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Vitamin D alleviates airway remodeling in asthma by down-regulating the activity of Wnt/β-catenin signaling pathway

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ABSTRACT

Vitamin D exerts a protective role in asthma; however, the molecular mechanisms underlying the vitamin D-attenuated asthma airway remodeling are yet to be elucidated. In this study, Sprague–Dawley (SD) rats were randomly divided into four groups: control, asthma, vitamin D 50 ng/mL, and vitamin D 100 ng/mL. The treatment with 100 ng/mL vitamin D remarkably reduced the thickness of the airway smooth muscle, collagen deposition, and the alpha-smooth muscle actin (α-SMA) mass and airway inflammation. Conversely, the treatment by vitamin D significantly up-regulated the serum levels of $25(OH)_2D_3$ that were decreased in asthma. The putative signaling pathway of vitamin D was based on Wnt5a and β-catenin expression assessed by quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) and Western blot, which revealed that the administration of vitamin D significantly decreased the activity of Wnt/β-catenin signaling pathway. These results suggested that administration of vitamin D alleviated the airway remodeling in asthma by down-regulating the activity of Wnt/β-catenin signaling pathway.

1. Introduction

Asthma is a chronic inflammatory disease of the airway, which is characterized by airway inflammation, airway hyperresponsiveness (AHR), mucin hypersecretion, and airway remodeling [1]. Airway remodeling is irreversible and includes airway wall thickening, increased airway muscle mass, and subepithelial fibrosis, which restricts the constant airflow as well as high morbidity and mortality of asthma [2,3]. However, the precise mechanism underlying the airway remodeling in asthma has not yet been elucidated.

Vitamin D is a fat-soluble vitamin, which is produced by skin exposure. A previous study demonstrated that vitamin D was involved in the regulation of innate and adaptive immune processes and exerted a great influence on allergy diseases such as asthma [4]. Lower vitamin D is typically associated with increased asthma severity and reduced glucocorticoid (GC) response [5–8]. In addition, $1,25(OH)_2D_3$ is the bioactive form of vitamin D, also a major circulating vitamin D metabolite that could be individually used for evaluating the status of vitamin D [9]. A number of studies found that $1,25(OH)_2D_3$ could slow down the multiplication of cells and arrest the cell cycle in G1-phase of airway smooth muscle cells (ASMCs) [10]. However, the molecular mechanisms are yet unknown. Thus, in the present study, we investigated the regulation of airway remodeling using vitamin D on asthma airway remodeling rats and investigated the underlying mechanisms.

The Wnt signaling pathway is conversed across evolution and classified into the canonical Wnt/β-catenin pathway and the non-canonical Wnt pathway [11,12]. The canonical Wnt/β-catenin pathway plays a critical role in cell proliferation, cell migration, stem cell self-renewal, organogenesis, and tissue homeostasis under a physiological conditions and tissue repair of injuries [13]. Furthermore, dysregulated Wnt signaling pathway is inextricably linked to the pathogenesis of asthmatic airway remodeling [14]. The intracellular aggregation and nuclear transfer of Wnt/β-catenin greatly influenced the lung maturity and structural adaptation, such as developing airway smooth muscle precursor cell, maintaining the growth of airway smooth muscle, as well as regulating its shrinkage [15,16]. Indeed, our previous studies have demonstrated that the activation of Wnt signaling pathway accelerated the proliferation of ASMCs that are the major structural cells involved in airway remodeling [17,18].

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Some studies found that $1,25(OH)_2D_3$ promoted the translocation of β-catenin from the nucleus to the plasma membrane, repressed the β-catenin-TCF-4 transcriptional activity, and activated the transcription of *DICKKOPF-1* gene which encodes an extracellular Wnt inhibitor in colon carcinoma cells [19,20]. Yurt et al. [21] demonstrated that vitamin D supplement reduced the airway resistance, following methacholine challenge, by down-regulating the expression of β-catenin. However, sufficient evidence of whether vitamin D down-regulates the Wnt signaling pathway in asthmatic airway remodeling is yet lacking. In this study, we hypothesized that the Wnt signaling pathway could be regulated by vitamin D to alleviate the airway remodeling. Thus, our results revealed the potential of vitamin D as a dietary supplement beneficial in preventing the development of airway remodeling in asthma.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley (SD) rats, weighing 120–160 g, aged 4–6 weeks, were purchased from Shanghai SLAC Laboratory Animal Center (wydw2017–0036, Shanghai, China). Rats were maintained in a pathogen-free environment under 12 h/12 h standard light/dark conditions at 22 ± 1 °C and 50 ± 1% relative humidity. All experiments were performed according to the regulations and guidelines of the Institutional Animal Care and Use Committee (IACUC) of Wenzhou Medical University (Wenzhou Medical University) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

2.2. Airway remodeling models and drug treatments

Rats were randomly divided into four groups: control, asthma, vitamin D 50 ng/mL, and vitamin D 100 ng/mL. Rats were sensitized by intraperitoneal (i.p.) injection of 1 mg ovalbumin (OVA, Sigma-Aldrich, St. Louis, MO, USA) emulsified in 100 mg $AL(OH)_3$ gel in 1.5 mL normal saline (NS) on days 1 and 8. Subsequently, the animals were challenged with OVA (0.1 mg/mL) aerosol for 30 min every alternate day for 60 days starting from day 15 by a Jet nebulizer (Pari IS-2 Jet nebulizer, PARI Respiratory. Equipment, Richmond, VA, USA). The control group was sensitized and challenged by normal saline.

Vitamin D (Calcitriol Soft Capsules, Roche, China) was obtained from the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University (Wenzhou, Zhejiang province, China) and solubilized in corn oil at 50 and 100 ng/mL, respectively. From day 31, vitamin D was administered orally for another 30 days. The control group was administrated with corn oil intragastrically. Fig. 1 shows the study schedule.

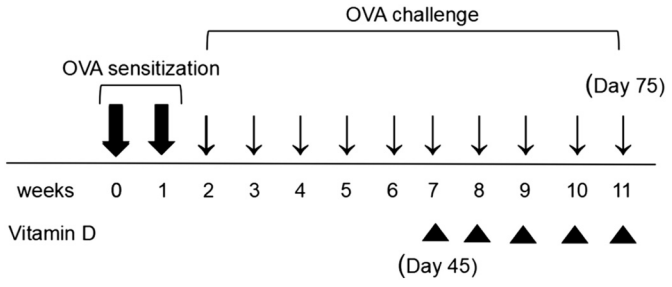

Fig. 1. Rats model of chronic asthmatic airway remodeling.
Thick arrows indicated the sensitization on days 1 and 8. The thin arrows indicated the OVA challenge for every alternate day. The triangle indicated the vitamin D supplement from day 31.

2.3. Estimation of serum $25(OH)_2D_3$

Blood samples were withdrawn from the abdominal aorta of rats, maintained at room temperature for 3 h, and centrifuged at 5000 rpm for 20 min. Sera were separated, and the $25(OH)_2D_3$ levels measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions using rats $25(OH)_2D_3$ kit (Shanghai Boyun Biotech Co. Ltd., Shanghai, China).

2.4. Morphological changes in the airways

The animals were sacrificed, and the left middle lobe from the lung was fixed in 4% paraformaldehyde for 4 h, followed by dehydration in ethylic alcohol, paraffin embedding, and slicing in 4-μm sections. Hematoxylin and eosin (HE) and Masson's trichrome stainings were performed following the standard protocol recommended by the manufacturer (Solarbio Life Science, Beijing, China). Then, the tissue slices were evaluated under a light microscope (Nikon, Japan) by a trained technician in a blinded manner.

In HE-stained sections, the degree of airway remodeling was assessed based on the bronchioles with 150–200 μm inner diameter. The total area of airway wall (Wat) and area of smooth muscle (Wam) were assessed by morphometric analysis (Image-Pro Plus, Media Cybernetics Co., Bethesda, MD, USA). The perimeter of the basement membrane (Pbm) was used to normalize the Wat and Wam. Thus, the ratios of Wat to Pbm (Wat/Pbm) and Wam to Pbm (Wam/Pbm) were used for evaluating the airway remodeling [22].

The inflammation cells were quantified in the H&E-stained sections by a sample blinded pathologist. The degree of allergic airway inflammation was scored based on the following histologic grading system (scored 0–4): 0 = normal, absence of inflammation cells; 1 = mild inflammation cells accumulation, < 25% of the circumference of the bronchus was involved; 2 = moderate inflammation cells accumulation, about 25%–75% of the circumference of the bronchus was involved; 3 = the peribronchial inflammation cells were accumulated surrounded the bronchus; 4 = sever inflammation cells accumulation, 2 or more layers of inflammatory cells completely surrounding bronchus. The terminal score of each section of the lung tissue was determined by the sum of the every individual bronchus in the section and divided by the number of bronchioles [23].

The subepithelial collagen deposition was identified by Masson's trichrome-staining and quantified under a light microscope (Nikon, Japan) attached to an image analysis system. Briefly, the epithelial basement membranes of bronchioles with 150–200 μm-diameter, were selected, the fibrotic area was divided by the basement membrane perimeter, and the results were expressed as the area of trichrome staining in μm/length [24].

2.5. Immunohistochemistry

The sections of lung tissues were stained by α-smooth muscle actin (α-SMA) antibody (Cell Signaling Technology, Danvers, MA, USA) and counterstained using hematoxylin (Solarbio Life Science). The expression of α-SMA was evaluated using Image-Pro Plus 6.0 via optical density analysis.

2.6. Western blot analysis

Lung tissues were fragmented and lysed in RIPA buffer containing protease inhibitor (Beyotime Institute of Biotechnology, China). Then, 40 μg total protein was resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the membrane. Subsequently, the membrane was probed with Wnt5a (Affinity Biosciences, OH, USA) and β-catenin (Abcam, Cambridge, UK) primary antibodies, and then for 1 h with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody. GAPDH (Bioworld

Table 1
Primer sequences (from 5′–3′) for measuring mRNAs by RT-qPCR.

mRNA	Forward primer (5′–3′)	Reverse primer (5′–3′)
Wnt5a	TCGACTATGGCTACCGCTTC	CGACCTGCTTCATTGTTGTG
β-Catenin	CAAGATGATGGTGTGCCAAG	TGGTCAGATGACGAAGAGCA
GAPDH	TCTCTGCTCCTCCCTGTTC	ACACCGACCTTCACCATCT

Technology, Inc., St. Louis Park, MN, USA) was used as the loading control. The G-box gel documentation system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the Image-Lab software was employed for visualizing and quantitating the density of the immunoreactive bands using enhanced chemiluminescent reagents.

2.7. Quantitative real-time RT-PCR

Total RNA was extracted from the lung tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and assessed for purity at 260/280 nm. Subsequently, 1 μg RNA was utilized to synthesize the complementary DNA (cDNA) using with a Maxime RT PreMix Kit (Takara-Bio, Kusatsu, Japan). LightCycler 480 System (Roche-Applied Science, Switzerland) and SYBR Green (Roche-Applied Science) were used for quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The expression of the target mRNA was normalized against that of *GAPDH* and expressed using the delta-delta Ct (ΔΔCt) method. The primer sequences of the genes are listed in Table 1.

2.8. Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Student–Newman–Keuls test were used to determine multiple comparisons. Kruskal–Wallis test followed by Dunnett's test were used for posthoc multiple comparisons in inflammation score. $P < 0.05$ was considered as statistically significant. Statistical analyses were conducted using SPSS 18.0 software (IBM, Armonk, NY, USA).

3. Results

3.1. Vitamin D attenuates the structural changes of the airway in chronic asthma

Compared to the control group, the OVA-challenged rats developed an obvious increase in the thickness of the airway wall and the layer of airway smooth muscle, whereas, 100 ng/mL vitamin D could significantly reduced this increase (Fig. 2A). Actually, we found that administration of 25 ng/mL vitamin D cannot significantly alleviate the degree of airway remodeling as that of 50 ng/mL treatment did (Supplementary Fig. S). Thus, we abandoned the 25 ng/mL group in the following experiments. Moreover, the morphometric measurements corrected by Pbm (Wat/pbm and Wam/pbm) in the vitamin D 100 ng/mL group were lower than those in the asthma group ($P < 0.01$). As shown in Fig. 2B, a large area of peribronchial collagen deposition was observed in the asthma rats. Interestingly, the vitamin D treatment at the dose of 100 ng/mL significantly inhibited the collagen deposition ($P < 0.01$). In addition, α-SMA mass was increased in the asthma group, and treatment with vitamin D inhibited this increase ($P < 0.01$, Fig. 2C).

3.2. Vitamin D reduces the airway inflammation in chronic asthma

In chronic asthma, airway remodeling was always accompanied by airway inflammation. Herein, the degree of accumulation of inflammatory cells was quantified in the HE-stained lung sections. Consequently, a robust accumulation of airway inflammatory cells was observed in the asthma group as compared to the control group ($P < 0.01$). As shown in Fig. 3, significant infiltration of inflammation cells was detected in the asthma group, while treatment with vitamin D at 100 ng/mL dose reduced these accumulations ($P < 0.01$).

3.3. Vitamin D up-regulates the serum 25(OH)$_2$D$_3$ level that was reduced in asthma

To determine the influence of asthmatic airway remodeling on vitamin D expression, the 25(OH)$_2$D$_3$ level in serum was measured (Fig. 4). The results showed a significant decline in the concentration of serum vitamin D in the asthma group (5.51 ± 1.12 ng/mL) as compared to the control group (14.19 ± 1.96 ng/mL). Moreover, the vitamin D 100 ng/mL group (8.88 ± 2.38 ng/mL, $P < 0.01$) showed a significant increase in this level, while rats treated with 50 ng/mL vitamin D did not show an effective increase.

3.4. Vitamin D alleviates airway remodeling by down-regulating the activity of Wnt/β-catenin signaling pathway

Furthermore, we assessed whether vitamin D alleviated the airway remodeling by inhibiting the Wnt signaling pathway. As illustrated in Fig. 5A–B, chronic OVA-challenging resulted in an increased expression of Wnt5a mRNA ($P < 0.05$) and β-catenin ($P < 0.01$), while treatment with 100 ng/mL vitamin D significantly inhibited this increase. Similar effects were detected with respect to the expression of Wnt5a and β-catenin ($P < 0.01$) protein as assessed by Western blot in Fig. 5C–D. These results demonstrated that the treatment by vitamin D inhibited the Wnt/β-catenin signaling pathway that was activated in asthma.

4. Discussion

In the present study, we found that the level of serum 25(OH)$_2$D$_3$ was correlated with the severity of airway remodeling. In addition, treatment with 100 ng/mL vitamin D ameliorated the airway remodeling by down-regulating the activity of Wnt/β-catenin signaling pathway. These findings demonstrated the beneficial effects of vitamin D in the prevention and treatment of asthma.

Vitamin D plays a critical role in asthma. The vitamin D insufficiency is associated with poorer asthma control, increase in the mass of airway smooth muscle, and the increased requirement of inhaled corticosteroids [25,26]. A prospective study about high-asthma-risk children in their initial 10 years postulated that the deficiency of vitamin D during the infantile and pre-school period (0.5–2–3 years) significantly increased the incidence rate of asthma, as well as wheezing and allergic symptoms in the following periods [27,28]. Conversely, clinical beneficial effects have been found in steroid-resistant asthma patients by administrating of 0.25 μg calcitriol twice every day [29]. Previous studies have defined vitamin D insufficiency as the 25(OH)D level < 30 ng/mL, which includes > 35% asthmatic patients [5,6,30,31]. When the concentration of serum vitamin D declines as per each ng/mL, the prevalence of asthma is increased by 0.79-fold [25]. However, the exact value of vitamin D insufficiency in rats varied according to the different modeling methods, while the normal range of 25(OH)$_2$D$_3$ comes to 14.04–23.2 ng/mL [21,32–36]. In the current study, the altered concentration in serum 25(OH)$_2$D$_3$ revealed that the vitamin D 100 ng/mL group showed a significant increase in the level of 25(OH)$_2$D$_3$ and relieved airway remodeling, indicating that serum vitamin D level was associated with the degree of asthma airway remodeling.

The thickened airway wall, collagen deposition, and increased mass of α-SMA were the main features of airway remodeling. Lai et al. [37] found that 1,25(OH)$_2$D$_3$ inhibited the proliferation of human airway

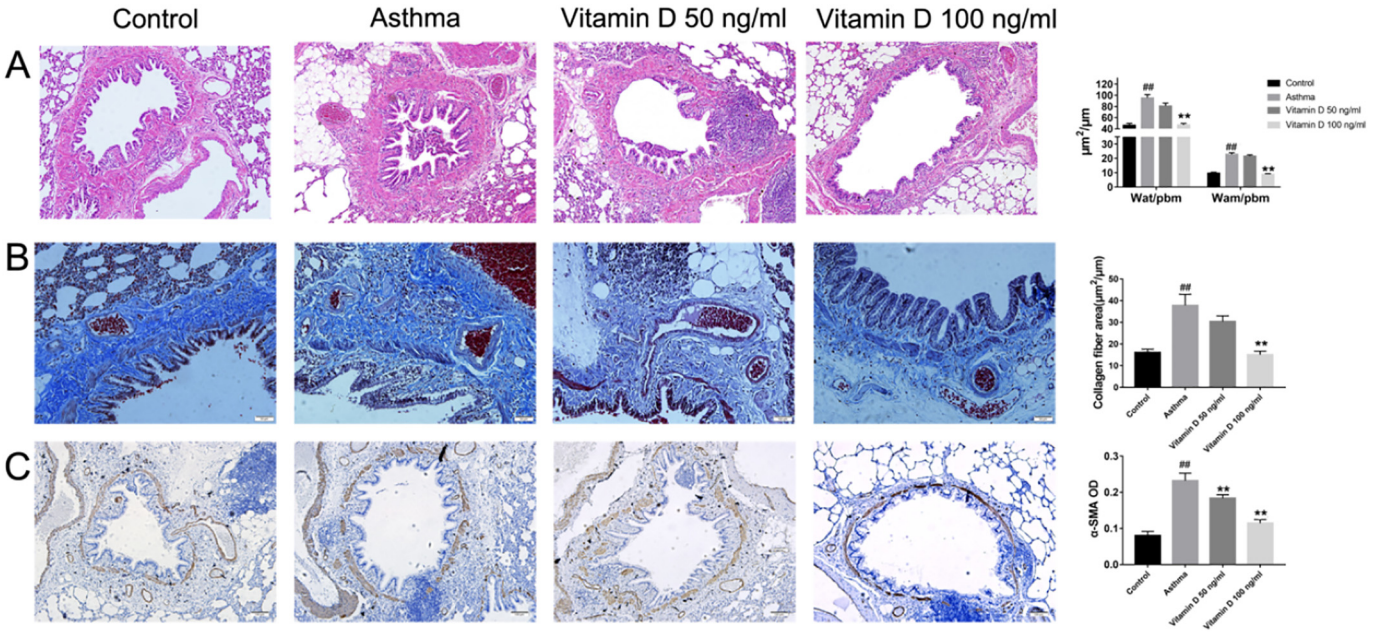

Fig. 2. Vitamin D ameliorates airway remodeling in chronic asthma rats.
HE staining of lung tissues at 100× magnification and the quantitative analysis of the thickness of airway wall and smooth muscle layer (A). Masson's trichrome staining of lung tissue at 200× magnification displays collagen deposition and the quantitative analysis (B). IHC of α-SMA at 100× magnification and the semi-quantitative analysis of α-SMA expression using Image-Pro Plus software (C). Data are expressed as mean ± SEM based on 6 random individual rats. $^{\#\#}P < 0.01$ compared to normal control group, $^{**}P < 0.01$ compared to the asthma group.

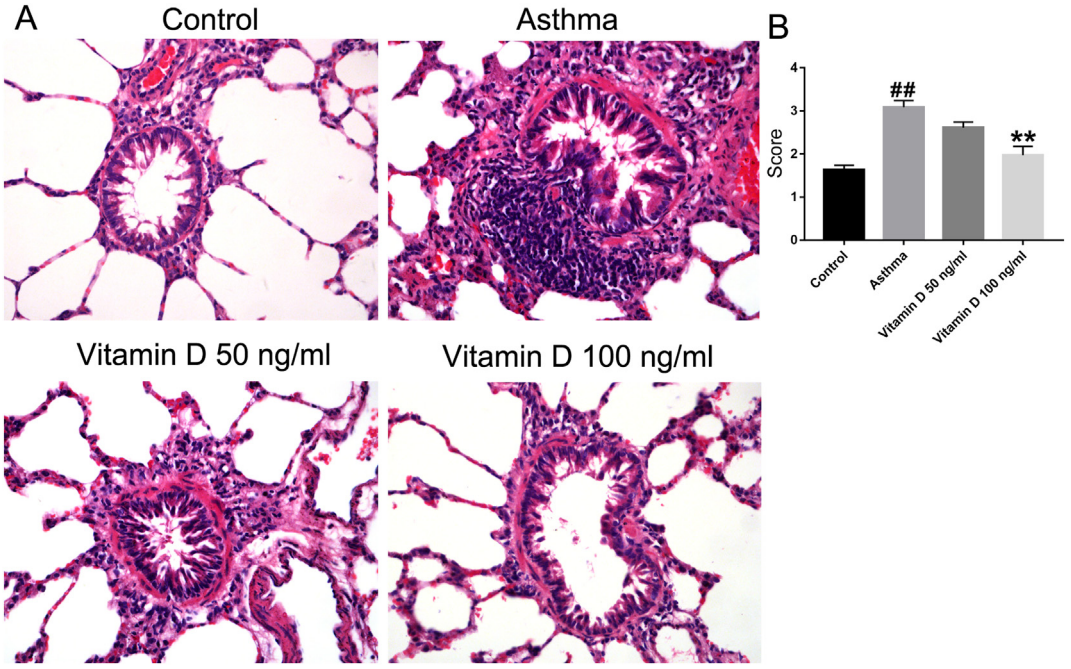

Fig. 3. Vitamin D reduced airway inflammation in chronic asthma rats.
HE staining of lung tissues at 400× magnification (A); The quantification results of the inflammation degree in the groups (B). $^{\#\#}P < 0.01$ compared to the normal control group, $^{**}P < 0.01$ compared to the asthma group (Kruskal-Wallis test, $P < 0.01$).

smooth muscle cells (HAMSCs), and reduced the mass of α-SMA. However, feeding vitamin D as supplemental diet (10,000 IU/kg) or changing the vitamin D-deficient diet to the supplemental diet ameliorated AHR, reduced airway inflammation, and inhibited the epithelia-mesenchymal transition [9, 38–40]. Rilatha et al. [41] have demonstrated that ingesting of vitamin D_3-supplemented diet (5000 IU/kg) exerted a role similar to that with the administration of 0.05 μg/mouse calcitriol, the hormonally active vitamin D metabolite. Accordingly, in this study, we used 100 ng/mL of calcitriol, orally gavage 1 mL per rat, which was equally to the 10,000 IU/kg vitamin D supplement diets. Combining with our preliminary experiment results (Supplementary Fig. S), we demonstrated that supplement of vitamin D at the dose of 100 ng/mL, other than 25 or 50 ng/mL, significantly reduced the airway remodeling in rats. Thus, our experiment may be help to provide the basis for the following clinic treatment in asthma.

Airway inflammation is one of the characteristics of asthma, which

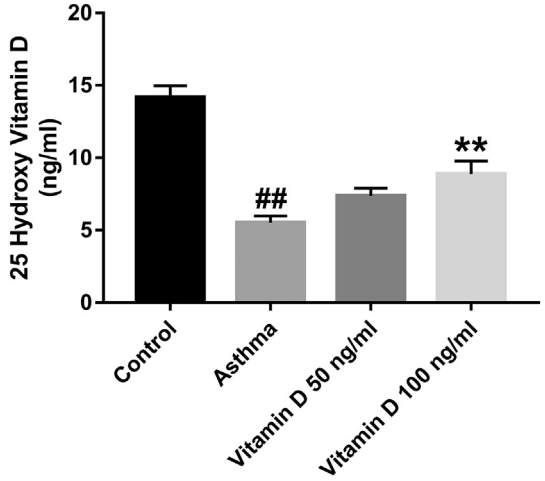

Fig. 4. The concentration of serum vitamin D was measured by ELISA.
The results are expressed as mean ± SEM based on 8 random individual rats. $^{\#\#}P < 0.01$ compared to the control group, and $^{**}P < 0.01$ compared to the asthma group.

behaved as enormous infiltration of inflammation cells [42]. We have previously elucidated that airway inflammation and airway remodeling were parallel but independent segments in asthma [1]. ASMCs, the major structural cells involved in airway remodeling, were proved to attract inflammation cells when they were stimulated [43,44]. In addition, the airway inflammation in chronic asthma has been suggested to promote airway remodeling in some way because of permanent destruction and persistent repair in airway tissues [45–47]. Researchers showed that vitamin D deficient asthma mice owned severer inflammation accompanied by airway remodeling, and supplement of vitamin D reduced the pro-inflammatory effects [37]. Inline with the previous study, we demonstrated that supplement of 100 ng/mL vitamin D significantly reduced the airway inflammation and airway remodeling in chronic asthma.

Mechanistically, we found that vitamin D reduced the expression of Wnt5a and β-catenin, thereby effectively inhibiting the activity of Wnt/β-catenin signaling pathway in chronic asthma. Wnt signaling pathway is conserved across evolution, and β-catenin acts as an adhesion-associated protein, which is crucial for the adhesion to the cytoskeleton. Recent studies have shown a significant increase in the target mRNA expression and the activation of β-catenin in asthma [48]. Gene expression analysis among asthma patients demonstrated that Wnt

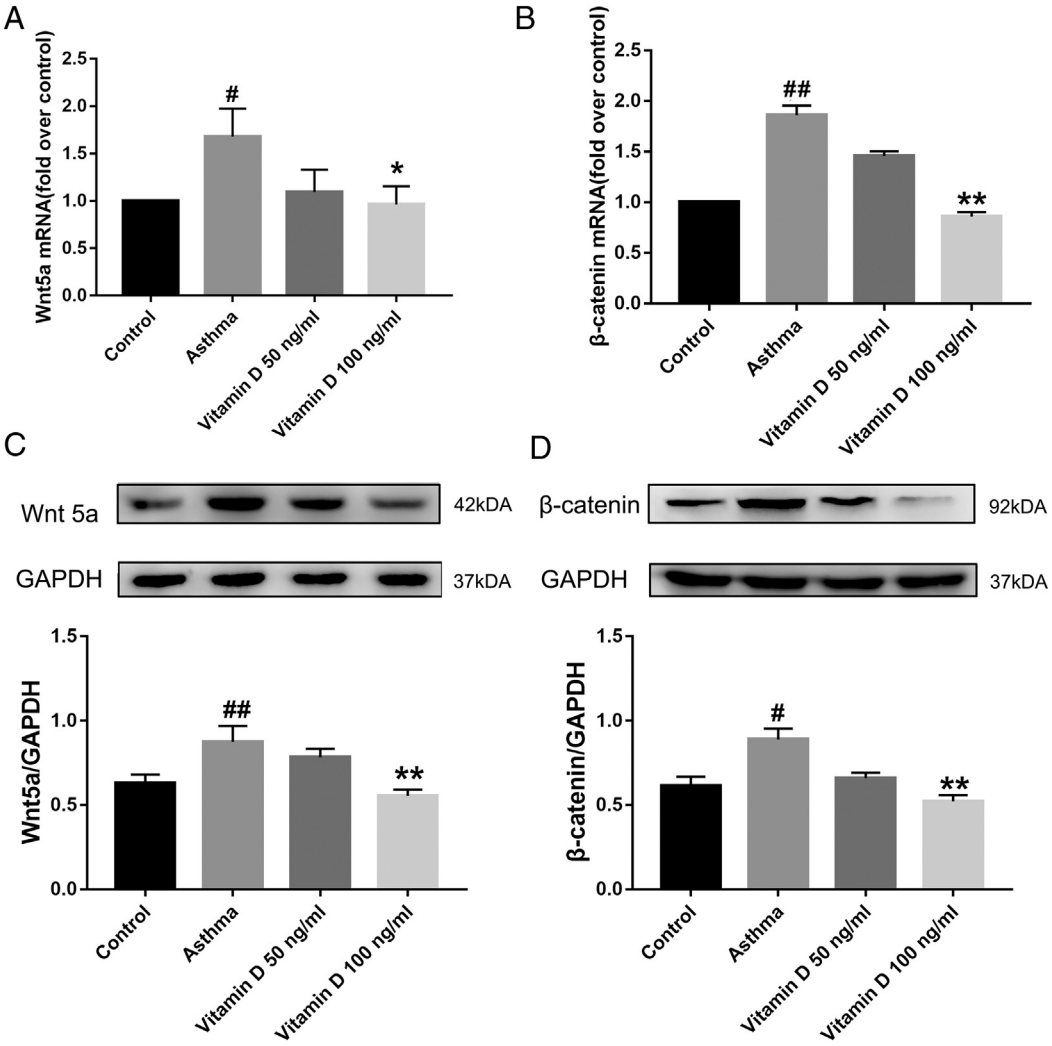

Fig. 5. Vitamin D reduced the mRNA and protein expression of Wnt5a and β-catenin.
The expression of Wnt5a and β-catenin mRNAs in the lung was determined by RT-qPCR (A and B); the expression of Wnt5a and β-catenin was determined by Western blot, which was normalized to the levels of GAPDH, including the quantitation of the ratio between Wnt5a/GAPDH and β-catenin/GAPDH (C and D). The results are expressed as mean ± SEM based on 6 random individual rats. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the asthma group.

signaling gene variants were associated with the impaired lung function [49,50]. In consistent with our study, previous study reported that vitamin D supplementation could reduce the expression of Wnt and β-catenin [51]. While inhibiting the expression of vitamin D receptor increased the expression of Wnt signaling, shortened the cell growth cycle, and promoted the production of extracellular matrix and fibers, which may aggravate the airway remodeling [52].

In summary, the current study demonstrated that in asthmatic rats, the serum vitamin D level remarkably decreased. The administration of 100 ng/mL vitamin D alleviated the OVA-induced asthmatic airway remodeling, airway inflammation and reduced the expression of Wnt5a, β-catenin, and α-SMA. Thus, vitamin D might be a promising candidate for the systemic therapy of airway remodeling in asthma. However, further trials exploring the clinical activity of vitamin D are essential to support its potential application for the treatment of airway remodeling in asthma.

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.12.061.

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