Original Article

Effect of different 1, 25-(OH)\(_2\)D\(_3\) doses on high mobility group box1 and toll-like receptors 4 expression in lung tissue of asthmatic mice

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Abstract: We established a mouse model of asthmatic airway remodeling. To investigate the effects of different doses of 1,2 5-(OH)\(_2\)D\(_3\) 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)\(_2\)D\(_3\) 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)\(_2\)D\(_3\) low, middle and high dose group was given different doses of 1,2 5-(OH)\(_2\)D\(_3\) respectively. Changes in mice airway structure were observed by hematoxylin-eosin (H&E). The expression of HMGB1 and TLR4 in molecular lever 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)\(_2\)D\(_3\), but were more pronounced in mice receiving the highest dose of 1,2 5-(OH)\(_2\)D\(_3\). 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)\(_2\)D\(_3\) 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)\(_2\)D\(_3\), 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)\(_2\)D\(_3\) is the active form of vitamin D3 [6]. In addition to regulating calcium and phosphorus metabolism, 1,2 5-(OH)\(_2\)D\(_3\) 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.
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The role of 1,25-(OH)$_2$D$_3$ 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 1,25-(OH)$_2$D$_3$ and series changes in the airway remodeling process were observed. We also examined the impact of 1,25-(OH)$_2$D$_3$ 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 1,25-(OH)$_2$D$_3$ 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.

Preparation of lung tissue specimens

All groups of mice were anesthetized by ether inhalation within 24 hours of the last treatment with 1,25-(OH)$_2$D$_3$ 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
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Figure 1. Pathologic changes in the lung tissue of mice (hematoxylin-eosin staining, ×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. 1.2 5-(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} ±s)

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

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 peroxidase complex solution in turn, Diaminobenzidine (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 (10×40). 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...
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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 μLcDNA was added to the reaction mixture containing 12.5 μL 2×GC-rich PCR Master Mix (Gold Biotechnology), 9.5 μL ddH\textsubscript{2}O and 2 μL (10 μM) forward and reverse primers optimized for each gene of interest in preliminary experiments. For all genes, denaturation step at 94°C for 3 min, modification at 94°C for 30 s, annealing at 55°C for 30 s, followed by 72°C for 1 min for a total of 35 cycles. Extension was at 72°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 ± standard deviation (±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 (10×40). 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 thicken and damaged in OVA-induced asthma mice. Airway lumen had stenosis. Epithelial cells were disorder and detached; numbers of metaplastic goblet cells was increased. Airway smooth muscle was thickened, and inflammatory cells infiltrated around the bronchi (Figure 1B). 1, 25-(OH)\textsubscript{2}D\textsubscript{3} 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, 25-(OH)\textsubscript{2}D\textsubscript{3} 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
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showed only sight expression of HMGB1 and TLR4 protein (Figure 2A), while mice in the OVA-induced asthma group showed strong expression of HMGB1 and TLR4 (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)$_2$D$_3$ 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)$_2$D$_3$ 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)$_2$D$_3$ high dose group was significantly higher than the OVA-induced asthma group (Figure 2E, P<0.05).

**HMGB1 mRNA and TLR4 mRNA 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 the 1,2 5-(OH)$_2$D$_3$ 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)$_2$D$_3$ high dose group
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 coordinat-ed 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
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)_2D_3 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)_2D_3, low and medium-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)_2D_3 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)_2D_3 dose. Proper amount of 1, 2 5-(OH)_2D_3 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)_2D_3 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)_2D_3 intervention can effectively relieve asthma, not only reduce asthma inflammation and remodeling, but also influence the expression of HMGB1 and TLR4 in lungs. Low or middle dose of 1,2 5-(OH)_2D_3 may suppress the downstream inflammatory cytokines release by blocking HMGB1-TLR4-NF-κB signaling pathway; 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)_2D_3 on action of asthma mice is not clear. These still to be researched at the next step.

Above all, 1,2 5-(OH)_2D_3 can effectively improve the airway inflammation and airway remodeling. Its effect has correlation with dose. The mechanisms may be related to HMGB1-TLR4-NF-κB signaling pathway. 1,2 5-(OH)_2D_3 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)_2D_3 needs further study.

Disclosure of conflict of interest

None.

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