

Original Article

Effect of different 1, 25-(OH)₂D₃ doses on high mobility group box1 and toll-like receptors 4 expression in lung tissue of asthmatic mice

Junying Qiao, Bin Luan, Huiru Gu, Yanli Zhang

Department of Pediatrics, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China

Received January 4, 2015; Accepted February 21, 2015; Epub March 15, 2015; Published March 30, 2015

Abstract: We established a mouse model of asthmatic airway remodeling. To investigate the effects of different doses of 1,2 5-(OH)₂D₃ on airway remodeling, expression of high mobility group box 1 (HMGB1) and Toll-like receptors 4 (TLR4) in asthmatic mice. The female mice (BALB/c) groups consisted of a control group, asthma group and 1,2 5-(OH)₂D₃ low, middle, high dose group. Each group contained 10 mice. An asthmatic mice model was induced by ovalbumin. The control group and asthma group used physiological saline instead. 1,2 5-(OH)₂D₃ low, middle and high dose group was given different doses of 1,2 5-(OH)₂D₃ respectively. Changes in mice airway structure were observed by hematoxylin-eosin (H&E). The expression of HMGB1 and TLR4 in molecular level were monitored by RT-PCR. We used immunohistochemistry to test HMGB1 and TLR4 protein levels. Obvious changes were noted in the airway of OVA-induced asthma mice compared with the control group by HE. These changes were less pronounced in mice receiving the low and middle doses of 1,2 5-(OH)₂D₃, but were more pronounced in mice receiving the highest dose of 1,2 5-(OH)₂D₃. Immunohistochemistry showed that expression of HMGB1 and TLR4 in the asthma group was higher than the control group. And low and middle dose group was decreased compared with asthma group, while higher than the control group; high dose group had an increased expression compared with the asthma group. From RT-PCR we got the same results as immunohistochemistry. In the asthmatic airway remodeling animal model, the appropriate amount of 1,2 5-(OH)₂D₃ reduced airway remodeling in asthmatic mice, and decreased the expression of HMGB1 and TLR4 in the asthmatic mice. However, over dose might play detrimental effect.

Keywords: 1,2 5-(OH)₂D₃, asthma, HMGB1, TLR4, mouse

Introduction

Asthma is a chronic disease characterized by airway hyper-responsiveness, chronic airway inflammation and airway remodeling. In recent years, the incidence of childhood asthma, and particularly fatal cases of this disease, have increased on a yearly basis [1]. And this situation seriously affects the quality of life for many children.

HMGB1 is a widespread and highly conserved nucleoprotein which is mainly released upon stimulation by inflammatory factors in pituitary cells, monocytes, macrophages, and can also be released by dead cells [2]. HMGB1 can stimulate the immune system to produce inflammatory response to certain types of stress. HMGB1 is not only involved in signal transduction, but

also participates in inflammatory responses caused by a variety of cytokines and chemotaxis of pro-inflammatory cells. HMGB1 is an endogenous immune adjuvant. It participated in TLR2/4 mediated diseases causing immune response [3, 4]. Recent studies on signal transduction mechanisms have confirmed that HMGB1 can interact with its receptor (TLR4) by activating NF-κB, and then induced release of downstream inflammatory mediators [5].

The molecule 1,2 5-(OH)₂D₃ is the active form of vitamin D3 [6]. In addition to regulating calcium and phosphorus metabolism, 1,2 5-(OH)₂D₃ also exerts an immunomodulatory effect. It primarily influences the immune status through activating dendritic cells (DCs) and monocytes [7, 8] meanwhile this protein also can affect cell growth and differentiation.

Different 1, 25-(OH)₂D₃ doses effect on HMGB1 and TLR4

Table 1. cDNA primer sequences used for reverse transcriptase-polymerase chain reaction to test the expression of HMGB1 and TLR4 gene in asthmatic mice lung tissues

Gene	Forward primer	Reverse primer	Product length
HMGB1	CGGATGCTTCTGTCAACT	TCAGCTTGGCAGCTTTCT	360 bp
TLR4	GGTGAGAAATGAGCTGGTA	TCTGCTAAGAAGCGGATA	313 bp
GAPDH	CCACTTGAAGGGTGGAGC	TGAAGTCGCAGGAGACAA	530 bp

The role of 1,2 5-(OH)₂D₃ in the pathogenesis of asthma is not understood. Therefore, in our former studies, we had examined its potential role on airway inflammation and remodeling in established mouse model of asthma. But the specific mechanism and dose-response relationship are not clear. In this study, our animal model with ovalbumin-induced asthma were injected with various doses of 1,2 5-(OH)₂D₃ and series changes in the airway remodeling process were observed. We also examined the impact of 1,2 5-(OH)₂D₃ on mRNA expression of HMGB1 and TLR4 proteins. This study will provide the basis for clinical drug treatment of asthma.

Materials and methods

Animals and reagents

Fifty SPF BALB/c female mice (aged six weeks, weight 20±2 g) were purchased from the experimental animal center of Henan Province, and used after 2 weeks of quarantine and acclimatization. The mice were given sterilized tap water and standard rodent chow. All experimental procedures were approved by the experimental animal center of Zhengzhou University experimental animal ethics committee and were performed in compliance with the National Institutes of Health Guidelines for the care. In this study, we used laboratory animals and national laws for animal welfare. License: SCXK (yu): 2010-0002s.

Ovalbumin (OVA) and 1,2 5-(OH)₂D₃ were purchased from Sigma (St. Louis, Mo, USA). Trizol, PCR reagent kits, primary antibodies, and immunohistochemical staining materials were purchased from Gold Biotechnology. Secondary antibodies and chromogenic agents were purchased from Zhong Shan JinQiao Biotechnology in Beijing.

Experimental method

Animal groups and mouse asthma model: A total of 50 BALB/c mice were randomly assigned to 5 groups. We prepared model animals as described previously [9-11]. These groups consisted of control group, asthma group,

1,2 5-(OH)₂D₃ low dose group, 1,2 5-(OH)₂D₃ middle dose group and 1,2 5-(OH)₂D₃ high dose group. The low, middle and high dose groups were treated with 1,2 5-(OH)₂D₃ at doses of 1 µg/kg, 4 µg/kg, and 10 µg/kg, respectively. Upon arrival, and during development of the animal model, the mice were feed for adaptation about 1 week.

On days 1, 8, 15 after arrival, mice in the 1,2 5-(OH)₂D₃ intervention and asthma groups were injected with 0.2 ml of an antigen mixture which containing 50 µg OVA, 0.15 mL 10% aluminum hydroxide and 0.05 ml saline in the abdominal cavity. To make them sensitize, the control group given 0.2 ml saline instead. Starting on day 22, mice in asthma group and 1,2 5-(OH)₂D₃ intervention groups were treated by inhalation of 1% OVA. This treatment was conducted once a day, for a period of 30 minutes, and continued until day 35. Following inhalation of OVA, the mice in low dose group were injected in the abdominal cavity with 0.02 µg 1,2 5-(OH)₂D₃ dissolved in 0.5 µl anhydrous ethanol and 0.02 ml saline. Mice in middle dose group were injected with 0.08 µg of 1,2 5-(OH)₂D₃ in a total volume of 0.08 ml. Mice in high dose group were injected with 0.2 µg of 1,2 5-(OH)₂D₃ in a total volume of 0.2 ml. Mice in asthma group were injected with 0.2 ml saline in the abdominal following OVA inhalation. Mice in control group were treated by inhalation of atomized saline and injection of physiological saline.

Preparation of lung tissue specimens

All groups of mice were anesthetized by ether inhalation within 24 hours of the last treatment with 1,2 5-(OH)₂D₃ or vehicle. Following inhalation of ether, the chest wall was opened and the left lung was removed. Lungs were cryopreserved for later analysis by RT-PCR. The rest of lung tissue samples were rinsed with physiological saline and fixed with 4% formaldehyde solution. The right lung of each mouse was fixed in

Different 1, 25-(OH)₂D₃ doses effect on HMGB1 and TLR4

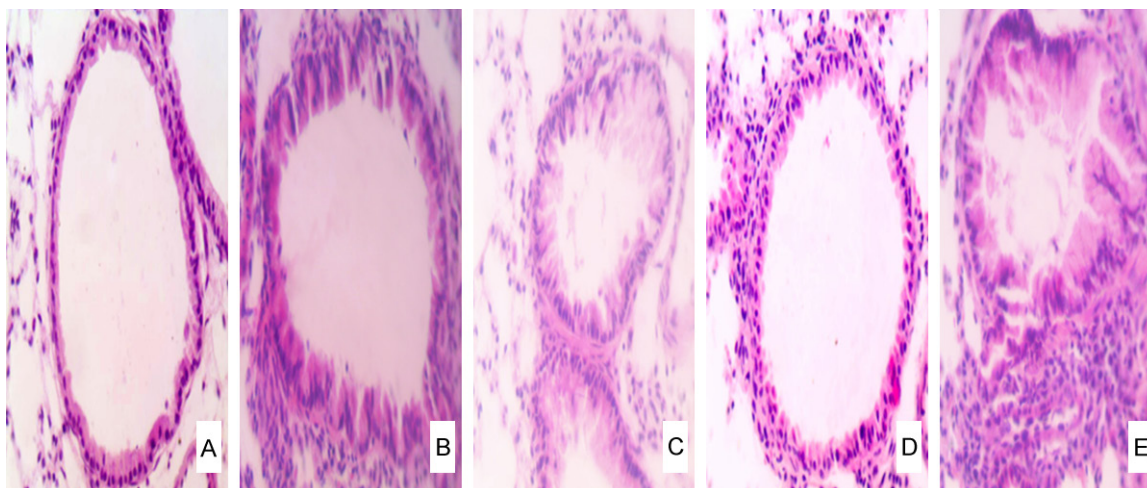


Figure 1. Pathologic changes in the lung tissue of mice (hematoxylin-eosin staining, $\times 400$). A. Control group mice bronchial wall tissue showed smooth structural integrity, with epithelial cells in alignment. Airway wall thickness was moderate and there was only slight inflammatory cell infiltration, and no evidence of non-metaplastic goblet cells. B. Asthma mice bronchial wall was thickened and damaged. Airway lumen showed evidence of stenosis. Epithelial cells showed a disordered arrangement and falling off. Metaplastic goblet cells increased in number. Airway smooth muscle was thickened, and numerous inflammatory cells were seen around the bronchi. C. 1,25-(OH)₂D₃ low dose showed lesser airway wall thickness, lumen stenosis, inflammatory cell infiltration, and fewer metaplastic goblet cells than the asthma group. Airway wall thickness in low dose group was slightly reduced and cells showed a regular arrangement; inflammatory cell infiltration decreased. D. Airway wall thickness in middle dose group significantly reduced, and cells were well arranged; inflammatory cells clearly decreased. E. High dose group airway changed more obviously than asthma mice.

Table 2. Expression of HMGB1/TLR4 mRNA/protein in different groups mice and airway thickness of them (n=10, $\bar{x} \pm s$)

group	n	HMGB1mRNA	TLR4mRNA	HMGB-protein	TLR4-protein	Airway thickness (μm)
high dose group	10	0.404 \pm 0.037	0.684 \pm 0.094	80.972 \pm 3.935	68.902 \pm 1.960	150.4 \pm 7.53
middle dose group	10	0.197 \pm 0.012	0.231 \pm 0.028	62.978 \pm 1.632	49.569 \pm 1.720	84.9 \pm 3.95
low dose group	10	0.251 \pm 0.033	0.336 \pm 0.033	70.253 \pm 2.920	52.869 \pm 1.361	105 \pm 5.73
asthma group	10	0.296 \pm 0.026	0.435 \pm 0.041	79.684 \pm 3.267	65.841 \pm 1.765	131.8 \pm 4.34
control group	10	0.054 \pm 0.005	0.082 \pm 0.011	51.711 \pm 2.913	38.61 \pm 0.955	45.5 \pm 3.53
F value		636.283	432.588	16.141	60.431	614.267
P value		<0.001	<0.001	<0.001	<0.001	<0.001

4% formaldehyde solution for 48 h, dehydrated with alcohol solutions, sliced at 4 μm thickness, and the sections were stained with H&E.

Histological and immunohistochemistry

Tissue sections embedded in paraffin were prepared for H&E staining. Stained sections were observed for morphological changes in the bronchial wall, arrangement of epithelial cells, evidence of stenosis and inflammatory cell infiltration. Drops primary antibodies rabbit anti-mouse HMGB1 (Beijing Biosynthesis Biotechnology, 1:200) and rabbit anti-mouse TLR4 (Beijing Biosynthesis Biotechnology, 1:200), second antibodies, horseradish peroxi-

dase complex solution in turn, Diaminbenzidine (DAB) developing, re-staining, hydrochloric acid differentiation, dehydrated and sealed piece. And then we made immunohistochemical operation. Using computer pathological image analysis system observed the expression of protein positive cells under the high magnification view (10 \times 40). We selected more than five view of high magnification randomly from each section and got protein semi-quantitative result.

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from the left lung tissue of each mouse by Trizol reagent (Gold

Different 1, 25-(OH)₂D₃ doses effect on HMGB1 and TLR4

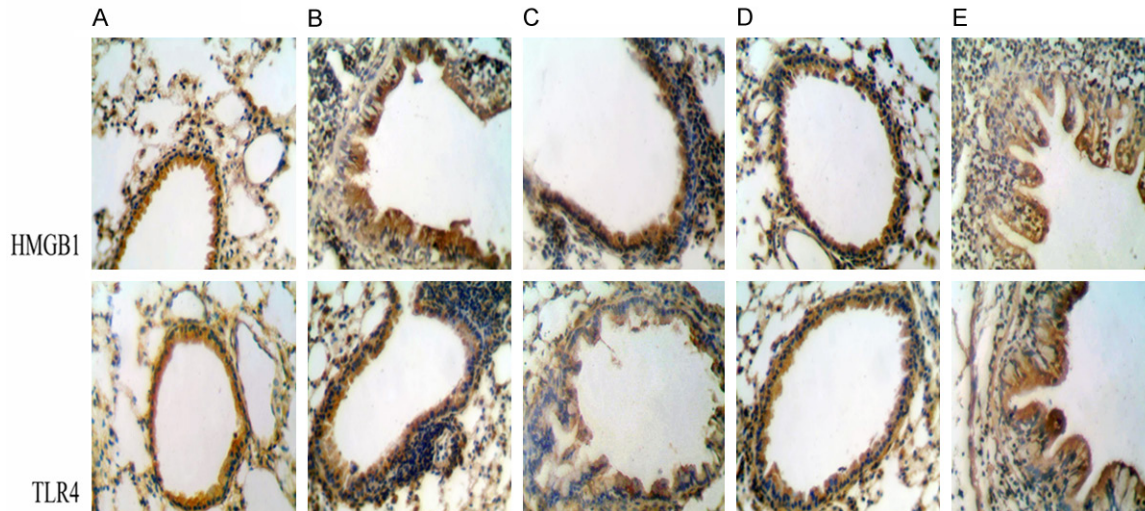


Figure 2. Expression of HMGB1 and TLR4 in mice lung tissue (DAB chromogenic, $\times 400$). A. HMGB1/TLR4 expression in control group was weak. B. HMGB1/TLR4 expression in asthma group was stronger than the control group. C. HMGB1/TLR4 expression in low dose group was slightly decreased compared with asthma group. D. HMGB1/TLR4 expression in middle dose group was clearly decreased compared with asthma group. E. HMGB1/TLR4 expression in high dose group was increased compared with asthma group. The deep tan color showed the expression of HMGB1/TLR4.

Biotechnology). A reverse transcription kit (Gold Biotechnology) was utilized to synthesize single strand cDNA. According to the manufacturer's instructions; the resultant complimentary DNA was used for PCR amplification. Briefly, 1 μ L cDNA was added to the reaction mixture containing 12.5 μ L 2 \times GC-rich PCR Master Mix (Gold Biotechnology), 9.5 μ L ddH₂O and 2 μ L (10 μ M) forward and reverse primers optimized for each gene of interest in preliminary experiments. For all genes, denaturation step at 94 $^{\circ}$ C for 3 min, modification at 94 $^{\circ}$ C for 30 s, annealing at 55 $^{\circ}$ C for 30 s, followed by 72 $^{\circ}$ C for 1 min for a total of 35 cycles. Extension was at 72 $^{\circ}$ C for 10 min. Negative controls run for all PCR reactions included no reverse transcription samples to check for genomic DNA, as well as reactions without the addition of the cDNA templates. Amplification products were separated by electrophoresis on a 1.5% agarose gel and detected by gel electrophoresis imaging systems. The primer sequences used in expression of HMGB1 and TLR4 are shown in **Table 1**.

Statistical analysis

Statistical analyses were conducted using SPSS 17.0 statistical software. Data are expressed as the mean value \pm standard deviation (\pm s). Comparison was done by using single factor analysis. *P* value < 0.05 indicated statistical significance.

Results

Mice lung tissue pathology

We observed the bronchial walls of model mice by optical microscopy (10 \times 40). These structures were smooth and showed alignment of epithelial cells in the control group. The thickness of airway wall was moderate, with little evidence of inflammatory cell infiltration. No metaplastic goblet cells were observed (**Figure 1A**). Bronchial walls showed being thickened and damaged in OVA-induced asthma mice. Airway lumen had stenosis. Epithelial cells were disordered and detached; numbers of metaplastic goblet cells was increased. Airway smooth muscle was thickened, and inflammatory cells infiltrated around the bronchi (**Figure 1B**). 1, 2 5-(OH)₂D₃ low and middle dose groups showed less extent damage in the case of airway wall thickness, lumen stenosis, inflammatory cell infiltration, and fewer metaplastic goblet cells (**Figure 1C** and **1D**). Injection of the high dose 1, 2 5-(OH)₂D₃ produced adverse effects (**Figure 1E**; **Table 2**).

HMGB1/TLR4 immunohistochemical

HMGB1 and TLR4 protein are primarily located in the nucleus and cytoplasm of inflammatory and epithelial cells. Mice in the control group

Different 1, 25-(OH)₂D₃ doses effect on HMGB1 and TLR4

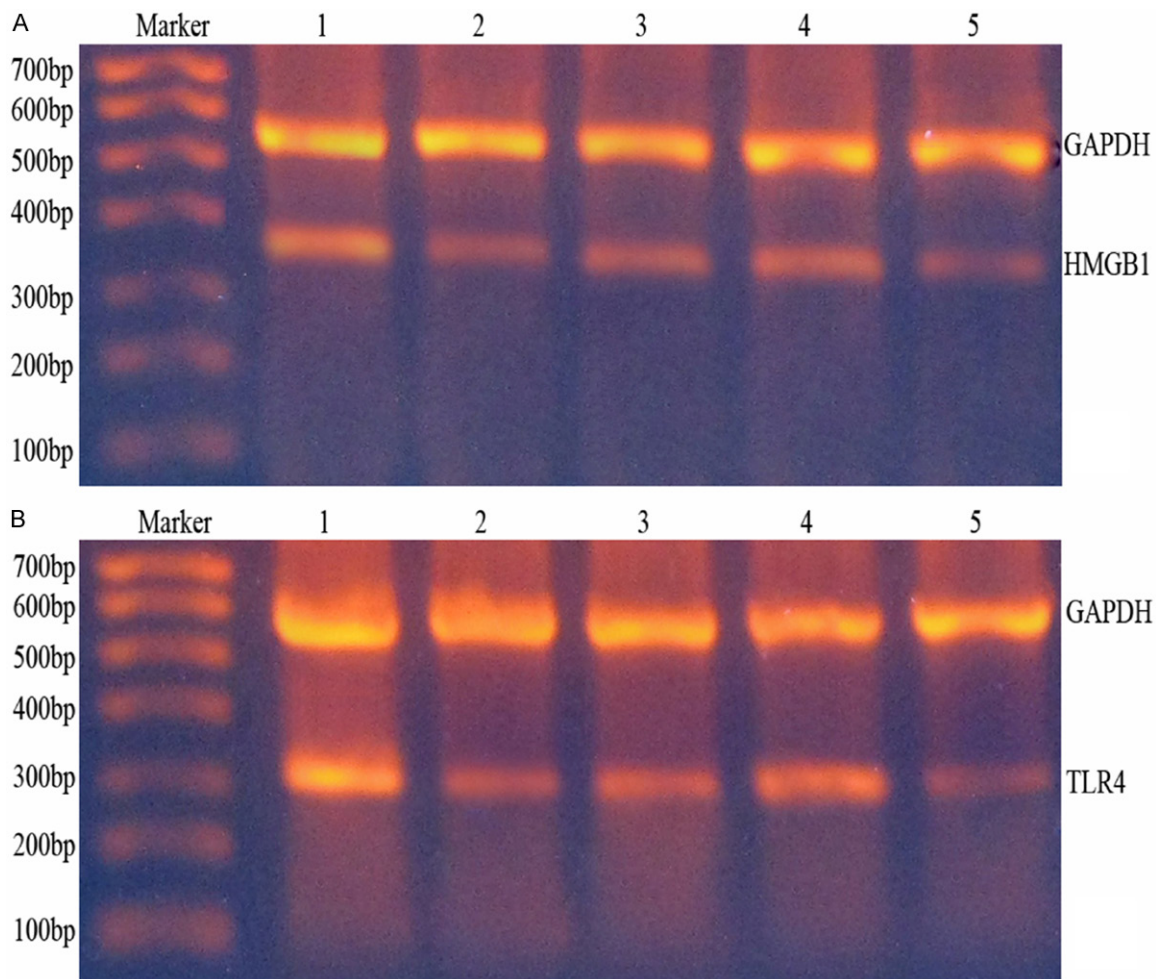


Figure 3. RT-PCR detection of HMGB1/TLR4 mRNA expression in all groups. A. HMGB1. B. TLR4 The expression of HMGB1 mRNA and TLR4 mRNA in the lungs of asthma mice was significantly greater than expression in control mice. These two mRNA in 1,2 5-(OH)₂D₃ low and middle dose groups were significantly lower than the asthma group. Expression of these two mRNA in the 1,2 5-(OH)₂D₃ high dose group were significantly greater than the asthma group. M: DNA markers; lane 1: high dose group; lane 2: middle dose group; lane 3: low dose group; lane 4: asthma group; lane 5: control group.

showed only slight expression of HMGB1 and TLR4 protein (**Figure 2A**), while mice in the OVA-induced asthma group showed strong expression of HMGB1 and TLR4 n (**Figure 2B**), and the difference between these two groups was statistically significant ($P < 0.05$). Immunohistochemically, HMGB1 and TLR4 positive cells in the 1,2 5-(OH)₂D₃ low and middle dose groups were less than the asthma group (**Figure 2C** and **2D**). The expression of HMGB1 and TLR4 protein in the 1,2 5-(OH)₂D₃ low and middle dose groups was significantly lower than expression in the asthma group ($P < 0.05$). The expression of HMGB1 and TLR4 protein in the 1,2 5-(OH)₂D₃ high dose group was significantly

higher than the OVA-induced asthma group (**Figure 2E**, $P < 0.05$).

HMGB1 mRNA and TLR4mRNA expression

RT-PCR detection results showed that expression of HMGB1 mRNA and TLR4 mRNA in the lungs of asthma mice was significantly greater than expression in control mice (**Figure 3A** and **3B** lane 4 and 5; **Table 2**). Expression of HMGB1 mRNA and TLR4 mRNA in 1,2 5-(OH)₂D₃ low and middle dose groups were significantly lower than the asthma group (**Figure 3A** and **3B** lane 2, 3 and 4; **Table 2**). Expression of these two mRNA in the 1,2 5-(OH)₂D₃ high dose group

Different 1, 25-(OH)₂D₃ doses effect on HMGB1 and TLR4

were significantly greater than the asthma group (**Figure 3A** and **3B** lane 1 and 4; **Table 2**).

Discussion

In recent years, a growing number of studies have shown that vitamin D may participate in the body's immune response [12-14]. To allergic diseases and autoimmune diseases vitamin D has regulating effect, such as 1,2 5-(OH)₂D₃. Through binding with intracellular vitamin D receptor 1,2 5-(OH)₂D₃ plays role in biological effects. Its regulation of the immune mechanism in asthma has become the hot spot at present [15, 16].

Studies confirm that 1,2 5-(OH)₂D₃ can down regulate MHC-II type and molecules coordinated stimulus on the surface of the APC; then inhibit its antigen presented function, thereby inhibiting T cell immune response to alleviate autoimmune reaction [17, 18]. Inhibition of IL-4, IFN-γ and IL-5, 1,2 5-(OH)₂D₃ reduced airway inflammation [11]. It reduces airway remodeling through inhibiting activity of MMP-9, NF-κB and fibrinolytic enzyme such as the original activators inhibitor-1. During pregnancy increasing vitamin D intake can obviously reduce the risk of children with early stage asthmatic disease [19, 20]. Animal experiments also showed that during pregnancy and lactation 1,2 5-(OH)₂D₃ intervention can reduce rats airway inflammation and play the role in immune regulation; different doses of 1,2 5-(OH)₂D₃ interact with inflammatory mediators and cytokines had much more differences, the influence on airway inflammation and airway remodeling was not consistent [11].

Current researches on HMGB1 and TLR4 in asthma are less. HMGB1 widely exists in nucleus and cytoplasm of the nucleated cells. The activated immune cells, damage and necrotic tissue release HMGB1, then it stimulate "necrosis induced inflammation". HMGB1 can promote the inflammatory cell activation and stimulate the production of proinflammatory factor and secretion, such as TNFα, IL-1, IL-6, IL-8; induce the dendritic cells mature and lead them to secrete a variety of proinflammatory cytokines; promote expression of DC surface stimulating molecules CD80, CD83, CD86 and MHCII [21]. TLR4 is a kind of pattern recognition receptors; it expresses in airway epithelial cells, endothelial cells, smooth muscle cells, macrophages, skeletal muscle cells, and so on.

Study showed that asthma was chronic airway inflammatory diseases mediated by DC and featured Th2 immune enhancement [22]. The most important biological function of is inducing dendritic cells mature and IL-12 production. TLRs has an important effect on inducing Th0 cell differentiation to type Th1 immune response [23]. So TLR4 closely associated with asthma, it can promote the synthesis and release of cytokines caused inflammation [24, 25]; mediate macrophages producing MMPs, cracking structural protein, participating in asthmatic airway remodeling. In our study, TLR4 and its mRNA had a higher expression in asthma mice (**Figures 2** and **3**). These results were consistent with previous studies above mentioned. HMGB1 mainly produces effects with its receptors TLR2, RAGE and TLR4. This interaction leads to downstream molecules NF-κB activation, prompting release downstream inflammatory factor, inducing the immune activation and immune response [26-28]. In our study, HMGB1 and its mRNA also had a higher expression in model animals (**Figures 2** and **3**). So we speculated that HMGB1-TLR4-NF-κB signaling pathway may play an important role in occurrence and development process in asthma. Foreign study found that 1,2 5-(OH)₂D₃ reduced the expression of monocytes TLRs and the production of inflammatory factor TNFα [29]. Exogenous and endogenous synthesis of 1,2 5-(OH)₂D₃ unlock the function of immune defense to relieve asthma airway inflammation through combining with VDR on the surface of epithelial cells and expressing cell toll-like receptors. But we got little system studies on 1,2 5-(OH)₂D₃ effecting HMGB1 and TLR4.

Our study found that asthma symptoms and the lung tissue pathological changes were similar to our previous study results in animal model. Asthma mice presented damaged bronchial wall thickening and luminal stenosis, epithelial cells arrange disorder, fall off, bronchial inflammatory cells infiltration; 1,2 5-(OH)₂D₃ low and media dose group reduced airway wall thickness, luminal stenosis, inflammatory cells infiltration quantity compared with the asthma group; high dose group had more obvious phenomenon, such as airway wall thickening and inflammatory cells infiltration (**Figure 1**). Therefore a suitable amount of vitamin D supplements may improve asthma mice lung tissue inflammation and airway remodeling, but

Different 1, 25-(OH)₂D₃ doses effect on HMGB1 and TLR4

excessive could aggravate airway inflammation and remodeling. These results were consistent with some domestic research [11]. We used immunohistochemistry and fluorescence quantitative PCR to detect protein and mRNA level of HMGB1 and TLR4 after different doses of 1,2 5-(OH)₂D₃ intervention. The result indicated that the expression of HMGB1 and TLR4 increased in asthma mice airway epithelial cells, mononuclear macrophage, B cells, T cells and DC significantly (**Figure 2; Table 2**). These two proteins in 1,2 5-(OH)₂D₃, low and middle-dose group was lower than those in asthma group (**Figure 2; Table 2**). Importantly, the expression of HMGB1 and TLR4 high in high dose group was obviously higher than the asthma group (**Figure 2; Table 2**). These results illuminated that dose of 1,2 5-(OH)₂D₃ had effect on the expression of HMGB1 and TLR4 in asthma mice. RT-PCR test had the same trends as immunohistochemistry (**Figure 3; Table 2**)

From our study, we deduced that the expression of HMGB1 and TLR4 had relationship with 1, 2 5-(OH)₂D₃ dose. Proper amount of 1, 2 5-(OH)₂D₃ can improve to reduce the expression of HMGB1 and TLR4, meanwhile it could reduce lung tissue inflammation and airway remodeling. But excessive dose of 1,2 5-(OH)₂D₃ increased the expression of HMGB1 and TLR4, meanwhile high dose aggravated airway inflammation and remodeling. In addition, our results showed that the expression of HMGB1 and TLR4 increased significantly in asthma mice, and airway inflammation and airway remodeling had the same direction changes. So we thought HMGB1 and TLR4 involved in airway inflammation and reconstruction process. 1,2 5-(OH)₂D₃ intervention can effectively relieve asthma, not only reduce asthma inflammation cells infiltration and airway wall thickness, but also influence the expression of HMGB1 and TLR4 in lungs. Low or middle dose of 1,2 5-(OH)₂D₃ may suppress the downstream inflammatory cytokines release by blocking HMGB1-TLR4-NF-κB signaling pathways; and then relieve asthma airway inflammation and airway remodeling in mice. But the exact mechanism and mutual relationship is unclear, and the mechanism of high dose 1,2 5-(OH)₂D₃ on action of asthma mice is not clear. These still to be researched at the next step.

Above all, 1,2 5-(OH)₂D₃ can effectively improve the airway inflammation and airway remodel-

ing. Its effect has correlation with dose. The mechanisms may be related to HMGB1-TLR4-NF-κB signaling pathway. 1,2 5-(OH)₂D₃ belongs to the natural biological agents, small side effects, is expected to become new treatment methods for asthma. This study adopted the low, middle and high dose based on the literature at home and abroad recommended vitamin D intake and maximum tolerated dose conversion. But it was lack of prospective clinical study. Practical clinical value of 1,2 5-(OH)₂D₃ needs further study.

Disclosure of conflict of interest

None.

Address correspondence to: Junying Qiao, Department of Pediatrics, The Third Affiliated Hospital of Zhengzhou University, NO. 7 Kangfuqian Street, Zhengzhou 450052, Henan, P. R. China. Tel: +8637166903236; Fax: +8637166903236; E-mail: junyingqiao2015@163.com

References

- [1] Ding G, Ji R, Bao Y. Risk and Protective Factors for the Development of Childhood Asthma. *Paediatr Respir Rev* 2015; 16: 133-139.
- [2] Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol* 2005; 5: 331-342.
- [3] Raucci A, Palumbo R, Bianchi ME. HMGB1: a signal of necrosis. *Autoimmunity* 2007; 40: 285-289.
- [4] Ek M, Popovic K, Harris HE, Naucner CS, Wahren-Herlenius M. Increased extracellular levels of the novel proinflammatory cytokine high mobility group box chromosomal protein 1 in minor salivary glands of patients with Sjogren's syndrome. *Arthritis Rheum* 2006; 54: 2289-2294.
- [5] Perros F, Lambrecht BN, Hammad H. TLR4 signalling in pulmonary stromal cells is critical for inflammation and immunity in the airways. *Respir Res* 2011; 12: 125.
- [6] Tian WM, Yang YG, Shang YX, Cai XX, Chen WW, Zhang H. Role of 1,25-dihydroxyvitamin D3 in the treatment of asthma. *Eur Rev Med Pharmacol Sci* 2014; 18: 1762-1769.
- [7] Barkauskaite V, Ek M, Popovic K, Harris HE, Wahren-Herlenius M, Nyberg F. Translocation of the novel cytokine HMGB1 to the cytoplasm and extracellular space coincides with the peak of clinical activity in experimentally UV-induced lesions of cutaneous lupus erythematosus. *Lupus* 2007; 16: 794-802.

Different 1, 25-(OH)₂D₃ doses effect on HMGB1 and TLR4

- [8] Hansdottir S, Monick MM. Vitamin D effects on lung immunity and respiratory diseases. *Vitam Horm* 2011; 86: 217-237.
- [9] Luan B, Wang YZ, Zhang YL, Gu HR, Li YL, Zhao J. [Effect of 1,25-(OH)₂D₃ on expression of TIM-4 in the lungs of asthmatic mice]. *Zhongguo Dang Dai Er Ke Za Zhi* 2013; 15: 67-70.
- [10] Gu HR, Luan B, Qiao JY, Wang YZ, Li Q. [Effect of 1,25-(OH)₂D₃ on expression of HMGB1 and TLR4 in the lungs of asthmatic mice]. *Zhongguo Dang Dai Er Ke Za Zhi* 2014; 16: 301-305.
- [11] Liu PY, Chen X, Jiang ZQ, Leng L, Wang XQ, Ji GY. [Effect of early vitamin D supplementation on lung inflammatory factors in baby rat with asthma]. *Zhonghua Yu Fang Yi Xue Za Zhi* 2011; 45: 645-649.
- [12] Wu AC, Tantisira K, Li L, Fuhlbrigge AL, Weiss ST, Litonjua A. Effect of vitamin D and inhaled corticosteroid treatment on lung function in children. *Am J Respir Crit Care Med* 2012; 186: 508-513.
- [13] Goleva E, Searing DA, Jackson LP, Richers BN, Leung DY. Steroid requirements and immune associations with vitamin D are stronger in children than adults with asthma. *J Allergy Clin Immunol* 2012; 129: 1243-1251.
- [14] Brehm JM, Celedon JC, Soto-Quiros ME, Avila L, Hunninghake GM, Forno E, Laskey D, Sylvia JS, Hollis BW, Weiss ST, Litonjua AA. Serum vitamin D levels and markers of severity of childhood asthma in Costa Rica. *Am J Respir Crit Care Med* 2009; 179: 765-771.
- [15] Majak P, Olszowiec-Chlebna M, Smejda K, Stelmach I. Vitamin D supplementation in children may prevent asthma exacerbation triggered by acute respiratory infection. *J Allergy Clin Immunol* 2011; 127: 1294-1296.
- [16] Wittke A, Chang A, Froicu M, Harandi OF, Weaver V, August A, Paulson RF, Cantorna MT. Vitamin D receptor expression by the lung micro-environment is required for maximal induction of lung inflammation. *Arch Biochem Biophys* 2007; 460: 306-313.
- [17] Sandhu MS, Casale TB. The role of vitamin D in asthma. *Ann Allergy Asthma Immunol* 2010; 105: 191-199; quiz 200-192, 217.
- [18] Clavreul A, D'Hellencourt CL, Montero-Menei C, Potron G, Couez D. Vitamin D differentially regulates B7.1 and B7.2 expression on human peripheral blood monocytes. *Immunology* 1998; 95: 272-277.
- [19] Miyake Y, Sasaki S, Tanaka K, Hirota Y. Dairy food, calcium and vitamin D intake in pregnancy, and wheeze and eczema in infants. *Eur Respir J* 2010; 35: 1228-1234.
- [20] Camargo CA Jr, Rifas-Shiman SL, Litonjua AA, Rich-Edwards JW, Weiss ST, Gold DR, Kleinman K, Gillman MW. Maternal intake of vitamin D during pregnancy and risk of recurrent wheeze in children at 3 y of age. *Am J Clin Nutr* 2007; 85: 788-795.
- [21] Dumitriu IE, Bianchi ME, Bacci M, Manfredi AA, Rovere-Querini P. The secretion of HMGB1 is required for the migration of maturing dendritic cells. *J Leukoc Biol* 2007; 81: 84-91.
- [22] Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 2010; 11: 373-384.
- [23] MacLeod H, Wetzler LM. T cell activation by TLRs: a role for TLRs in the adaptive immune response. *Sci STKE* 2007; 2007: pe48.
- [24] Crespo-Lessmann A, Juarez-Rubio C, Plaza-Moral V. [Role of toll-like receptors in respiratory diseases]. *Arch Bronconeumol* 2010; 46: 135-142.
- [25] Lafferty EI, Qureshi ST, Schnare M. The role of toll-like receptors in acute and chronic lung inflammation. *J Inflamm (Lond)* 2010; 7: 57.
- [26] Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, Strassheim D, Sohn JW, Yamada S, Maruyama I, Banerjee A, Ishizaka A, Abraham E. High mobility group box 1 protein interacts with multiple Toll-like receptors. *Am J Physiol Cell Physiol* 2006; 290: C917-924.
- [27] Yu M, Wang H, Ding A, Golenbock DT, Latz E, Czura CJ, Fenton MJ, Tracey KJ, Yang H. HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock* 2006; 26: 174-179.
- [28] vanBeijnum JR, Buurman WA, Griffioen AW. Convergence and amplification of toll-like receptor (TLR) and receptor for advanced glycation end products (RAGE) signaling pathways via high mobility group B1 (HMGB1). *Angiogenesis* 2008; 11: 91-99.
- [29] Sadeghi K, Wessner B, Laggner U, Ploder M, Tamandl D, Friedl J, Zugel U, Steinmeyer A, Pollak A, Roth E, Boltz-Nitulescu G, Spittler A. Vitamin D3 down-regulates monocyte TLR expression and triggers hyporesponsiveness to pathogen-associated molecular patterns. *Eur J Immunol* 2006; 36: 361-370.