The protective role of vitamin D3 in a murine model of asthma via the suppression of TGF-β/Smad signaling and activation of the Nrf2/HO-1 pathway

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Abstract. Asthma is a common worldwide health burden, the prevalence of which is increasing. Recently, the biologically active form of vitamin D3, 1,25-dihydroxyvitamin D3, has been reported to have a protective role in murine asthma; however, the molecular mechanisms by which vitamin D3 attenuates asthma-associated airway injury remain elusive. In the present study, BALB/c mice were sensitized to ovalbumin (OVA) and were administered 100 ng 1,25-dihydroxyvitamin D3 (intraperitoneal injection) 30 min prior to each airway challenge. The inflammatory responses were measured by ELISA, airway damage was analyzed by hematoxylin and eosin staining, airway remodeling was analyzed by Masson staining and periodic acid-Schiff staining, markers of oxidative stress were measured by commercial kits, and the expression levels of α-smooth muscle actin (α-SMA) and the activity of the NF-E2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) and the transforming growth factor-β (TGF-β)/Smad signaling pathways were measured by immunohistochemistry and western blotting. The results demonstrated that OVA-induced airway inflammation and immunoglobulin E overexpression were significantly reduced by vitamin D3 treatment. In addition, treatment with vitamin D3 decreased α-SMA expression, collagen deposition and goblet cell hyperplasia, and inhibited TGF-β/Smad signaling in the asthmatic airway. The upregulated levels of malondialdehyde and the reduced activities of superoxide dismutase and glutathione in OVA-challenged mice were also markedly restored following vitamin D3 treatment. Furthermore, treatment with vitamin D3 enhanced activation of the Nrf2/HO-1 pathway in the airways of asthmatic mice.

In conclusion, these findings suggest that vitamin D3 may protect airways from asthmatic damage via the suppression of TGF-β/Smad signaling and activation of the Nrf2/HO-1 pathway; however, these protective effects were shown to be accompanied by hypercalcemia.

Introduction

Bronchial asthma, commonly abbreviated to asthma, is one of the most common respiratory diseases, which is characterized by infiltration of the airways with mast cells, eosinophils and activated T helper lymphocytes (1). Asthma affects >300 million individuals worldwide, and by 2025 the prevalence is predicted to increase by 100 million (2,3). At present, inhaled corticosteroids are the standard therapy for persistent asthma; however, the antioxidant effects of corticosteroids are not satisfactory. Furthermore, this treatment is hindered when steroid dependence or steroid resistance occurs (4). Therefore, the development of novel and efficient therapeutic strategies is of great significance in the control of asthma.

Vitamin D3 is a fat-soluble vitamin that can be produced in the skin, liver and kidney, or can be absorbed from food. Vitamin D3 is widely known to act as a regulator of calcium homeostasis, and has an important role in bone formation and resorption (5). Furthermore, it has been reported that vitamin D3 exhibits several pharmaceutical properties, including anti-inflammatory (6), anticancer (7-9), antimicrobial (10) and immunoregulatory activities (11,12). Recently, Rigo et al (13) reported that vitamin D3 is associated with modulation of the innate immune defense of airway epithelium, and vitamin D3 deficiency has been shown to result in the exaggerated features of airway disease in ovalbumin (OVA)-induced asthmatic mice (14). In addition, Lai et al (15) reported that the biologically active metabolite of vitamin D3, 1,25-dihydroxyvitamin D3, could protect OVA-sensitized mice from airway remodeling. Another study also demonstrated that 1,25-dihydroxyvitamin D3 may attenuate airway inflammation in asthmatic rats (16). These findings suggest that vitamin D3 may contribute to the control of asthma; however, the underlying mechanism remains to be fully elucidated.

In the present study, mice were sensitized to OVA, and were then treated with the biologically active form of
vitamin D3, 1,25-dihydroxyvitamin D3, in order to examine the protective effects of vitamin D3 on murine asthma. The results demonstrated that treatment with vitamin D3 reduced airway inflammation in asthmatic mice via the inhibition of inflammatory cell infiltration. Airway remodeling was also alleviated in the vitamin D3-treated group, as characterized by decreased α-smooth muscle actin (α-SMA) and hydroxyproline levels, collagen deposition and goblet cell hyperplasia. Furthermore, OVA-induced activation of transforming growth factor-β (TGF-β)/Smad was inhibited following vitamin D3 treatment. Vitamin D3 treatment also alleviated oxidative stress via activation of the NF-E2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway. These data preliminarily revealed the mechanisms by which vitamin D3 protects against airway damage in OVA-induced asthma.

Materials and methods

OVA-induced murine model of asthma. A total of 36 BALB/c female mice (age, 8-10 weeks; weight, 22±2 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in a controlled environment with 40-50% humidity at 22±1°C under a 12-h light/dark cycle. All mice had access to food and water ad libitum. All animal experiments in the present study were conducted in compliance with the Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Harbin Medical University (Harbin, China). Asthma was induced using OVA as previously described (15). Briefly, mice were sensitized with 50 μg OVA (Sigma-Aldrich, St. Louis, MO, USA), which was emulsified with 5 mg aluminum hydroxide (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), via intraperitoneal injection on days 0, 7 and 14. From day 15, the mice received an airway challenge with 1% OVA for 30 min, three times a week for 9 weeks.

Animal groups and vitamin D3 treatment. Following a week of adjustable feeding, the mice were randomly divided into three groups (n=12/group): i) Control group, mice received the same volume of phosphate-buffered saline (PBS) by intraperitoneal injection, and did not undergo airway challenge; ii) OVA group, mice were injected intraperitoneally with 300 μl PBS containing 0.9% ethanol 30 min prior to each airway challenge; iii) vitamin D3 group, mice were intraperitoneally injected with 100 ng 1,25-dihydroxyvitamin D3 (Sigma-Aldrich) dissolved in ethanol, and further diluted to 0.9% by 300 μl PBS, 30 min prior to each airway challenge. A total of 24 h after the last challenge, mice were anesthetized with 3.0 ml/kg 10% chloral hydrate (Sinopharm Chemical Reagent Co., Ltd.) by intraperitoneal injection and lungs were lavaged with 1.5 ml saline three times; the bronchoalveolar lavage fluid (BALF) was collected for further analysis. Subsequently, the mice were sacrificed by 5.0 ml/kg 10% chloral hydrate, and the lungs were harvested for further experimentation.

Analysis of BALF. BALF samples were centrifuged at 156 x g for 10 min at 4°C, the supernatant was stored at -80°C, and the cell pellet was resuspended in 1 ml PBS. The number of total cells was counted using a hemocytometer, and the remaining cells were centrifuged onto slides and stained with Wright-Giemsa in order to count the number of eosinophils.

Measurement of immunoglobulin (Ig)E, TGF-β1 and calcium. The levels of total IgE and TGF-β1 in the BALF samples were measured using ELISA kit for Immunoglobulin E and ELISA kit for TGF-β1 (USCN Life Science, Inc., Wuhan, China) according to the manufacturer’s protocol. The optical density (450 nm) was measured using a microplate reader (ELx800; Biotek Instruments, Inc., Winooski, VT, USA), and the concentrations of IgE and TGF-β1 was calculated from a standard curve. The serum levels of calcium were measured using a calcium colorimetric assay kit (Sigma-Aldrich) according to the manufacturer’s protocol.

Lung histological and immunohistochemical analyses. For histological analysis, lung tissues were fixed with 10% buffered neutral formalin, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin (H&E) using 0.2% hematoxylin for 5 min and 0.35% eosin for 3 min; periodic-acid Schiff (PAS), using 0.5% Schiff staining solution for 15 min; or Masson, using 1% hematoxylin for 6 min and ponceaude-acid fuchsin staining solution (0.7% ponceau, 0.3% acid fuchsin) for 1 min. For immunohistochemical analysis, the sections were initially incubated with anti-α-SMA mouse monoclonal antibody (1:200; Wuhan Boster Biological Technology, Ltd., Wuhan, China; cat. no. BM0002), anti-Nrf2 mouse polyclonal antibody (1:50; Wuhan Boster Biological Technology, Ltd.; cat. no. BA3790) or anti-HO-1 mouse polyclonal antibody (1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. sc-390991) at 4°C overnight, and were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:50; cat. no. A0286) and goat anti-rabbit (1:50; cat. no. A0277) secondary antibodies (Beyotime Institute of Biotechnology, Haimen, China) for 30 min at 37°C. Positive staining was detected with HRP-conjugated streptavidin, visualized with 3,3’-diaminobenzidine and counterstained with hematoxylin. Finally, the sections were mounted, cover-slipped, and examined under a light microscope (DP73; Olympus Corporation, Tokyo, Japan). The extent of lung infiltration as detected by H&E staining, and goblet cell hyperplasia as detected by PAS staining were graded using a semi-quantitative scoring system (17). The collagen area of similar size bronchioles and the α-SMA positive area were analyzed using Image-Pro Plus 4.5 (Media Cybernetics, Inc., Rockville, MD, USA) and are presented as positive area/total area of bronchioles (18). The integrated optical density (IOD) of Nrf2 and HO-1 was also measured by Image-Pro Plus 4.5, and the mean density was calculated as IOD / area.

Western blot analysis. Total proteins were extracted using radioimmunoprecipitation assay lysis solution (Beyotime Institute of Biotechnology). Nuclear and cytoplasmic protein extracts were prepared using a Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology). Protein concentration was determined using the BCA Protein assay kit (Beyotime Institute of Biotechnology) and 40 μg protein were separated by 5, 10 or 14% sodium dodecyl-sulfate polyacrylamide gel electrophoresis, and were transferred onto polyvinylidene fluoride membranes (EMD Millipore, Bedford, MA, USA).
After blocking with 5% fat-free milk, the membranes were immunoblotted with primary antibodies as follows: Mouse α-SMA (1:400), rabbit HO-1 (1:200; Santa Cruz Biotechnology, Inc.; cat. no. sc-10789), mouse Nrf2 (1:400), rabbit TGF-β1 (1:200; Santa Cruz Biotechnology, Inc.; cat. no. sc-146), rabbit Smad2 (1:500; BIOSS, Beijing, China; cat. no. bs-0718R), rabbit phosphorylated (p)-Smad2 (1:500; BIOSS; cat. no. bs-5618R), rabbit Smad3 (1:500; BIOSS; cat. no. bs-3484R), rabbit p-Smad3 (1:500; BIOSS; cat. no. bs-5459R), mouse β-actin (1:1,000; Santa Cruz Biotechnology, Inc.; cat. no. sc-7778) and rabbit histone H3 (1:1,000; BIOSS; cat. no. bs-17422R) at 4°C overnight. Subsequently, the membranes were incubated with HRP-conjugated goat anti-rabbit (1:5,000; cat. no. A0216) or goat anti-mouse (1:5,000; cat. no. A0208) IgG (Beyotime Institute of Biotechnology) at 37°C for 45 min. The specific bands were visualized using an enhanced chemiluminescence (ECL) kit (7Sea Biotech, Shanghai, China) according to the manufacturer's protocol. The semi-quantitative analysis was performed using the Gel-Pro Analyzer version 3.0 (Media Cybernetics, Inc.; cat. no. sc-10789), mouse Nrf2 (1:400), β-actin or histone H3 was employed as an internal control and the relative protein levels were quantified by the comparison to the control group.

**Measurement of hydroxyproline, HO-1, malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) in lungs.** Lung tissues from each group were homogenized in pre-cooled PBS, and were frozen in liquid nitrogen and thawed three times. Following centrifugation at 10,000 x g for 10 min at 4°C, the supernatant was collected, in order to determine the concentrations of hydroxyproline, MDA and GSH, and the activity of SOD. HO-1 activity was evaluated by determining the amount of bilirubin, as previously described (19). All measurements were performed using commercial kits (Hydroxyproline assay kit, Malondialdehyde assay kit, Glutathione Peroxidase assay kit, Total Superoxide Dismutase assay kit, and Total Bilirubin kit) obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China) according to the manufacturer's manual.

**Electrophoretic mobility shift assay (EMSA).** To detect the DNA-binding activity of Nrf2, EMSA was performed using a Chemiluminescent EMSA kit (Viagene Biotech, Inc., Tampa, FL, USA). Briefly, 25 µg nuclear protein from each group were combined with biotin-labeled double-stranded oligonucleotide probes containing the specific recognition sequence of Nrf2 (sense strand, 5'-GGGGAACCTGTTGCTAGTCACTGGA-3'; anti-sense strand: 5'-TCCAGTGACCTAGACAGGTTCCCC-3'). Following separation on a 6.5% polyacrylamide gel, the complex was transferred onto a nylon membrane and was exposed to ultraviolet light for 10 min. Subsequently, the specific bands were detected using HRP-conjugated streptavidin and an ECL kit (7Sea Biotech).

**Statistical analysis.** Data are expressed as mean ± standard deviation. Differences between the groups were compared by one-way analysis of variance followed by Bonferroni post-hoc test, or nonparametric Kruskal-Wallis test followed by Dunn's multiple comparisons test using SPSS version 16.0 software for Windows (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Vitamin D3 inhibits OVA-induced airway inflammation.** To investigate the effects of vitamin D3 on asthma, mice were sensitized and challenged with OVA, and the inflammatory cells in the BALF were counted (Fig. 1A and B). As compared with the control group, the number of total cells and eosinophils in the OVA group was significantly increased (P<0.05), whereas these were markedly decreased by vitamin D3 treatment (P<0.05). The concentration of IgE, which is another feature of asthma, was also determined; as shown in Fig. 1C and D, the elevated levels of IgE in the BALF and serum of the OVA group were significantly reduced following vitamin D3 treatment. H&E staining further confirmed that inflammatory cells intensively infiltrated the peribronchial space of OVA-challenged mice, which was accompanied by thickening of the lung mesenchyme; however, these alterations were markedly alleviated by treatment with vitamin D3 (Fig. 1E). These results clearly indicate that vitamin D3 may effectively inhibit OVA-induced airway inflammation.

**Vitamin D3 attenuates OVA-induced airway remodeling.** To determine the effects of vitamin D3 on OVA-induced airway remodeling, the expression of α-SMA, a marker of smooth muscle cells, was detected by immunohistochemistry. As shown in Fig. 2A, OVA challenge resulted in a marked increase in the expression of α-SMA, which was decreased by vitamin D3 treatment. This result was further confirmed by western blotting (Fig. 2B and C). In addition, the amount of hydroxyproline was determined, in order to assess collagen content (Fig. 2D); OVA challenge significantly elevated hydroxyproline levels (P<0.05), which were reversed following treatment with vitamin D3 (P<0.05). Consistent with these results, Masson's staining indicated that OVA-induced collagen deposition in the lungs was markedly reduced by vitamin D3 treatment (Fig. 2E). Furthermore, PAS staining revealed that OVA-induced goblet cell hyperplasia in the airway epithelium was inhibited by vitamin D3 (Fig. 2F). Collectively, these results suggest that vitamin D3 attenuates OVA-induced airway remodeling.

**Vitamin D3 suppresses OVA-induced activity of the TGF-β/Smad pathway.** Previous studies have suggested that TGF-β1 is an important mediator of airway disease (20,21); therefore, the activities of TGF-β1 and members of its signal transducing system, Smad2 and Smad3, were determined, in order to investigate whether TGF-β1/Smad pathway inhibition is involved in the protective effects of vitamin D3 on OVA-induced airway remodeling. As shown in Fig. 3A-C, OVA challenge resulted in a significant increase in the expression of TGF-β1 in BALF and lung tissues; however, it was markedly reversed by vitamin D3 treatment. Furthermore, the levels of p-Smad2 and p-Smad3 in the lungs were markedly upregulated by OVA challenge and were further dampened by vitamin D3 treatment (Fig. 3D-F). Overall, these data indicate that the TGF-β1/Smad pathway is associated with vitamin D3-mediated protection of OVA-induced airway remodeling.

**Vitamin D3 inhibits OVA-induced oxidative stress via activation of the Nrf2/HO-1 signaling pathway.** To evaluate the
Figure 1. Effects of vitamin D3 on inflammatory cell recruitment and airway damage in ovalbumin (OVA)-induced asthma. (A) Total cell counts in bronchoalveolar lavage fluid (BALF). (B) Number of eosinophils in BALF. IgE levels in (C) BALF and (D) serum, as determined by enzyme-linked immunosorbent assay. (E) Representative hematoxylin and eosin staining of lung tissues, and the average infiltration score of each group. Scale bar=100 µm. Data are presented as the mean ± standard deviation of at least three repeat experiments, n=6. **P<0.01 vs. the control group; # P<0.05, ## P<0.01 vs. the OVA group.

Figure 2. Effects of vitamin D3 on ovalbumin (OVA)-induced airway remodeling. (A) Representative α-smooth muscle actin (α-SMA) expression determined by immunohistochemistry; the α-SMA-positive area was calculated as α-SMA-positive area/total bronchiole area in similar size bronchioles from each group. Scale bar=100 µm. (B) Representative protein bands of α-SMA as determined by western blotting. (C) Relative protein levels of α-SMA in lung tissue. (D) Hydroxyproline levels in lung tissues. (E) Representative Masson staining of lung tissues; the collagen-positive area was calculated as the collagen-positive area/total bronchiole area in similar size bronchioles from each group. Scale bar=100 µm. (F) Representative periodic acid-Schiff (PAS) staining of lung tissues and the PAS score of each group. Scale bar=100 µm. Data are presented as the mean ± standard deviation of three repeat experiments, n=6. **P<0.01 vs. the control group; # P<0.05, ## P<0.01 vs. the OVA group.
effects of vitamin D3 on OVA-induced oxidative stress, MDA and GSH content, and SOD activity were detected in the lungs using commercial kits. As shown in Fig. 4, MDA content was significantly increased in the OVA group (P<0.05), which was accompanied by a marked decrease in SOD activity and GSH content (P<0.05); however, these effects were significantly attenuated by vitamin D3 treatment.

The Nrf2/HO-1 signaling pathway has a pivotal role in antioxidant reactions (22,23); therefore, to investigate whether it is associated with the protective effects of vitamin D3 on OVA-induced oxidative damage to the lungs, the expression and activity of Nrf2 and HO-1 were examined. As expected, immunohistochemistry demonstrated that the expression of Nrf2 was significantly increased in the bronchial epithelial cells of the OVA group (Fig. 5A; P<0.01), and was further upregulated in the vitamin D3 group (P<0.01). In addition, nuclear Nrf2 levels were significantly higher in the OVA group compared with the control group (Fig. 5B; P<0.01), and were further upregulated following vitamin D3 treatment (P<0.01). Similar results were observed by EMSA (Fig. 5C); Nrf2 binding activity was elevated in the OVA group, and it was intensified by vitamin D3 treatment. In accordance with Nrf2, HO-1 levels were also markedly upregulated in airway epithelial cells from the OVA group, the levels of which were further enhanced by vitamin D3 treatment (Fig. 5D and E). Furthermore, OVA challenge resulted in a marked increase in HO-1 activity, which was markedly enhanced by vitamin D3 treatment (Fig. 5F). These data indicate that vitamin D3 protects airways from OVA-induced oxidative injury, at least partially via activation of the Nrf2/HO-1 signaling pathway.

Vitamin D3 protects asthmatic airways, accompanied by hypercalcemia. A previous study demonstrated that high
doses of vitamin D3 may result in a surfeit of calcium in the blood (24); therefore, the serum levels of calcium were measured to evaluate the effects of vitamin D3 on blood calcium levels. The results demonstrated that calcium concentration in the vitamin D3 group was significantly increased (Fig. 6; P<0.01). These results suggest that the asthma protective effects of vitamin D3 are accompanied by hypercalcemia.

Discussion

The results of the present study demonstrated that vitamin D3 effectively suppressed airway inflammation in a murine model of asthma. In addition, the overexpression of α-SMA,
hydroxyproline and collagen deposition in the airways of asthmatic mice was reversed by vitamin D3 treatment. Vitamin D3 treatment also suppressed the OVA-induced activation of TGF-β/Smad. Furthermore, vitamin D3 suppressed airway oxidative stress in asthmatic mice, alongside increased activation of the Nrf2/HO-1 pathway. Taken together, these results suggested that vitamin D3 may protect airway injury from allergic asthma via the suppression of TGF-β/Smad signaling and activation of the Nrf2/HO-1 pathway; therefore, vitamin D3 may be considered effective in the control of asthma.

Epidemiological studies have demonstrated that vitamin D deficiency is a cause of increased asthma prevalence (25); however, Wittke et al (26) reported that vitamin D receptor deficiency blocked asthma-induced airway injury and suggested that the vitamin D endocrine system is implicated in the pathogenesis of asthma. Subsequently, other studies have demonstrated that the active metabolite of vitamin D has a protective effect on asthmatic rodents (15,16). Airway inflammation, eosinophil infiltration and IgE production are prominent features of asthma (27). In the present study, asthma was induced by OVA challenge, as evidenced by an increased number of eosinophils, upregulated IgE levels in BALF and increased inflammatory cell infiltration in the airways. Conversely, these alterations were markedly reversed following vitamin D3 treatment. These results further confirmed that vitamin D3 may protect airways from OVA-induced inflammatory damage.

Airway remodeling is the most common pathophysiological feature of asthma, which is characterized by subepithelial fibrosis and airway collagen deposition (28). Furthermore, goblet cell hyperplasia that leads to excessive mucin secretion is also a typical pathological alteration common in asthma (29). The present study detected a marked increase in α-SMA, airway collagen deposition and goblet cell hyperplasia in OVA-challenged mice; however, these effects were all significantly reduced by vitamin D3 treatment. These results are consistent with a previous study, which suggested that vitamin D3 could protect airways from asthmatic remodeling (15). The TGF-β/Smad pathway has been demonstrated to be a main mediator in asthmatic lung remodeling, due to its effect on epithelial alterations, subepithelial fibrosis, goblet cell hyperplasia and smooth muscle proliferation (20,30). A clinical study reported that TGF-β/Smad signaling was significantly activated in bronchial biopsy specimens from asthmatic subjects, and was closely related with basement membrane thickness (31). The results of the present study demonstrated that an OVA challenge resulted in the obvious activation of TGF-β/Smad signaling, which was markedly suppressed by vitamin D3 treatment. Therefore, vitamin D3 may attenuate OVA-induced airway remodeling via the inhibition of TGF-β/Smad signaling.

Mounting evidence has demonstrated that oxidative stress is involved in increased lipid peroxidation, aggravated airway reactivity and overexpression of chemoattractants in asthma (32,33). In the present study, OVA challenge significantly increased the levels of MDA, which is the end product of lipid peroxidation, and decreased the activity of antioxidant enzymes, SOD and GSH. However, these changes were markedly attenuated by vitamin D3 treatment. These results suggested that vitamin D3 may limit airway damage by reducing oxidative stress; these results were in accordance with those of previous studies, which suggested that vitamin D3 is a potent antioxidant (34,35). In addition, the present study detected enhanced Nrf2/HO-1 pathway activity in the airways of the vitamin D3 treatment group. Nrf2 is a cellular sensor of oxidative stress, which is associated with transcriptional activation of antioxidant-response element genes (23). Nrf2 deficiency has been reported to result in an exaggerated airway inflammation in asthmatic mice (36). HO-1 is considered an important endogenous antioxidant and cytoprotective enzyme, which may be upregulated by Nrf2 (37,38). Therefore, the airway protective effects of vitamin D3 in OVA-induced asthma may partly depend on activation of the Nrf2/HO-1 pathway. However, the asthmatic protective effects of vitamin D3 are accompanied by a non-lethal, but significant increase in serum calcium levels.

In conclusion, the present study reported a protective role of vitamin D3 in allergic asthma. In addition, inhibition of TGF-β/Smad signaling and activation of the Nrf2/HO-1 pathway are potential mechanisms by which vitamin D3 protects against asthma-induced airway damage. However, treatment with vitamin D3 may result in hypercalcemia.

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