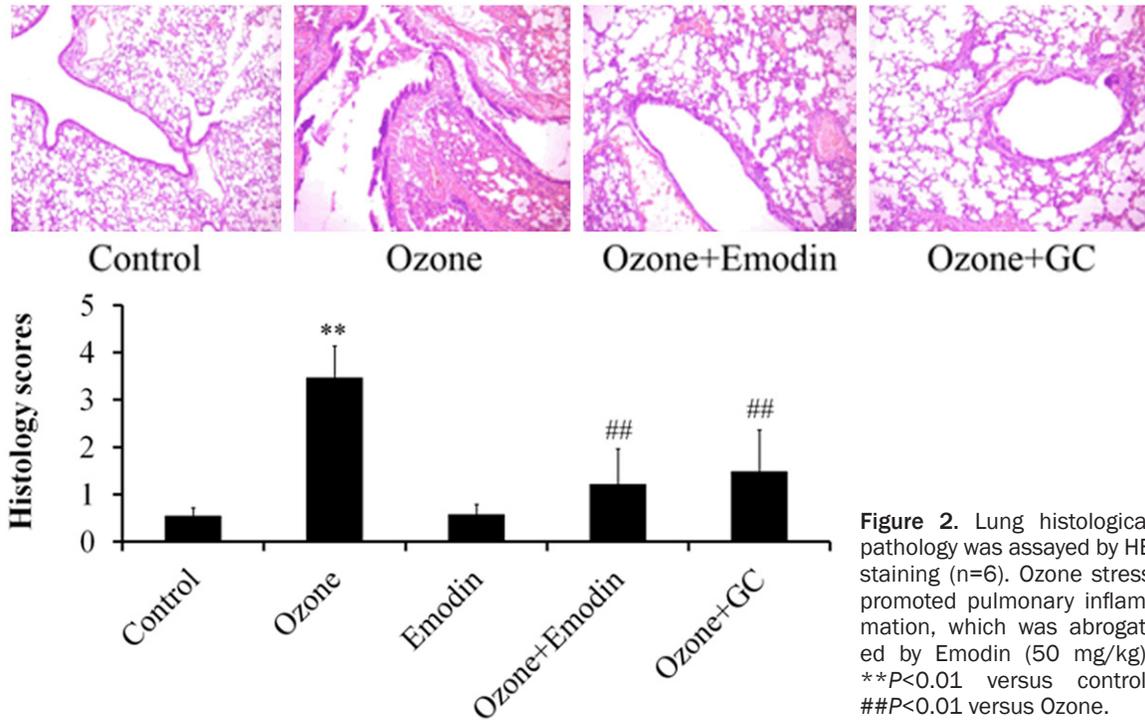


Figure 2. Lung histological pathology was assayed by HE staining (n=6). Ozone stress promoted pulmonary inflammation, which was abrogated by Emodin (50 mg/kg). ** $P < 0.01$ versus control; ## $P < 0.01$ versus Ozone.

Table 1. Total and differential cells counts in BALF

Group	Total cells	Macrophage	Eosinophils	Neutrophils	Lymphocytes
Control	2.45±0.47	0.74±0.35	0.18±0.12	0.51±0.35	0.77±0.31
Ozone	4.98±0.99**	1.25±0.62**	0.24±0.19	1.03±0.82**	1.97±0.55**
GC	2.37±1.01##	0.89±0.43##	0.23±0.15	0.76±0.62##	0.98±0.48##
Control + Emodin	2.44±0.92	0.79±0.45	0.19±0.23	0.49±0.26	0.76±0.46
Ozone + Emodin	2.05±1.33##	0.93±0.53##	0.22±0.16	0.81±0.61##	0.88±0.73##

Data are presented as (Mean ± SE) × 10⁵ cells/ml. ** $P < 0.01$, significantly different from control group (n=6). ## $P < 0.01$, significantly different from ozone group (n=6).

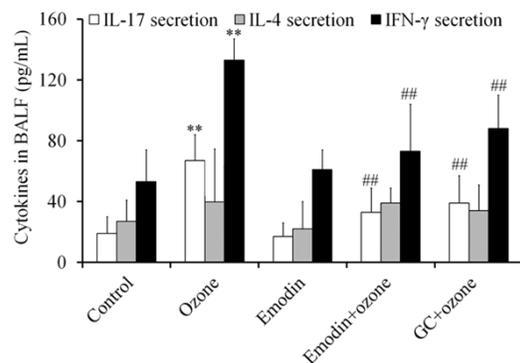


Figure 3. Effects of Emodin on IFN γ , IL-4 and IL-17 production in BALF (n=6). Ozone (50 mg/kg) promoted the production of IFN γ and IL-17. Emodin decreased ozone-induced effects (** $P < 0.01$ versus control group; ## $P < 0.01$ versus ozone group).

same as those of hormone therapy group. Emodin had no effects on normal airway.

Lung histological analysis

The results showed that the control group only showed mild alveolar hyperemia and pulmonary interstitial hyperplasia. In the ozone-stressed group, the alveolar walls are incomplete with a large number of inflammatory cells infiltration, stroma hyperplasia and congestion. Inhaled corticosteroid could effectively inhibit inflammatory cells infiltration by showing mild pulmonary interstitial hyperemia and inflammatory cells infiltration. Emodin (50 mg/kg) group also showed obvious inhibition on pulmonary inflammation induced by ozone (**Figure 2**).

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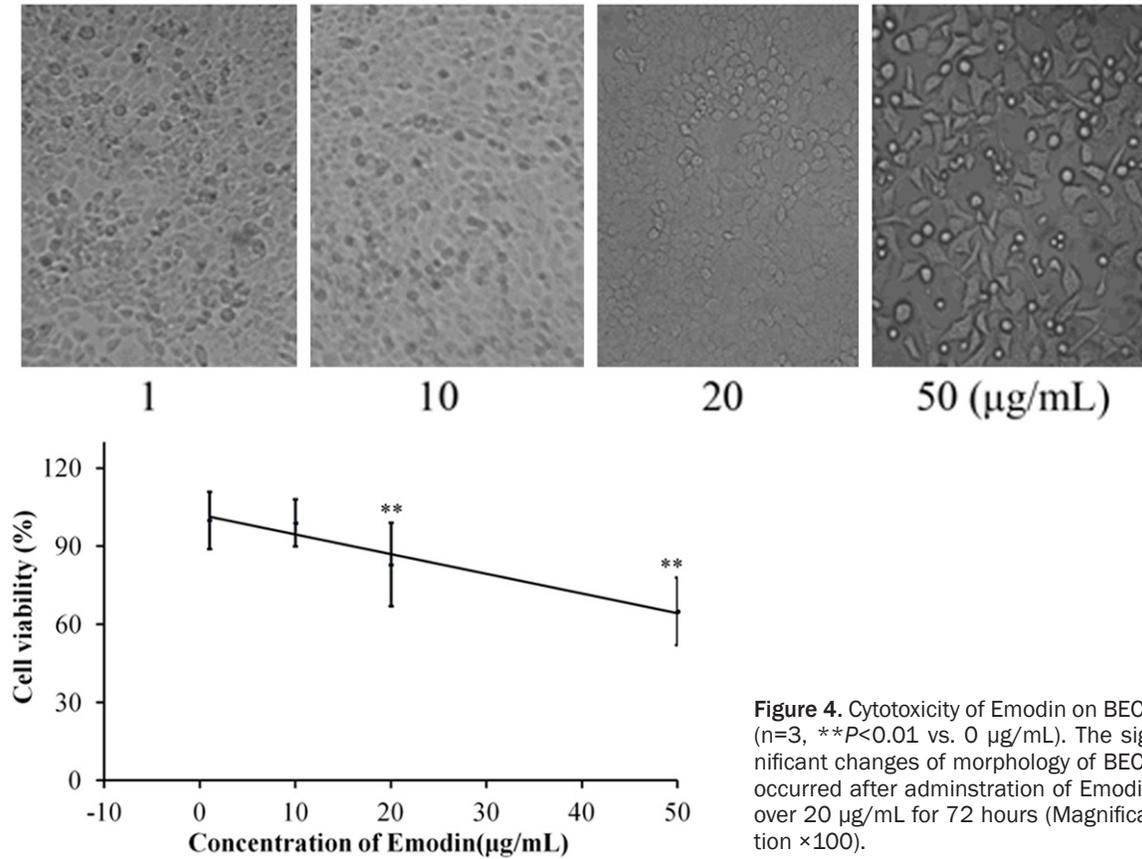


Figure 4. Cytotoxicity of Emodin on BECs (n=3, ** $P<0.01$ vs. 0 µg/mL). The significant changes of morphology of BECs occurred after administration of Emodin over 20 µg/mL for 72 hours (Magnification $\times 100$).

Cell enumeration of BALF

The lungs of animals treated with ozone showed a significant increase in the number of total cellular score, macrophages, neutrophils and lymphocytes. Inhaled corticosteroid could effectively inhibit the number of total cellular score, macrophages, neutrophils and lymphocytes in BALF induced by ozone. Emodin (50 mg/kg) also had similar inhibitory effects on total cellular score, macrophages, neutrophils and lymphocytes (**Table 1**).

ELISA assay of BALF

The lungs of animals treated with ozone showed a significant increase in the production of IFN γ and IL-17, but not in IL-4. Emodin (50 mg/kg) decreased ozone-induced IFN γ and IL-17 production (**Figure 3**).

Cytotoxicity of Emodin on BECs

Under microscope, the proliferation decreased and significant changes of morphology of BECs including cell shrinkage and more suspension

cells occurred after the administration of 20 µg/mL of Emodin at 72 hours (**Figure 4**). In the control, the cells were clearly outlined and tightly packed with vigorous growth.

The levels of regulatory mediators released from BECs

In order to find out primarily the possible molecular signal mechanism in the airway protection, two of major representative regulatory mediators involved in airway remodeling released from BECs were measured using ELISA. The results showed that the secretion of TGF- β increased rapidly and PGE2 production reduced with time with ozone stress. Emodin (10 µg/mL) inhibited the production of TGF- β and promoted PGE2 production induced by ozone (**Figure 5**). Inhaled corticosteroid only inhibited the production of TGF- β .

Determination of protective mechanism against injury

The spontaneously ^3H release rate was $40.33 \pm 1.95\%$ in the control. O_3 stress promoted ^3H

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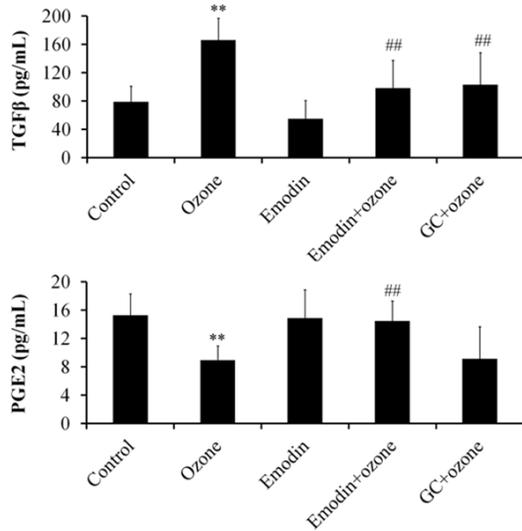


Figure 5. Effects of Emodin on TGF- β and PGE2 production from BECs (n=6). Ozone promoted the production of TGF- β and inhibited the production of PEG2. Emodin (10 μ g/mL) decreased ozone-induced effects (** P <0.01 versus control group; ## P <0.01 versus ozone group).

release rate to $61.54 \pm 10.79\%$ (P <0.01), indicating that the damage effect was obvious. Emodin (10 μ g/mL) treatment can reduce BECs injury by decreasing ^3H release rate to $37.98 \pm 11.05\%$ (P <0.01, **Figure 6A**). The spontaneous activity of LDH release in the control was 82 ± 38 U. O_3 stress promoted the release of LDH to 141 ± 32 U (P <0.01). Emodin (10 μ g/mL) treatment significantly reduced the O_3 stress-induced LDH release with its value was 81 ± 19 U. The trend was consistent with ^3H release rate (**Figure 6B**). After O_3 stress, catalase activity in cells was 185.15 ± 12.39 mU, and the catalase activity after Emodin (10 μ g/mL) treatment increased to 257.87 ± 14.85 mU (**Figure 6C**). Inhaled corticosteroid only inhibited the secretion of ^3H .

Discussion

AHR usually shows overreaction to general physical and chemical stimuli, drugs and endogenous immune or inflammatory media, low doses of antigens, so leading to decreased resistance to pathogenic microorganisms and toxic gas and being prone to increased airway resistance and repeated infection which are the common clinical manifestations and pathological features of asthma, emphysema, bronchitis and COPD. Several investigations have

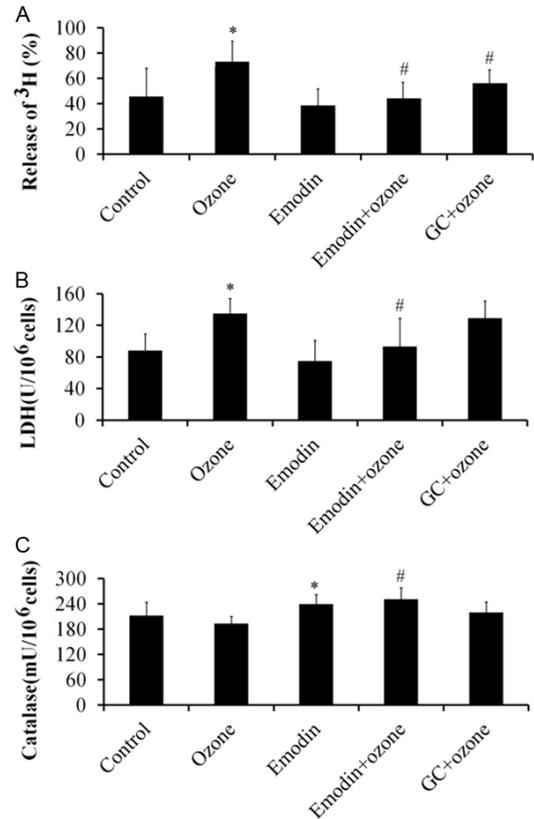


Figure 6. Determination of protective mechanism against injury (n=6). (A) Emodin (10 μ g/mL) inhibited the release of ^3H (A) and LDH (B) and promoted the release of catalase from O_3 -stressed BECs (C). * P <0.05 vs Control group; # P <0.05 vs O_3 group.

demonstrated that BECs are the main initiating factors for immune imbalance and airway inflammation [8, 9]. When stressed, BEC can express adhesion molecules, release a variety of cytokines to attract and recruit a variety of inflammation cells, such as neutrophils and eosinophil, which may be related to the increase of chronic inflammation of respiratory tract, respiratory tract mucous and airway remodeling [1]. BECs also drive the proliferation and differentiation of T helper cells [10]. The present experiments showed that an ozone-stressed animal model was built successfully by ozone challenge. The AR of ozone-stressed animals increased significantly compared with that of the control animals. The cellular population in BAL fluid was altered. Especially significant are the increases in macrophages, neutrophils and lymphocytes. IFN γ and IL-17 in BALF increased significantly. Emodin prevented the development of AHR, airway inflammatory cell infiltra-

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tion and decreased IFN γ and IL-17 in BALF. These results demonstrate that Emodin has profound regulatory effects on the development of airway inflammation in the ozone-stressed model.

As described earlier, TGF β promotes fibroblast into myofibroblast. The latter can secrete collagen and growth factor, which further promote the hyperplasia of the smooth muscle and blood vessel endothelial cell [11]. So, the remodeling signal from damaged epithelial cells was extended and amplified to the deep layer of mucous membrane. It has also reported that TGF- β which is expressed in airways of asthmatics may contribute to irreversible airway remodeling by enhancing ASMC proliferation, which is mediated by phosphorylation of p38 and ERK1/2 [12]. PGE2 is a lipid mediator that can be derived from cell membrane phospholipids synthesized from arachidonic acid by cyclooxygenases and synthases. PGE2 plays an important counter-regulatory role by suppressing fibroblast proliferation and collagen production [13] and promoting the proliferation of bronchial epithelial cells [14] in previous study. PGE2 inhibits fibroblast activation in primary lung fibroblasts via binding of EP2 receptor and production of cAMP [15]. Besides, PGE2 released from bronchial epithelial cells can directly relax airway smooth muscle or lower sensitivity of airway smooth muscle, inhibit the proliferation of smooth muscle cells and fibroblasts and strengthen the tolerance of lung epithelial cells of damage [16]. We observed that the secretion of TGF β increased and the secretion of PGE2 decreased with ozone stress. Emodin inhibited the secretion of TGF β and promoted the secretion of PEG2, indicating that Emodin has the potential to inhibit the airway remodeling and promote airway relaxation.

The destruction of the structure induced by apoptosis or necrosis can lead to release of LDH or labeled ^3H into the culture. This experiment also assessed the injury of cells by detecting ^3H and LDH release. The results showed that Emodin can alleviate the injury of ozone and reduce lipid peroxidation with obvious protective effects against injury. Catalase can directly degraded hydrogen peroxide and is the antioxidant enzymes inside the BECs to maintain homeostasis. Emodin can improve the Catalase activity of ozone-stressed BECs, indi-

cating that the protective mechanism against injury is related to improved antioxidant capacity of cells.

In conclusion, these findings suggest that Emodin has strong anti-AHR effects, which was related with improvement of epithelial protection against injury.

Disclosure of conflict of interest

None.

Abbreviations

BALF, Bronchoalveolar lavage fluid; AR, airway resistance; AHR, airway hyperresponsiveness.

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