Vitamin D reduces inflammatory response in asthmatic mice through HMGB1/TLR4/NF-κB signaling pathway

HAN ZHANG, NAN YANG, TIANYUE WANG, BING DAI and YUNXIAO SHANG

Department of Pediatrics, Shengjing Hospital of China Medical University, Shenyang, Liaoning 110004, P.R. China

Abstract. The present study aimed to investigate the effects of vitamin D (VD) on inflammatory responses in asthmatic mice and the underlying mechanism, providing a theoretical basis for clinical application of targeted drug therapy, and the development of novel drugs against asthma. Mouse models of asthma were established. Hematoxylin-eosin staining was performed to observe the pathological changes of the lung tissue. Pulmonary function tests were conducted to determine airway resistance in asthmatic mice. ELISA was performed to measure the serum levels of inflammatory factors. Western blot analysis and reverse transcription-quantitative polymerase chain reaction were performed to determine the changes in apoptosis-inducing factors, and high mobility group box 1 protein (HMGB1)/Toll-like receptor-4 (TLR4)/nuclear factor (NF)-κB signaling pathway-related proteins. VD reduced infiltrated inflammatory factors, attenuated the airway resistance of asthmatic mice, decreased serum levels of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, increased serum levels of IL-10, decreased apoptotic factor Bcl-2-associated X and caspase-3 expression, downregulated HMGB1 and TLR4, NF-κB and phosphorylated-NF-κB p65 expression. When TLR4 expression was inhibited, the anti-inflammatory effects of VD were attenuated, and HMGB1, TLR4, NF-κB and p-NF-κB p65 expression was increased. VD was able reduce the inflammatory response of asthmatic mice and apoptosis in lung tissue through the HMGB1/TLR4/NF-κB signaling pathway.

Introduction

Bronchial asthma is a chronic inflammatory respiratory disease that seriously affects human health (1). It develops from a chronic airway inflammation that involves eosinophils, lymphocytes, mast cells, neutrophils and other inflammatory cells and cell components (2-4). Approximately 300 million people worldwide currently suffer from asthma. The World Health Organization sponsored the World Asthma Day (the first Tuesday in May each year) to remind the public awareness of the disease and strengthen its prevention and treatment. At present, many asthma-related issues remain poorly understood. Therefore, it remains very important to investigate the pathogenesis of asthma and to look for new preventive and therapeutic targets in asthma.

High mobility group box 1 protein (HMGB1) is a highly conserved nuclear protein that can be used as an immunomodulatory factor and an inflammatory factor to participate in airway inflammation (5). Intranuclear HMGB1 mainly regulates DNA recombination, replication, repair and transcription (6). Extranuclear HMGB1 can be actively released by immune cells including monocytes/macrophages under the stimulation of inflammatory factors such as lipopolysaccharide, tumor necrosis factor (TNF)-α, and interleukin (IL)-1 and it can be passively released by necrotic cells in the early stage of tissue injury and necrosis (7-9). Extranuclear HMGB1, as an important endogenous proinflammatory factor and an inflammatory mediator, participates in the pathological process of various diseases such as sepsis, pneumonia, and arthritis (10-12).

Toll-like receptor-4 (TLR4) is an important immune pattern recognition receptor that controls innate and adaptive immune responses and plays an important role in initiating and regulating airway inflammation (13,14). TLR4 is the main receptor of HMGB1 (15). Nuclear factor (NF)-κB is located on the downstream TLR4 signaling pathway and is also in a pivotal position to regulate the immune response, cell proliferation and differentiation (16). Therefore, HMGB1/TLR4/NF-κB signaling pathway is an important part of immunoregulatory processes. It is also considered an important pathological mechanism underlying asthma. Regulation and intervention of any part of the signaling pathway may affect the occurrence and development of asthma.

Vitamin D (VD) is reportedly to be involved in many aspects of allergic and autoimmune diseases, exhibiting a remarkable immunoregulatory effect (17,18). There is evidence that VD receptor (VDR) gene is a new susceptibility gene for asthma (19). VD exhibits biological effects via binding to intracellular VDR, and its immunoregulatory effects on asthma has become an increasing area of interest. A previous study has demonstrated that $1,25\text{-}(OH)_2D_3$ can downregulate the expression of MHC class II molecules and co-stimulatory

Correspondence to: Professor Yunxiao Shang, Department of Pediatrics, Shengjing Hospital of China Medical University, 36 Sanhao Street, Shenyang, Liaoning 110004, P.R. China
E-mail: shangyunx@sina.com

molecules on antigen-presenting cell surface, prevent antigen presentation and T cell immune response, inhibit the expression of IL-4, interferon-γ, and IL-5, and thereby alleviate airway inflammation (20). In this study, we investigated the role of VD in a mouse model of asthma, and the mechanism of action of HMGB1/TLR4/NF-κB in asthma, providing a theoretical basis for clinical application of targeted drug therapy and development of new drugs against asthma.

Materials and methods

Animals and grouping. Healthy Balb/c mice, weighing 20-22 g, were purposed by Laboratory Animal Center, China Medical University, China. The experiments were approved by China Medical University Institutional Animal Care and Use Committee (IACUC; no. 20167642). Mice were randomly divided into six groups with 8 mice in each group: Normal control, asthma, asthma + high-dose VD (HVD), asthma + medium-dose VD (MVD), asthma + low-dose VD (LVD), asthma + VD + 40 μg/kg/d E5564 (EVD). Mice in the LVD group were daily administered a mixture of 0.1 μg/ml/20 g VD solution via the tail vein. Mice in the MVD group were identically administered a mixture of 0.4 μg/ml/20 g VD solution per day. Mice in the HVD group were identically injected with a mixture of a mixture of 1 μg/ml/20 g VD solution per day. Mice in the normal control group were daily injected with equal amounts of normal saline via the tail vein.

Establishment of mouse models of asthma. Mice in all groups with the exception of normal control group were intraperitoneally administered 0.2 ml antigen solution (50 μg ovalbumin + 0.15 ml 10% Al (OH)$_3$ + 0.05 ml normal saline). Mice were immunized on experimental days 1, 8 and 15. On day 21, mice were placed in a closed container and sprayed with 5% ovalbumin and normal saline, once a day, 45 min each time, for 7 successive days. In the normal control group, only equal amount of normal saline was used to spray the mice. Mice in the HVD, MVD, and LVD groups were injected with VD solution via the tail vein 30 min before irritation.

Sample collection. On the day of asthma model establishment and on day 28 of intervention, blood samples were collected from mouse vein. Serum was separated and ELISA assay was performed. Mouse bronchial alveolar tissue was isolated. One portion of bronchial alveolar tissue was fixed in 10% formaldehyde and the other portion was used for western blot assay and reverse transcription-quantitative polymerase chain reaction (RT-qPCR; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Measurement of airway resistance in asthmatic mice. The airway resistance in asthmatic mice was measured at 24 h after the last spraying. Briefly, after anesthesia with sodium pentobarbital, a 22G indwelling needle was positioned for tracheal intubation and it was connected with a pulmonary function testing instrument. The mice were placed in a closed incubator to maintain body temperature at 37°C and assisted mechanical ventilation was given. Airway resistance (cm $H_2O/L/s$) was measured. The airway resistance for mice inhaling PBS was designated as R (baseline) and the airway resistance for mice inhaling different concentrations of methacholine

Table I. RT-qPCR using gene primers.

Gene	Primer (5'→3')
Bax	Forward: GCGGCGACATGGAGACAG
	Reverse: GTGTGACCCGAACCAGAAG
Bcl2	Forward: GCAGGGTGGTGGCACTGT
	Reverse: ACAATTATCAGCTGGACC
Caspase-3	Forward: CACATGGCAGACGGTGGC
	Reverse: CTGGAGTTCTCACCACTG
GAPDH	Forward: AACATCGATCTCGAGGTC
	Reverse: TTCAACTGCCGCAGGGTT

Bax, Bcl-2-associated X; Bcl2, B-cell lymphoma 2; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

was designed as R (response) at the corresponding concentrations. The highest value of R at each concentration of methacholine was introduced into the following formula. The fold increase of R was used as an evaluation index of airway resistance: Fold increase of R=[R (response)-R (baseline)]/R (baseline).

Hematoxylin and eosin staining. Mouse bronchial alveolar tissue was dehydrated, cleared, embedded with paraffin, sliced, stained with hematoxylin for 5 min, washed with PBS, differentiated with hydrochloric acid-ethanol for 3 sec, stained with eosin for 2 min, and mounted with neutral resin. Finally, pathological change of mouse bronchial alveolar tissue was observed under the optical microscope.

ELISA assay. Serum levels of IL-1β (SEA563Mu, CCC), IL-6 (SEA079Mu, CCC), TNF-α (SEA134Mu, CCC) and IL-10 (SEA056Mu, CCC) were measured by ELISA assay according to kit instructions. A total of 100 μl standard sample and 100 μl diluted sample were added to the reaction plate and incubated at 37°C for 30 min. After washes, 100 μl tested sample was added to each well and then incubated at 37°C for 2 h. After washes, 100 μl horseradish peroxidase-labeled secondary antibody was added to each well. Samples were incubated at 37°C for 30 min. After washes, developer A and developer B, each 50 μl, was added. The tested sample was developed for 15 min in the dark. A stop solution was added at 50 μl per well to terminate the reaction. Optical density at 450 nm was read using an ELISA reader (EXL808; BioTek Instruments, Inc., Winooski, VT, USA). A standard curve was drawn. According to the curve equation, the concentration of the corresponding sample was calculated.

RT-qPCT. Mouse lung tissue was thoroughly ground into a powder and total RNA was extracted using 1 ml TRIzol (15596018; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the reagent operating instructions. First chain DNA was reverse transcribed. RT-qPCR was performed according to kit instructions (204057; Qiagen GmbH, Hilden, Germany). The relative gene expression data was analyzed with the $2^{-\Delta\Delta Cq}$ method. The primers used for RT-qPCR are listed in Table I.
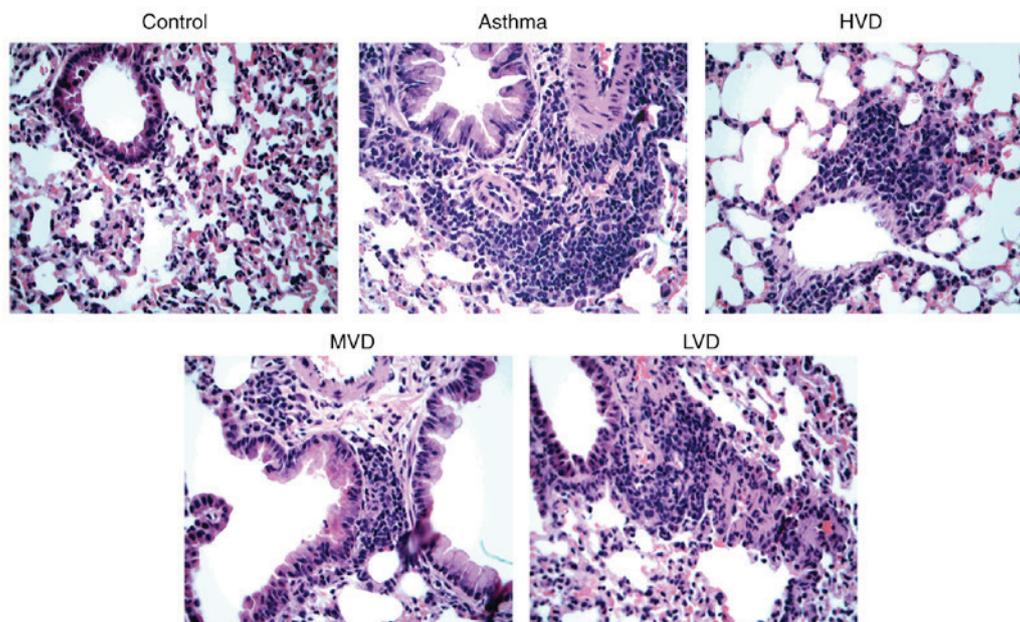
Figure 1. After the establishment of bronchial asthma model, high (H)-, medium (M)- and low (L)-dose of mice were treated with vitamin D (VD), lung tissue observed the pathological changes by hematoxylin and eosin staining in mice (magnification, x40).
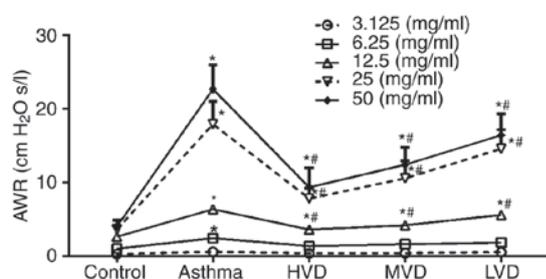

Figure 2. High (H)-, medium (M)- and low (L)-dose of vitamin D (VD) were treated, airway resistance was detected by Lung function instrument. Data collected from control group, asthma group, HVD group, MVD group and LVD group. Compared with control group, *P<0.05; Compared with asthma group, #P<0.05.

deviation. t-test was used for pairwise comparison. One-way analysis of variance was performed for comparisons among groups. P<0.05 was considered to indicate a statistically significant difference.

Results

VD improves asthma symptoms in asthmatic mice. Compared with normal control mice, asthmatic mice had reduced activities, were irritable, exhibited running nose, sneezing and lackluster hair as well as deep and fast breathing, nodding while breathing, slow action, and cyanosis in lips after spraying. LVD group mice showed obvious runny nose and other asthma symptoms. MVD group the asthma symptoms has improved compared with LVD group. After treatment with HVD, the abovementioned symptoms alleviated, and mice breathed better and slow action was improved. Hematoxylin and eosin staining revealed that airway injury was obvious in asthmatic mice, a large number of inflammatory cells were infiltrated, and epithelial cells were poorly arranged (Fig. 1). After treatment with HVD, the number of infiltrated inflammatory cells was decreased. MVD and LVD group mice still have a lot of inflammatory cell infiltration. HVD exhibited better obvious therapeutic effects than MVD and LVD.

VD attenuates airway resistance in asthmatic mice. After spayed with different concentrations of methacholine, the airway resistance of asthmatic mice was obviously increased, but after treatment with HVD, airway resistance of asthmatic mice was significantly decreased (P<0.05, vs. asthma group) (Fig. 2). Airway resistance of asthmatic in LVD group had no significantly decreased, and in MVD group airway resistance of asthmatic mice was decreased, but it was no significantly improved compared with HVD. All these results suggest that VD can obviously attenuate the airway resistance of asthmatic mice.
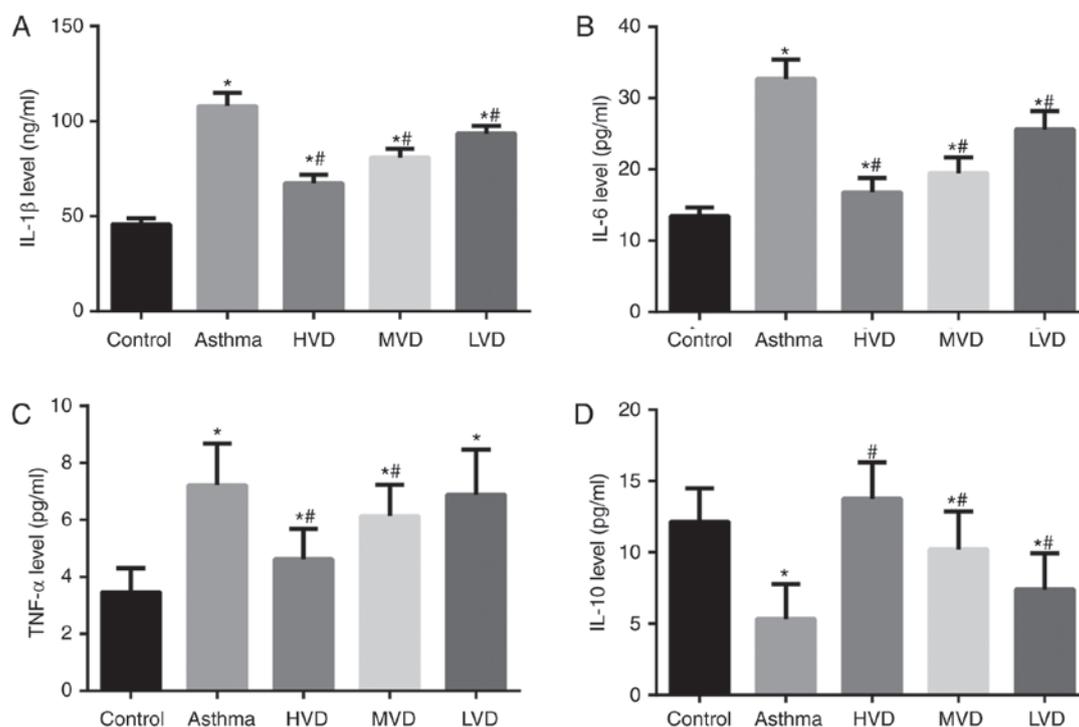
Western blot analysis. Mouse bronchial alveolar tissue was lysed using RIPA lysate containing protease inhibitor on ice and centrifuged. The supernatant was collected. Protein concentration was determined using a BCA protein assay kit. Protein samples were subjected to SDS-PAGE and then transferred onto PVDF membrane. Bax (ab32503; Abcam, Cambridge, UK), Bcl2 (ab59348; Abcam), caspase-3 (ab13847; Abcam), Active-caspase3 (ab47822; Abcam) HMGB1 (ab18256; Abcam), TLR4 (ab13867; Abcam), NF-κB (ab16502; Abcam) and p-NF-κB p65 (ab86299; Abcam) antibodies were added. Protein samples were incubated overnight at 4˚C. PVDF membrane was washed with TBST. After horseradish peroxidase labeled secondary antibody was added, protein samples were incubated at room temperature for 2 h. Protein bands were visualized using an ECL chemiluminescence (Bio-Rad Laboratories, Inc.) detection kit and a gel imaging system. Absorbance analysis was performed using Image Tools.

Statistical analysis. All data were statistically analyzed using SPSS 19.0 software and are expressed as the mean ± standard

Figure 3. ELISA used to detect the serum inflammatory factors. (A) Interleukin (IL)-1β, (B) IL-6, (C) tumor necrosis factor (TNF)-α and (D) IL-10 levels. Data collected from control group, asthma group, high-dose vitamin D (HVD) group, medium-dose vitamin D (MVD) group and low-dose vitamin D (LVD) group. Compared with control group, *P<0.05; Compared with asthma group, #P<0.05.

VD reduces inflammatory response in asthmatic mice. ELISA results showed that serum levels of IL-1β, IL-6, TNF-α were significantly increased (Fig. 3A-C) and IL-10 was significantly decreased in the asthma group than in the normal control group (P<0.05) (Fig. 3D). After VD treatment, serum levels of IL-1β, IL-6, TNF-α were significantly decreased and IL-10 was significantly increased in the HVD group than in the asthma group (P<0.05). These results suggest that HVD can effectively reduce the inflammatory response in asthmatic mice.

VD reduces cell apoptosis in the lung tissue of asthmatic mice. Western blot analysis revealed that Bax, caspase-3 and active-caspase3 expression in the lung tissue was significantly increased, while Bcl-2 expression was significantly decreased, in the asthma group than those in the normal control group (P<0.05; Fig. 4A). Bax and caspase-3 expression in the lung tissue was significantly, while Bcl-2 expression was significantly increased in the HVD, MVD and LVD groups than those in the asthma group (P<0.05; Fig. 4B). These data suggest that VD can reduce cell apoptosis in the lung tissue of asthmatic mice.

VD reduces inflammatory response in asthmatic mice through HMGB1/TLR4/NF-κB signaling pathway. To investigate the mechanism of action of VD, we used TLR4 inhibitor E5564. ELISA results showed that after TLR4 was inhibited, the protective effects of VD disappeared (Fig. 5A). Western blot analysis revealed that HMGB, TLR4, NF-κB and p-NF-κB p65 expression levels in the lung tissue were significantly increased in the asthma group than those in the normal control group (P<0.05; Fig. 5B). HMGB, TLR4,

NF-κB and p-NF-κB p65 expression levels were significantly decreased in the HVD, MVD and LVD groups than those in the asthma group (P<0.05). After the expression of TGF-β1 receptor was inhibited, HMGB, TLR4, NF-κB and p-NF-κB p65 expression levels in the lung tissue were significantly increased compared to those in the VD-treated groups (P<0.05). These data suggest that VD reduces inflammatory response in asthmatic mice through HMGB1/TLR4/NF-κB signaling pathway.

Discussion

In this study, asthma mouse model was established, data showed that HVD greatly attenuated the inflammatory response of bronchial alveolar cells and reduced apoptosis. This effect of VD is achieved through the HMGB1/TLR4/NF-κB signaling pathway.

Bronchial asthma is a common chronic respiratory inflammatory disease that occurs mostly in the autumn and is characterized by repeated and recurrent attacks and refractory treatment. Glucocorticoids are still the most effective drugs against bronchial asthma (21). But they do not really benefit asthma patients and only reduced the symptoms associated with asthma. Moreover, glucocorticoids have many adverse reactions and possess a number of health risks (22). Strong evidence exists that VD participates in the development of allergic and autoimmune diseases. Sadeghi *et al* found that VD can downregulate the expression of TLRs in the monocytes and reduce the production of pro-inflammatory cytokines such as TNF-α (23). Farhangi *et al* reported that VD effectively reduced the oxidative stress and inflammatory response of adipose tissue in a rat model of high fat diet-induced obesity (24). Our results revealed that VD treatment greatly
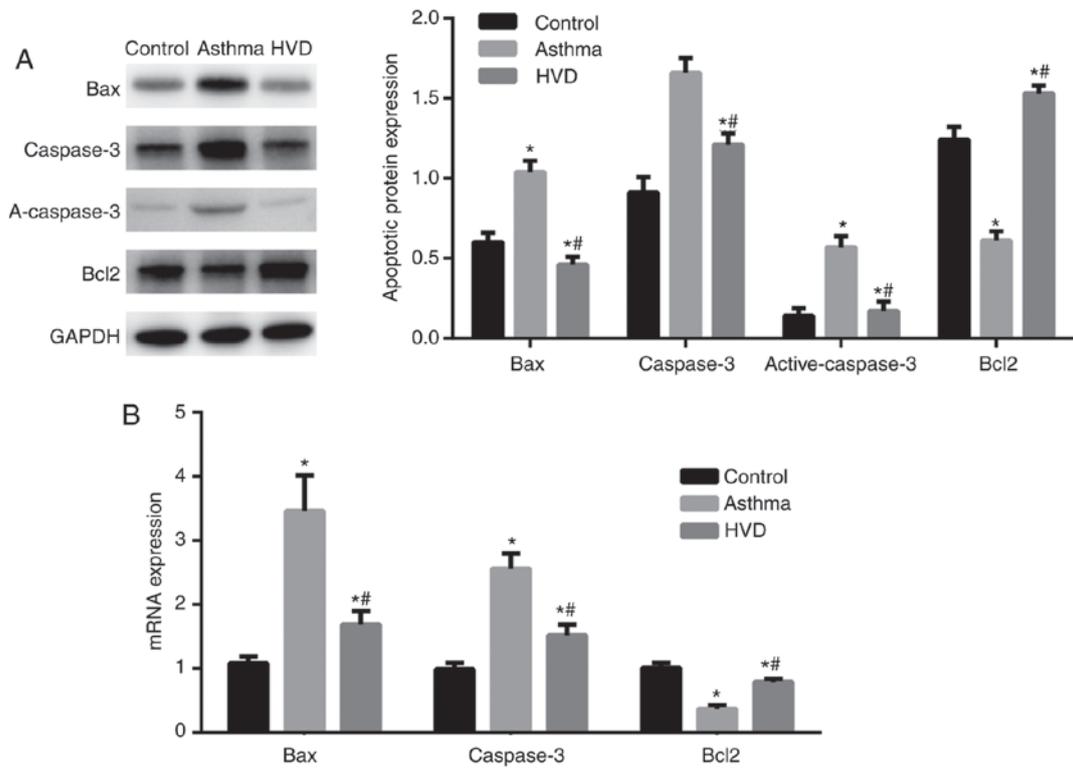
Figure 4. After high, medium and low dose of vitamin D (VD) were treated, the high dose of VD was choose to the treatment dose. Apoptosis factors Bcl-2-associated X (Bax), caspase-3, B-cell lymphoma 2 (Bcl2) expression in mice lung tissue. Data collected from control group, asthma group, HVD group. Compared with control group, *P<0.05; Compared with asthma group, #P<0.05. (A) Bax, caspase-3, Bcl2 expression by western blotting. (B) Bax, caspase-3, Bcl2 mRNA expression by RT-qPCR.
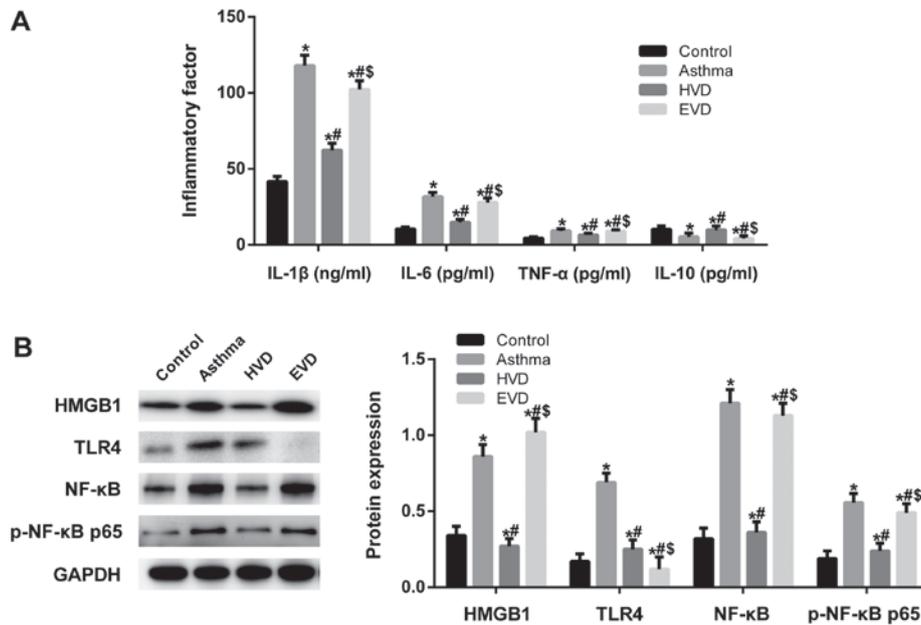

Figure 5. After used TLR4 inhibitor E5564, the inflammatory factors and the high mobility group box 1 protein (HMGB1)/ Toll-like receptor-4 (TLR4)/nuclear factor (NF)-κB pathway-related protein were detected. Data collected from control group, asthma group, HVD group and EVD group. Compared with control group, *P<0.05; Compared with asthma group, #P<0.05; Compared with HVD group, $P<0.05. (A) The level of interleukin (IL)-1β, IL-6, IL-10 and tumor necrosis factor (TNF)-α by ELISA. (B) The expression of HMGB1, TLR4 and NF-κB by western blotting.

reduced the airway inflammatory response and cellular apoptosis in the lung tissue of a mouse model of asthma.

The pathological mechanism of bronchial asthma is very complex and inflammation is the pathological basis. HMGB1

as a nucleoprotein was discovered through purification in the 1970s together with chromosomal DNA.

Intranuclear HMGB1 mainly regulates DNA replication, repair and transcription. When cells are stimulated by

inflammation, nuclear membrane permeability was increased, HMGB1 exudes into the cytoplasm, and then released into the extracellular environment by neutrophils, monocytes/macrophages, and other inflammatory cells, act as an extracellular signal transporter through binding to other receptors on the cell surface to exert pro-inflammatory effects (25).

TLR4 is an important pattern recognition receptor and it is highly expressed on immune cells including macrophages, neutrophils, and dendritic cells (26). TLR4 mainly functions to mediate the maturation of dendritic cells, releases interleukin-12, promotes the synthesis and release of cytokines, contributing to production of inflammatory response (27). TLR4 signaling is a process that activates NF-κB, initiates inflammatory mediators-related gene transcription, and promotes the production of inflammatory factors through myeloid differentiation proteins (MyD88)-dependent and -independent signaling pathway (28). HMGB1 can act as an endogenous ligand, binds to TLR4, activates downstream NF-κB, and induces immune response and inflammatory response (29). Lee *et al* found that HMGB1, TLR2 and TLR4 expression in the lung tissue was obviously increased in a mouse model of asthma-induced by ovalbumin (30). After anti-HMGB1 treatment, airway inflammation, mucous membrane formation, collagen deposition, and TLR2 and TLR4 expression were significantly reduced. Our results showed that after inhibition of TLR4, the anti-inflammatory effects of VD disappeared, presuming that VD may compete with HMGB1 to bind to TLR4, thus inhibiting the pro-inflammatory effects of HMGB1.

Our results showed that VD reduced the inflammatory response and apoptosis in lung tissue in asthmatic mice through HMGB1/TLR4/NF-κB signaling pathway. This provides a theoretical basis for clinical application of targeted drug therapy and development of new drugs against asthma.

Acknowledgements

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