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Effects of Connexin 43 Inhibition in an Ovalbumin-induced Mouse Model of Asthma

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ABSTRACT

Connexion 43 (Cx43), a gap junction protein, is expressed abundantly in the airway and has been implicated in the pathogenesis of asthma. However, the effects of blocking Cx43 in asthma remain unclear.

We investigated the therapeutic effects of two specific Cx43 inhibitors (Gap26 and Gap27) on the development of allergic airway disease in mice.

Allergic asthma was induced in BALB/c mice by sensitization and challenge with ovalbumin (OVA). Different doses of Cx43 inhibitors were administered by aerosol inhalation 1 h after OVA challenge on days 21 and 23. Airway hyperresponsiveness (AHR), lung pathology, mucus production, and inflammatory cells and cytokines in bronchoalveolar lavage fluid (BALF) were examined.

We found that Gap26 significantly inhibited OVA-induced AHR, inflammatory cell infiltration surrounding the bronchia, mucus production, inflammatory cells and cytokines in BALF, and OVA-specific IgE in the serum in a dose-dependent manner. Gap27 showed effects similar to those of Gap26 in inhibiting inflammatory cytokine production in BALF.

We conclude Cx43 inhibitor inhalation alleviates asthma features in mice and may be a promising therapy for clinical asthma.

Keywords: Asthma; Connexin 43; Inflammation; Ovalbumin

INTRODUCTION

Allergic asthma is an inflammatory disease of the airways that is characterized by bronchial hyperreactivity, chronic airway inflammation, inflammatory cell infiltration, and reversible airway obstruction, and affects 300 million people worldwide.[1,2] Despite advances in management, current standard medications, including inhaled corticosteroids, β2-agonists, and leukotriene modifiers, are ineffective in a large number of patients.[3,4]

Asthma has been traditionally ascribed to be driven by the T helper (Th) 2 immune response and subsequent eosinophil aggregation.[5,6] Allergic inflammation characterized by excess inflammatory cell infiltration develops rapidly across the entire lung or even both

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lungs. In this process, it appears that inflammatory cells respond in a coordinated but dysfunctional manner via an array of complex signaling pathways that facilitate communication between immune and structural cells, where gap junction channels (GJCs) may play an important role.

GJCs allow metabolites, second messengers, ions, and small hydrophilic molecules to pass freely and rapidly between neighboring cells in the lung.[7,8] GJCs are typically constituted by two hemichannels, each composed of a hexamer of connexin (Cx) protein, with Cx43 being among the most frequently expressed members of the Cx family.[9-11] The expression and role of Cx43 in the airway has recently received increasing attention. Cx43 was reported to be up-regulated and involved in the pathogenesis of chronic rhinosinusitis, acute lung injury (ALI), cystic fibrosis, and pulmonary arterial hypertension.[12-15] Sarieddine et al showed that increased expression of Cx43 in alveolar promotes neutrophil recruitment to the airspace in a mouse model of ALI.[13] They also found that instillation of Gap26 in inflamed lungs markedly reduced neutrophil transmigration, indicating that Cx43 represents a pharmacological target in lung diseases.[13] Although a recent study demonstrated that Cx43 is strongly up-regulated in the lung during asthma and correlated with airway inflammation,[16] the possible benefits of Cx43-specific inhibition in asthma therapy have not been investigated. In the present study, we examined the role of Cx43 inhibitors in lung inflammation in a murine model of asthma.

MATERIALS AND METHODS

Animals

60 female BALB/c mice (6 weeks old) were purchased from the Shanghai SLAC Laboratory Animal Center Limited Liability Company (Shanghai, China). Mice were maintained under specific pathogen-free conditions for 1 week prior to starting the experiments. All procedures were strictly conducted in accordance with protocols approved by Ethics Committee (No.2016050) for Animal Studies at the Second Affiliated Hospital of Fujian Medical University.

Experimental Protocols

For Gap26 (Alpha Diagnostic, San Antonio, TX, USA) blocking experiments, 36 mice were randomly separated into 6 groups as follows (n=6): control group, control+2 μg/kg Gap26 group, ovalbumin (OVA)-induced asthma model group, and Gap26 treatment groups (low dose: 0.5 μg/kg, middle dose: 1 μg/kg, high dose: 2 μg/kg). For Gap26 and Gap27 (Alpha Diagnostic, San Antonio, Texas, USA.) blocking experiments, 24 mice were randomly separated into 4 groups (n=6): control group, OVA-induced asthma model group, Gap26 treatment group (2 μg/kg), and Gap27 treatment group (2 μg/kg). Sensitization, challenge, and treatment protocols for the different groups in this study are summarized in Figure 1 and were performed according to a previous study.[17] Briefly, on days 0, 7, and 14, the mice were sensitized by intraperitoneal injection of 20 μg of OVA emulsified with 2 mg aluminum hydroxide in a total volume of 200 μL pyrogen-free phosphate-buffered saline (PBS, pH 7.3). Using an air-compressing nebulizer in a plexiglass chamber (403A, Yuyue, Danyang, Jiangsu, China), the mice were challenged by inhalation with 5% aerosolized OVA or PBS for 40 minon days 21, 22, 23, and 24. Gap26 or Gap27 was dissolved in PBS to concentrations of 0.5, 1, and 2 mg/kg. The doses of Gap26 or Gap27 used in this study were based on those used in previous studies.[13,18] From days 21 to 24, mice were made to inhale Gap26 or Gap27 daily for 30 min at 1 h after OVA challenge. Control group mice received equal amounts of PBS or/and Gap26. Model group mice received equal amounts of inactive scrambled versions of Gap26 or Gap27. All efforts were made to minimize the number of animals used and their suffering.

Evaluation of AHR

Airway responsiveness to aerosolized methacholine (Mch, Sigma-Aldrich, St. Louis, MO, USA) was measured in unrestrained and spontaneously breathing mice 24 h after the last OVA challenge by whole body plethysmograph (WBP system, Buxco Electronics, Wilmington, NC, USA) as previously described.[19] Mice were stabilized in the chamber for 15 min and then exposed to aerosolized PBS for 5 min as a control. Next, mice were challenged every 15 min with aerosolized Mch.

Increasing doses of aerosolized Mch were administered and enhanced pause (Penh) values for each Mch dosage were measured over the subsequent 5 min as an index of airway obstruction.

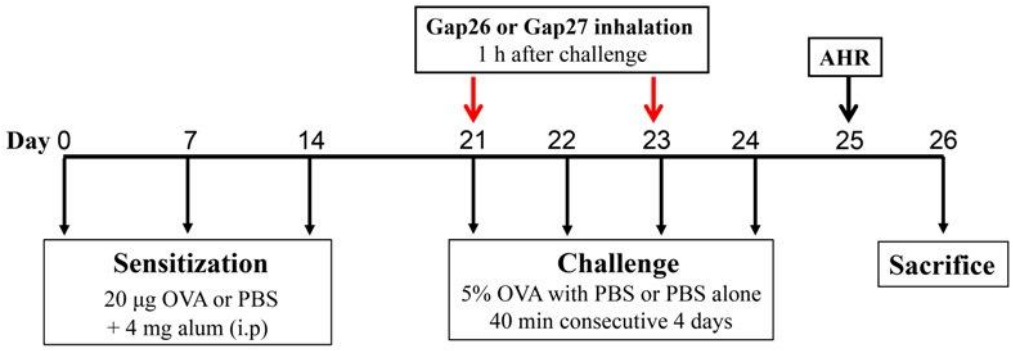

Figure 1. Time line representation of asthma model and intervention
PBS: phosphate-buffered saline; i.p: intraperitoneal; OVA: ovalbumin

Histology Analysis

The left lungs were harvested and fixed in 4% formalin overnight. The fixed tissue was embedded in paraffin for hematoxylin and eosin and periodic acid-Schiff (PAS) staining. A reproducible scoring system was used to assess mucus-containing goblet cells (PAS-positive cells) and lung inflammation in a blinded manner, as previously reported.[16] Goblet cells were quantified as the percentage of PAS-positive cells in epithelial areas from 8 to 10 tissue sections per mouse. Lung inflammation was scored based on the level of peribronchial inflammation and perivascular inflammation in five randomly selected sections across the main bronchus of each animal. The values ranged from 0 to 3 according to the following inflammatory parameters: 0, no inflammation was detectable; 1, occasional cuffing with inflammatory cells; 2, most bronchi or vessels surrounded by a thin layer (1–5 cells) of inflammatory cells; and 3, most bronchi or vessels were surrounded by a thick layer (>5 cells) of inflammatory cells.[16]

Bronchoalveolar Lavage Fluid (BALF) Cell Count and Cytokine Measurement

Mice were sacrificed by intraperitoneal administration of an overdose of pentobarbital sodium (Sigma-Aldrich) 24 h after AHR measurement, and tracheotomy was performed. Next, 1 mL cold PBS was added into the lung three times to collect the BALF. The collected BALF was centrifuged and resuspended for quantification. The cells present within the BALF were counted using a hemocytometer and then cytospun onto glass slides and stained with Diff-Quick (Baso Diagnostics, Inc., Zhuhai, Guangdong, China) according to the manufacturer's instructions. A least of 200 cells per slide were evaluated for eosinophils, macrophages, neutrophils, and lymphocytes as previously reported.[16]

The levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4, IL-5, and IL-13 (R&D Systems, Minneapolis, MN, USA) in the supernatants were measured by sandwich enzyme-linked immunosorbent assays (ELISA) following the manufacturer's instructions.

OVA-Specific Ig Protein Measurements

Serum was harvested after anesthesia. OVA-specific IgE and IgG1 were measured by ELISA, as previously described.[16] The samples were diluted 100,000-fold in PBS for IgG1 (BD Biosciences, Franklin Lakes, NJ, USA), while IgE (BD Biosciences, Franklin Lakes, NJ, USA) was not diluted. The optical density (OD) was read at 450 nm with a Power Wave X microplate absorbance reader (Bio-Tek Instruments, Winooski, VT, USA).

Statistical Analysis

All data are expressed as the mean±SEM. One-way analysis of variance with Tukey multiple comparison test was performed for statistical analysis. Statistical analyses were performed using SPSS version 19.0(IBM Corp., Armonk, N.Y., USA), and P values less than 0.05 were considered statistically significant.

RESULTS

Gap26 Reduced OVA-Induced AHR

AHR is one of the most important pathological features of asthma and is widely used to distinguish asthma from other airway inflammatory diseases. We first evaluated whether Gap26 treatment could improve AHR in allergic mice after OVA challenge (Figure 1). The effects of Gap26 on AHR were measured by noninvasive whole-body plethysmography in this study. As shown in Figure 2, the Penh value of OVA-challenged mice increased significantly compared to that in the control group at high Mch concentrations (25, 50, and 100 mg/mL) (p<0.01). Treatment with Gap26 significantly decreased the Penh value induced by OVA in a dose-dependent manner (p<0.05 or p<0.01), particularly at a dose of 100 mg/mL Mch (Figure 2, p<0.01).

Gap26 Prevented OVA-induced Lung Histopathological Changes

The effects of Gap26 on OVA-induced lung histological changes were assessed by hematoxylin and eosin and PAS staining. We observed obvious inflammatory cell infiltration in peribronchial areas and goblet cell hyperplasia in the bronchi of OVA-induced asthmatic lung tissue compared to that in control mice (all p<0.01). Gap26 inhalation at either dose significantly reduced inflammatory cell infiltration (p<0.05 in low and p<0.01 in mild and high) and goblet cell hyperplasia (all p<0.01), with the best effect observed for high-dose Gap26 inhalation (Figure 3, all p<0.01).

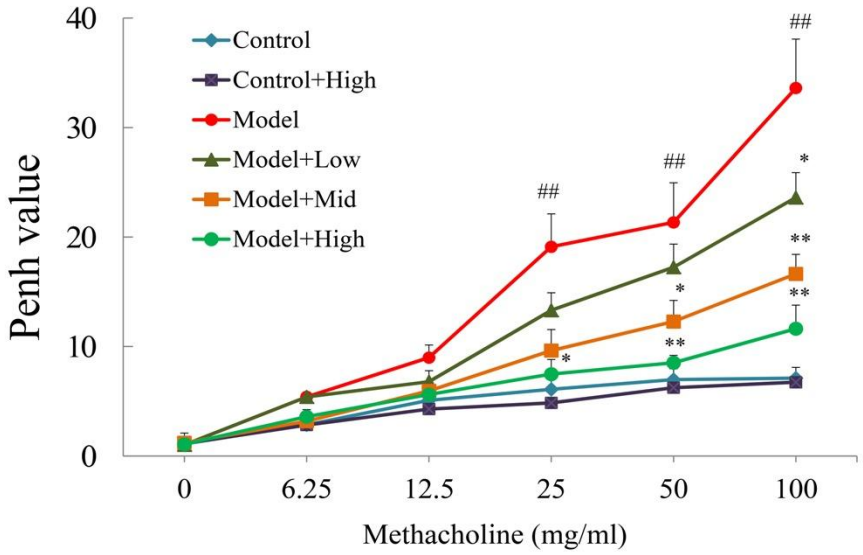

Figure 2. Gap26 reduced ovalbumin-induced airway hyper reactivity at all doses.

The effects of Gap26 on airway hyper reactivity were measured using noninvasive whole-body plethysmography. Control: phosphate-buffered saline controls; Control + High: phosphate-buffered saline controls treated with 2 µg/kg Gap26; Model: ovalbumin-induced asthma + inactive scramble of Gap26; Model + Low: ovalbumin-induced asthma treated with 0.5 µg/kg Gap26; Model + Mid: OVA-induced asthma treated with 1 µg/kg Gap26; Model + High: ovalbumin-induced asthma treated with 2 µg/kg Gap26. The Penh value of ovalbumin-challenged mice increased significantly compared to that in the control group at high Mch concentrations (25, 50, and 100 mg/mL) (p<0.01). Treatment with Gap26 significantly decreased the Penh value induced by ovalbumin in a dose-dependent manner (p<0.05 or p<0.01), particularly at a dose of 100 mg/mL Mch (p<0.01).

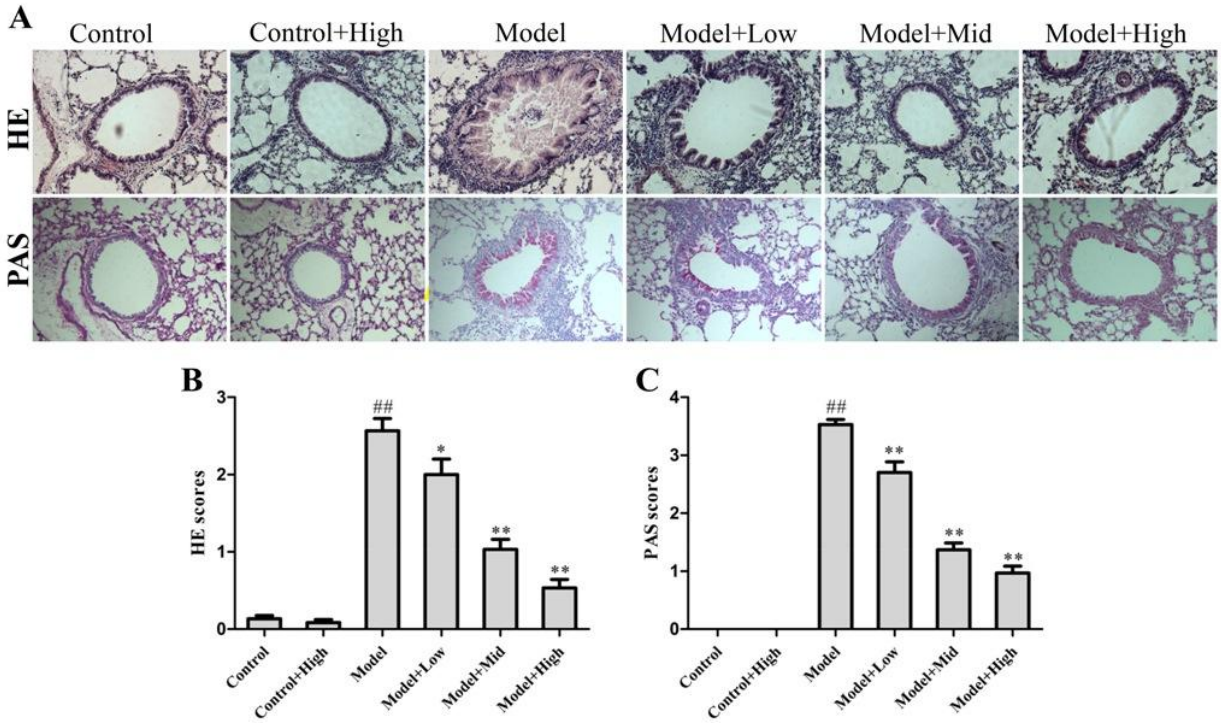

Figure 3. Gap26 prevented lung histopathologic changes in ovalbumin-induced asthma.
(A): Inflammatory cell infiltration in peribronchial areas and goblet cell hyperplasia in the bronchi of ovalbumin-induced asthmatic lung tissue compared to that in control mice (all $p<0.01$). Gap26 inhalation at either dose significantly reduced inflammatory cell infiltration ($p<0.05$ in low and $p<0.01$ in mild and high) and goblet cell hyperplasia (all $p<0.01$), with the best effect observed for high-dose Gap26 inhalation. Representative H&E and PAS stained lung section photomicrographs are shown for each group (×200); (B): The airway inflammation scores were based on the H&E staining;(C); The airway inflammation scores were based on the PAS staining. N=6 mice for each group. Data are shown as means±SEM. #$p<0.05$ versus control group; *$p<0.05$, **$p<0.01$ versus model group.

Gap26 Decreased Inflammatory Cell Infiltration in BALF

Compared to that in control mice, OVA sensitization and challenge induced a significant increase in the number of total cells as well as eosinophils, macrophages, neutrophils, and lymphocytes in the BALF (Figure 4, all $p<0.01$).

The total cell count indicated that all three doses of Gap26 significantly alleviated OVA-induced inflammation. Furthermore, Gap26 at all doses markedly decreased counts of OVA-induced eosinophils ($p<0.05$ in low and $p<0.01$ in mild and high), macrophages ($p<0.05$ in low and $p<0.01$ in mild and high), neutrophils ($p<0.05$ in low and $p<0.01$ in mild and high), and lymphocytes ($p<0.01$ in high), particularly eosinophils and neutrophils (Figure 4, $p<0.05$ in low and $p< 0.01$ in mild and high).

Gap26 Attenuated Levels of Th2 Cytokines in BALF and OVA-Specific IgE in Serum

Th2 cytokines and IgE have been reported to play important roles in the pathogenesis of asthma. In this study, the production of Th1 (IFN-γ and TNF-α) and Th2 cytokines (IL-4, IL-5, and IL-13) in the BALF and OVA-specific IgE and IgG1 in the serum were detected by ELISA. The results showed that Th1 and Th2 cytokine levels in the BALF and OVE-specific IgE and IgG1 levels in the serum were markedly elevated in OVA-challenged mice compared to that in control mice (all $p<0.01$). Gap26 dose-dependently suppressed OVA-induced IL-4, IL-5, and IL-13 production in the BALF and OVA-specific IgE production in the serum (Figure 5, all $p<0.05$ in low and $p<0.01$ in mild and high). However, Gap26 inhalation did not affect IFN-γ and TNF-α levels in the BALF and OVA-specific IgG1

levels in the serum (Figure 5).

Gap27 Decreased Inflammatory Cytokine Levels in BALF

To further confirm the treatment effects of Cx43 inhibition in asthma, we used another Cx43 inhibitor, Gap27, and compared the effects of Gap26 and Gap27 in the mouse asthma model. In the BALF, the IL-4, IL-5, and IL-13 levels were significantly increased by OVA challenge (all $p<0.05$), but were remarkably decreased in the Gap26- and Gap27-treated groups (Figure 6, all $p<0.05$). There were no differences in IL-4, IL-5, and IL-13 levels between the Gap26- and Gap27-treated groups, indicating that Gap26 and Gap27 had similar effects on OVA-induced mouse asthma.

DISCUSSION

This is the first study to demonstrate that Cx43 inhibitors have therapeutic potential for allergic asthma. Our results showed that Gap26 inhibited OVA-induced airway hyperresponsiveness, lung histopathological changes, inflammatory cells and Th2 cytokines in the BALF, and OVA-specific IgE in the serum. A similar decrease in Th2 cytokine levels in the BALF was observed following Gap26 and Gap27 administration compared to that in asthmatic mice. Cx43 inhibitors may be useful for preventing OVA-induced asthma.

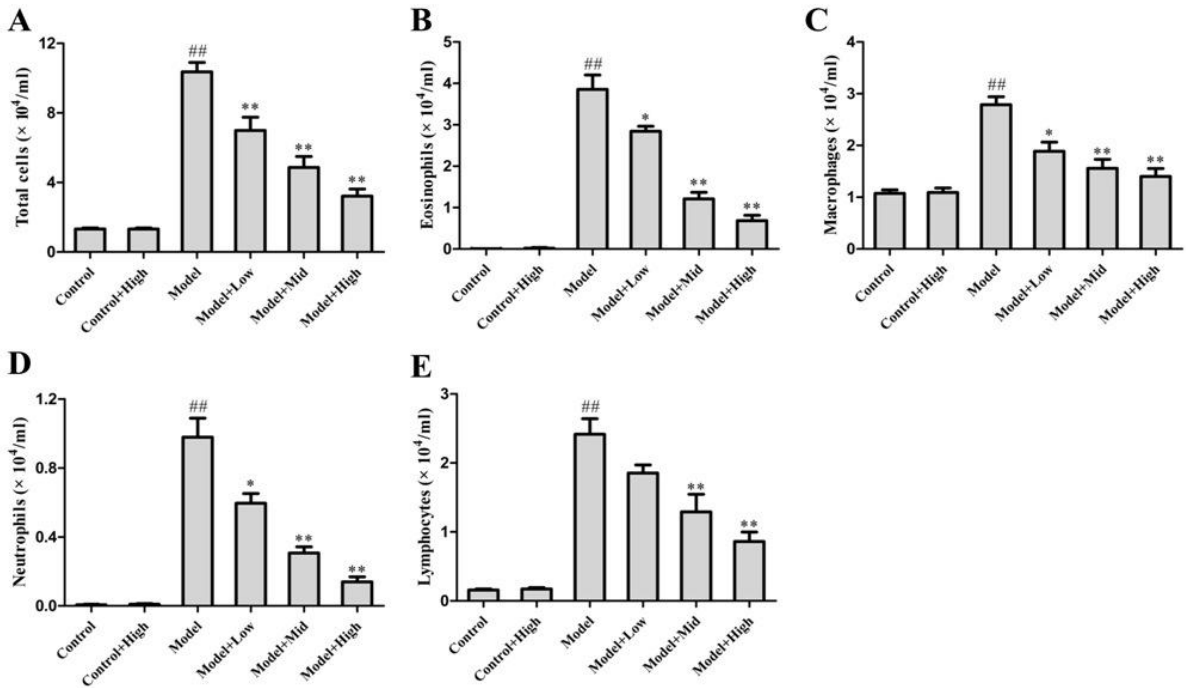

Figure 4. Effects of Gap26 on ovalbumin (OVA)-induced inflammation cell count in bronchoalveolar lavage fluid) BALF). (A): Compared to that in control mice, OVA sensitization and challenge induced a significant increase in the number of total cells in the BALF; (B): Compared to that in control mice, OVA sensitization and challenge induced a significant increase in eosinophils in the BALF; (C): Compared to that in control mice, OVA sensitization and challenge induced a significant increase in macrophages in the BALF; (D): Compared to that in control mice, OVA sensitization and challenge induced a significant increase in neutrophils in the BALF; (E): Compared to that in control mice, OVA sensitization and challenge induced a significant increase in lymphocytes in the BALF.N=6 mice for each group. Data are shown as means ± SEM. #$p<0.05$ versus control group; *$p<0.05$, **$p<0.01$ versus model group.

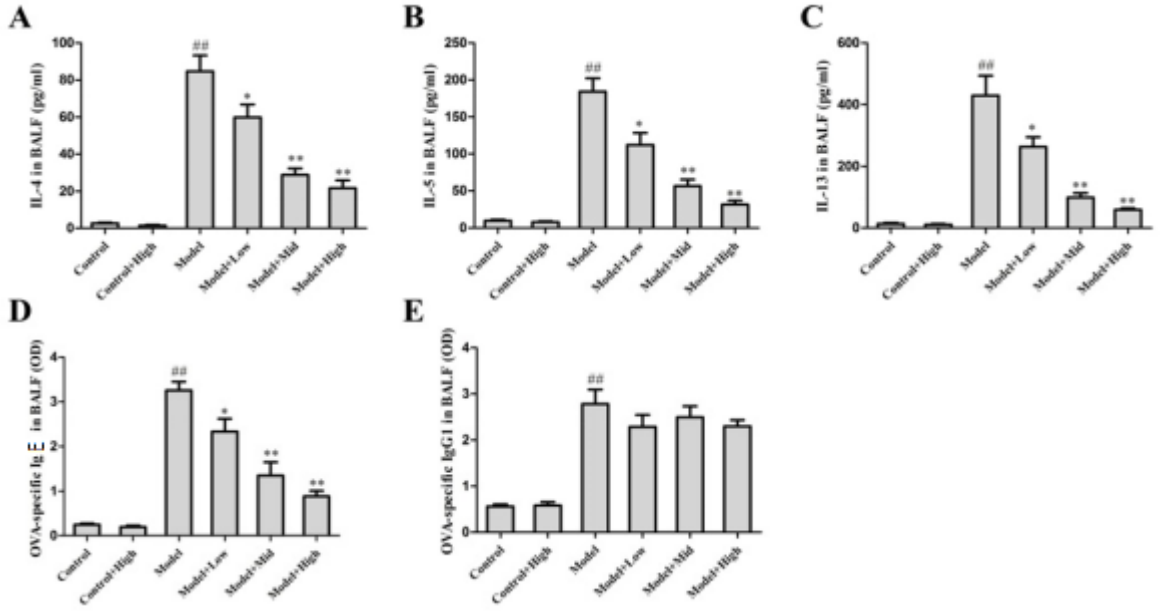

Figure 5. Gap26 attenuated Th2 cytokines in bronchoalveolar lavage fluid (BALF) and ovalbumin (OVA)-specific IgE in serum.

(A) : Gap26 dose-dependently suppressed OVA-induced IL-4 production levels in the BALF; (B): Gap26 dose-dependently suppressed OVA-induced IL-5 production levels in the BALF; (C): Gap26 dose-dependently suppressed OVA-induced IL-13 production levels in the BALF; (D): Gap26 dose-dependently suppressed OVA-specific IgE levels in the serum; (E): Gap26 inhalation did not affect OVA-specific IgG1 levels in the serum. N=6 mice for each group. Data are shown as means ± SEM. #p<0.05 versus control group; *p<0.05, **p<0.01 versus model group.

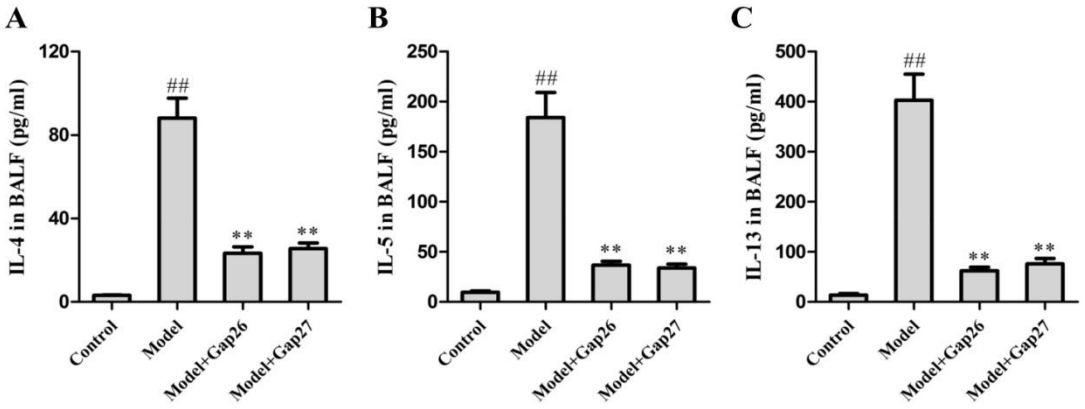

Figure 6. Gap27 decreased inflammation cytokines in bronchoalveolar lavage fluid (BALF) of ovalbumin (OVA)-induced mouse lung.

(A): In the BALF, the IL-4 levels was significantly increased by ovalbumin challenge (all p<0.05), but was remarkably decreased in the Gap26- and Gap27-treated groups; (B): In the BALF, the IL-5 levels was significantly increased by OVA challenge (all p<0.05), but was remarkably decreased in the Gap26- and Gap27-treated groups; (C): In the BALF, the IL-13 levels was significantly increased by ovalbumin challenge (all p<0.05), but was remarkably decreased in the Gap26- and Gap27-treated groups. N=6 mice for each group. Data are shown as means ± SEM. #p<0.05 versus control group; *p<0.05, **p<0.01 versus model group.

Asthma is a common chronic inflammatory disease of the airways and occurs in people of all ages.[2] According to Global Asthma Report, more than 300 million people suffer from this disease and its burden of disability is high.[2,5] Although a variety of drugs has been developed to treat asthma, conventional treatments are ineffective in a large number of patients.[1,4] Therefore, it is necessary to develop new treatments to cure this disease.

Gap junctions provide direct cell-to-cell communication between different cells constituting the tissue.[20] This communication is mediated by Cx channels that ensure a direct physical link between cells.[11] As the most widely and highly expressed gap junction protein, Cx43 is predominantly expressed in alveolar epithelial and endothelial cells in the lung.[13,15,21] Increasing evidence has suggested that Cx43 is involved in the pathogenesis of lung diseases such as ALI, cystic fibrosis, pulmonary arterial hypertension, and asthma.[14-16] Sarieddine et al showed that Cx43 in alveolar promoted neutrophil recruitment to the airspace in a mouse model of ALI.[13] A recent study showed that Cx43 was up-regulated in OVA-induced asthma mice model, and increased Cx43 expression in the lung was associated with infiltration of inflammatory cells, particularly eosinophils.[16] Importantly, previous studies showed that Cx43 inhibitors efficiently reduced neutrophil recruitment from the blood circulation into the lungs in a mouse model of ALI.[13] Moreover, the Cx43 inhibitors Gap26 and Gap27 have been widely used to treat other diseases, including spinal cord injury, myocardial ischemia injury, and wound repair, showing satisfactory effects.[15,22-26] However, the therapeutic effects of the Cx43 inhibitor in allergic airway inflammation have not been thoroughly studied.

OVA-induced asthma is characterized by airway hyperreactivity and excessive Th2-related cytokine production.[6,27] AHR is an important pathological marker that is a fundamental characteristic of asthma and contributes to morbidity and mortality.[27] AHR development is associated with inflammatory cytokines, such as IL-13, a crucial mediator in eosinophil-induced airway AHR.[28, 29]

IL-13, IL-4, and IL-5, which are secreted by T helper type 2 lymphocytes, were demonstrated to contribute to the development of allergic asthma.[6,30] For example, IL-5 is essential for the differentiation and aggregation of eosinophils and enhances IL-4-induced IgE production.[31]In the present study, IL-4, IL-5, and IL-13 levels were significantly decreased in the BALF of the Gap26 treatment groups, which contributed to the overall protective effects in the asthma model.

In the lung, infiltration of inflammatory cells, particularly eosinophils, promotes the development of allergic airway inflammatory dysfunction.[32] Increased neutrophil counts in asthma have been associated with severe asthma, corticosteroid insensitivity, and chronic airflow obstruction.[33] In addition, the increased number of eosinophils in the BALF is an established phenotype of asthma. The present results clearly demonstrated that Gap26 significantly reduced eosinophil and neutrophil numbers in BALF.

Allergic asthma is associated with increased serum IgE levels in response to inhaled allergens.[34] IgE is important in inducing and maintaining allergic inflammation, and represents a prime target for therapeutic intervention[35,36] We examined the effects of Gap26 on OVA-specific IgE levels. The data revealed that treatment with Gap26 significantly inhibited the production of IgE in OVA-induced asthma mice. Moreover, high levels of IgG1 were observed in asthmatic mice, and there were no differences after treatment with Gap26. This different effect suggests that Gap26 specifically regulates IgE production in allergic disease.

To further confirm the treatment effect of Cx43 inhibition on asthma, we compared the effects of Gap26 and Gap27 in a mouse model of asthma. Both inhibitors significantly reduced IL-4, IL-5, and IL-13 levels in the BALF, with no differences between the two inhibitor-treated groups. These results further demonstrate that Cx43 inhibitors reduced inflammation in our OVA-induced mouse asthma model.

Although we found that the inflammatory response of asthma was extensively suppressed by Cx43 inhibitors, the mechanism of this effect is unclear. A recent study reported that Cx43 controls the myofibroblastic differentiation of asthmatic fibroblasts, indicating that Cx43 inhibitors exert inhibitory effects by attenuating myofibroblast formation.[37] In this study, we measured airway responsiveness to Mch by whole body plethysmograph. This method has also been employed in previous asthma model studies.[16,38]

However, to further assess lung responsiveness, a more invasive measurement system would more accurately reflect airway responsiveness. In addition, the effects of Cx43 inhibitors when administered at other time points should be examined.

There were some limitations in our study, in addition to the measurement and drug given time, this was only an animal study which is still far from clinical application.

In summary, the current study demonstrated that Cx43 inhibitors effectively decreased OVA-induced AHR, airway inflammation, and OVA-specific IgE production, likely by down-regulating IL-4, IL-5, and IL-13. Our findings support the possible application of Cx43 inhibitors as therapeutic drugs for patients with allergic asthma. However, further studies are required to investigate other possible effects and side-effects of Cx43 inhibitors, becauseCx43is not only expressed in airways but also in many other organs, such as myocardium, brain, liver, prostate, and is also related to some cancers, including thyroid carcinoma, non-Hodgkin's lymphoma, and liver and laryngeal cancer.

ACKNOWLEDGEMENTS

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