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Inhalation with Vitamin D3 Metabolites—A Novel Strategy to Restore Vitamin D3 Deficiencies in Lung Tissue

Michał Chojnacki, Jakub Anisiewicz, Ilona Leśniowska and Marta Kinga Lemieszek *

Department of Medical Biology, Institute of Rural Health, Jaczewskiego 2, 20-090 Lublin, Poland; chojnacki.michal@imw.lublin.pl (M.C.)

* Correspondence: lemieszek.marta@imw.lublin.pl; Tel.: +48-81-7184-513

Abstract: Vitamin D3 deficiency has been recognized as a pandemic with serious health consequences including chronic respiratory diseases. Unfortunately, improvement in this situation by using vitamin D supplementation has failed. The direct delivery of 1,25$(OH)_2$-vitamin D3 and its precursor into the respiratory tract, by nebulization, seemed to be a better option, as verified in the presented study. To induce vitamin D deficiency, mice received a diet with 0.05 IU/g cholecalciferol, while control animals were given feed with 0.5 IU/g cholecalciferol. Vitamin-D-deficient mice were exposed to different doses of calcidiol or calcitriol via nebulization for at least 7 days. At the end of the experiment, whole-body plethysmography was conducted. Pulmonary and serum levels of calcitriol were examined using ELISA. The calcitriol concentrations in mice on standard vs. deficient diet were 30.31/18.20 pg/mg (lungs) and 132.24/98.61 pg/mL (serum), respectively. Restoration of the physiological level of calcitriol in vitamin-D-deficient mice required 1-week exposure to 100 pg/g of calcidiol or 5 pg/g of calcitriol. The inhalations did not cause any side changes in murine respiratory function. The presented study revealed the usefulness and safety of chronic inhalation with a bioactive form of vitamin D3 or its precursor for the restoration of physiological calcitriol levels in animals with vitamin D deficiencies.

Keywords: vitamin D; calcitriol; calcidiol; murine model; nebulization; respiratory system

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1. Introduction

Vitamin D3, also known as cholecalciferol, next to vitamin D2 (ergocalciferol; characteristic for mushrooms and plants) is the major form of vitamin D. Vitamin D is synthesized in the skin from 7-dehydrocholesterol in response to UVB radiation (290–315 nm) and/or is absorbed in the diet in the form of vitamin D2 or vitamin D3. Both the ergocalciferol and cholecalciferol forms undergo the same two-step activation to 25(OH)-vitamin D3 (calcidiol), with the participation of 25-hydroxylases CYP2R1 and CYP27A1 (liver enzymes containing cytochrome P450), and further to biologically active 1,25$(OH)_2$-vitamin D3 (calcitriol), with the participation of 1-hydroxylase CYP27B1—an enzyme found in the kidneys as well as extrarenal tissues [1].

Although there are two independent ways of obtaining vitamin D3 via the human body (endogenous synthesis and diet), its deficiency, defined as a serum concentration of 25(OH)-vitamin D below 50 nmol/L or 20 ng/mL [2], was recognized as a pandemic with serious health consequences including chronic respiratory diseases [3–9]. As revealed in scientific studies over the last two decades, vitamin D3 deficiency significantly elevated the risk of development and severity of asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), lung cancer, idiopathic pulmonary fibrosis (IPF), and allergic inflammation as well as several respiratory infections including COVID-19 or tuberculosis [3–5].

At first sight, the easiest way to obtain the proper vitamin D3 content seems to be sun exposure, but the growing awareness of society about the pro-carcinogenic and pro-aging effects of UVB radiation on the skin [7,10,11], as well as a multitude of factors disrupting

the process of endogenous synthesis of vitamin D3 (e.g., altitude, latitude, season, time of day, skin pigmentation, age, use of sunscreen, and solar filters) [2,4,6,7,11–13], resulted in most strategies for neutralizing vitamin D3 deficiencies being based on vitamin D supplementation through the digestive route, including the prevention and treatment the pulmonary diseases [2–4,7,13]. Nevertheless, revision of the most important clinical studies focused on vitamin D3 role in several respiratory disorders, provided ambiguous results on the effectiveness of therapy based on supplementation with vitamin D metabolites, and revealed that achieving and maintaining the physiological level of calcitriol in pulmonary compartments requires more than just increasing this compound intake with food, supplements, or drugs. The main reason for the failure of this therapeutic strategy was the fact that the investigated patients suffered from other comorbidities as well as metabolic disorders disrupting the calcitriol synthesis [3,4]. Furthermore, vitamin D supplementation is also burdened with the risk of vitamin D overdose, leading to hypercalcemia, hypercalciuria, or hyperphosphatemia, and in the case of their non-treatment to kidney stones, calcification of blood vessels, and organ parenchyma [7]. The increased risk of side effects after oral or parenteral administration of calcitriol or its direct precursor (calcidiol) is easy to understand when we realize that calcidiol to calcitriol transformation can occur not only in kidneys but also in many other sites [1,14], either locally produced or directly delivered $1,25(OH)_2$-vitamin D3 after binding with vitamin D receptor (VDR), the presence of which has been proven in almost every nucleated cell in our body to regulate the expression over 200 different genes [1,14,15]. Thus, direct delivery of calcidiol and/or calcitriol to the respiratory system in order to prevent or treat pulmonary diseases associated with vitamin D3 deficiencies seems to be an obvious option to restore the physiological level of this hormone, as well as revealing its beneficial pro-health effect without unnecessary and uncontrolled activation of vitamin-D-target genes in other body compartments. Direct targeting treatment and reduced systemic side effects are important but are not the only reasons for vitamin D3 nebulization being considered in the treatment of the above-indicated disorders. Among the advantages of inhalation, the following should be mentioned: (1) Direct delivery to the effector site allows to the use of lower doses of drug to obtain the therapeutic effects, compared to other drug delivery systems; (2) The inhaled drug could be absorbed quickly into the bloodstream through the highly-vascularized lung tissue, which can lead to faster therapeutic effects compared to other routes of administration; (3) Due to the almost 100% bioavailability and limited impact of the biotransformation processes, it is easier to determine the therapeutic dose and select it for the specific needs of a patient; (4) Inhalation is a convenient and non-invasive method of drug delivery, particularly for patients with chronic respiratory conditions who need regular medication; (5) It could be used simultaneously with oxygen therapy, which is very often required by patients with chronic respiratory conditions; (6) Inhalation is often more acceptable to patients than injections or oral administration, leading to better compliance with prescribed treatments.

Despite the many reasons for using inhalation to administer vitamin D3 metabolites in lung diseases, the occurrence and development of which have been associated with disturbances in the level of this hormone, no researcher has attempted to restore the physiological concentration of calcitriol in lung tissue via nebulization with this compound and/or its precursor. The presented study is the first attempt to explore this topic. The aim of the study was to determine the influence of calcidiol and calcitriol nebulization on the $1,25(OH)_2$-vitamin D3 level in lung tissue compartments in mice with vitamin D3 deficiencies induced by dietary cholecalciferol restriction.

2. Materials and Methods

2.1. Reagents

Unless otherwise indicated, the chemicals used in the study were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MI, USA).

2.2. Design of Study

Three-month-old male C57BL/6 mice were purchased from Mossakowski Medical Research Centre of the Polish Academy of Sciences in Warsaw, Poland. The mice were kept in colony cages with access to food and tap water ad libitum, under standardized housing conditions (natural light–dark cycle and temperature of 22–24 °C). The animals were given the diet with standard cholecalciferol (0.5 IU/g) or vitamin-D3-deficient diet with a reduced level of cholecalciferol level (0.05 IU/g); both types of diets were purchased from Altromin (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany). 25(OH)-vitamin D3 (100, 250, 500, 750, and 1000 pg/g) or 1,25$(OH)_2$-vitamin D3 (1, 5, 10, 25, and 50 pg/g) dissolved in phosphate-buffered saline were administered daily for 7, 14, or 28 days to mice via nebulization. Inhalations were conducted using the Buxco Inhalation Tower (Data Sciences International, St. Paul, MN, USA) under the following conditions: airflow 2.5 L/min; pressure −0.5 cm H_2O; room temperature; and average nebulization rate 295 µL/min. Mice pulmonary function was examined using a Non-invasive Airway Mechanics Plethysmograph Chamber (Data Sciences International, St. Paul, MN, USA) under the following conditions: airflow 0.6 L/min; pressure 0 cm H_2O; and room temperature. The frequency of breathing (F), tidal volume (TV), minute volume (MV), time of inspiratory (Ti), time of expiratory (Te), peak inspiratory flow (PIF), peak expiratory flow (PEF), and mid-tidal expiratory flow (EF50) were determined using DSI FinePointe Software v 2.3.1.21, while inspiratory flow rate (IFR), expiratory flow rate (EFR), duty cycles of inspiratory, and expiratory were calculated based on the above-mentioned respiratory parameters.

Both untreated (control) and treated mice were sacrificed via cervical dislocation with spinal cord rupture and blood and lung samples were collected for further examination. The research group description was presented in Table 1.

Table 1. Description of research groups.

Research Group (Number of Animals)		Vitamin D3 Content in the Diet	25(OH)-Vitamin D3		1,25$(OH)_2$-Vitamin D3	
			Concentration (Compound Dose per Mouse Weight)	Time of Exposure	Concentration (Compound Dose per Mouse Weight)	Time of Exposure
Group 1 (n = 6)		0.5 IU/g	-	-	-	-
Group 2 (n = 6)		0.05 IU/g	-	-	-	-
Group 3 (n = 15)	3A (n = 3)	0.05 IU/g	100 pg/g	30 min/day for 7 days	-	-
	3B (n = 3)	0.05 IU/g	250 pg/g	30 min/day for 7 days	-	-
	3C (n = 3)	0.05 IU/g	500 pg/g	30 min/day for 7 days	-	-
	3D (n = 3)	0.05 IU/g	750 pg/g	30 min/day for 7 days	-	-
	3E (n = 3)	0.05 IU/g	1000 pg/g	30 min/day for 7 days	-	-
Group 4 (n = 15)	4A (n = 3)	0.05 IU/g	-	-	1 pg/g	30 min/day for 7 days
	4B (n = 3)	0.05 IU/g	-	-	5 pg/g	30 min/day for 7 days
	4C (n = 3)	0.05 IU/g	-	-	10 pg/g	30 min/day for 7 days
	4D (n = 3)	0.05 IU/g	-	-	25 pg/g	30 min/day for 7 days
	4E (n = 3)	0.05 IU/g	-	-	50 pg/g	30 min/day for 7 daysNEW:

Table 1. *Cont.*

Research Group (Number of Animals)		Vitamin D3 Content in the Diet	25(OH)-Vitamin D3		$1,25(OH)_2$-Vitamin D3	
			Concentration (Compound Dose per Mouse Weight)	Time of Exposure	Concentration (Compound Dose per Mouse Weight)	Time of Exposure
Group 5 (n = 12)	5A (n = 6)	0.05 IU/g	100 pg/g	30 min/day for 14 days	-	-
	5B (n = 6)	0.05 IU/g	100 pg/g	30 min/day for 28 days	-	-
Group 6 (n = 12)	6A (n = 6)	0.05 IU/g	-	-	5 pg/g	30 min/day for 14 days
	6B (n = 6)	0.05 IU/g	-	-	5 pg/g	30 min/day for 28 days

2.3. Measurement of Calcitriol Concentration in Lung Tissue Homogenates—ELISA Method

Lung samples were placed in Lysing Matrix M tubes (MP Biomedicals, Solon, OH, USA) containing 5 mM EDTA solution in PBS (0.5 mL/tube). Tissues were then homogenized mechanically using a FastPrep-24 5G homogenizer (MP Biomedicals) under the following conditions: 6 m/s, 40 s, and 20 °C. The homogenates were further incubated on ice for 20 min and centrifugated (10,000× g, 5 min, 4 °C). The obtained supernatants were next passed through a 70 µm nylon mesh into new tubes and stored at −180 °C for later use. Determination of calcitriol concentration in lung tissue homogenates was preceded by its extraction using the Extraction Kit for $1,25(OH)_2$ Vitamin D ELISA (BioVendor R&D, Brno, Czech Republic). Calcitriol concentration was assessed using the $1,25(OH)_2$ vitamin D Total ELISA (BioVendor R&D) in strict accordance with the manufacturer instructions. Because of the significant differences in the weight of the lungs used in the assays, obtained results were normalized to the total protein content in lung homogenates determined using the standard BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA).

2.4. Measurement of Calcitriol Concentration in Serum–ELISA Method

After clotting, the blood samples were centrifugated (25,000× g, 10 min, and 20 °C) and the supernatants were collected. Because of the low amount of serums, all 3 samples collected within subgroups belonging to Groups 3 and 4 were pooled together, while in Groups 1, 2, 5, and 6, sera from 2 animals were pooled. The calcitriol concentration in serum samples was determined using the Mouse 1,25-dihydroxyvitamin D3(DVD/DHVD3) ELISA, according to the manufacturer's instructions (Shanghai Coon Koon Biotech Co., Ltd., Shanghai, China).

2.5. Statistical Analysis

The data were presented as the mean value and standard error of the mean (SEM) or standard deviation (SD). Statistical analysis was performed using a one-way ANOVA with a Tukey post hoc test; column statistics were used for comparisons. Significance was accepted at $p < 0.05$.

3. Results

As presented in Figure 1 and Table S1, the diets of the mice changed the calcitriol concentration in their lungs. Mice fed the diet with standard vitamin D3 level (0.5 IU/g) revealed a significantly higher calcitriol concentration (30.31 pg/mg) than mice on the diet with a 10-fold reduced vitamin D3 level (18.20 pg/mg). The calcitriol amount in murine lung tissue was also affected by inhalation with vitamin D3 metabolites. Mice exposure to calcidiol in the whole range of tested doses restored the physiological pulmonary level of calcitriol (level recorded in untreated mice on the diet with standard vitamin D3 content). Furthermore, calcitriol content in lung tissues of mice nebulized with calcidiol at

concentrations of 500, 750, and 1000 pg/g significantly exceeded the indicated physiological concentration (30.31 pg/mg) as follows: 37.73 pg/mg, 38.75 pg/mg, 37.90 pg/mg, respectively. Murine nebulization with exogenous calcitriol at the concentration of 1 pg/g was not able to restore the physiological level of the tested compound; on the contrary, this desired effect was observed in mice exposed to 1,25(OH)$_2$-vitamin D3 at a concentration of 5 pg/g. An additional increase in 1,25(OH)$_2$-vitamin D3 doses led to a further increase in endogenous calcitriol amount to the following levels: 33.16 pg/mg (10 pg/g), 33.52 pg/mg (25 pg/g), and 34.00 pg/mg (50 pg/g).

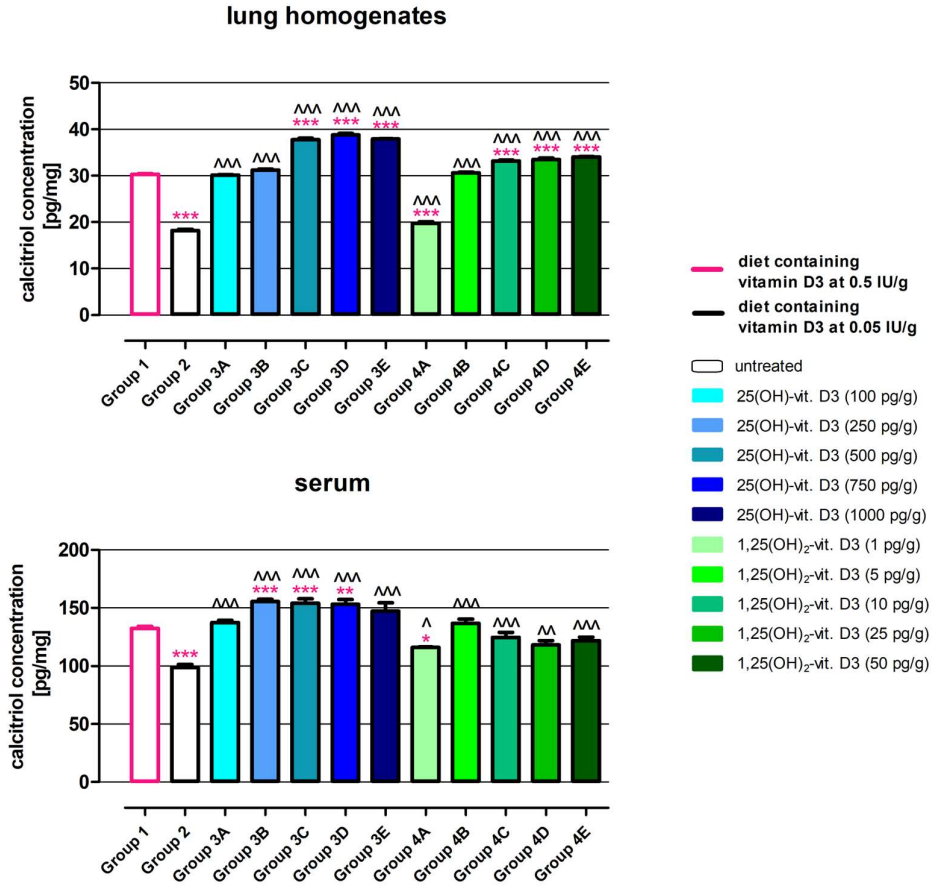

Figure 1. Alterations in pulmonary and serum levels of calcitriol in response to inhalation with vitamin D3 metabolites. The mice were exposed daily to 25(OH)-vitamin D3 (calcidiol) at the following concentrations: 100, 250, 500, 750, and 1000 pg/g or 1,25(OH)$_2$-vitamin D3 (calcitriol) at the following concentrations: 1, 5, 10, 25, and 50 pg/g. Inhalations were performed for 7 consecutive days. As the control, untreated mice from Group 1 (main control) and Group 2 were used. Animals from Group 1 were given the diet with a standard cholecalciferol level (0.5 IU/g), while the mice from other research groups were given a diet with a reduced cholecalciferol level (0.05 IU/g). Calcitriol concentration was determined using the ELISA method in both lung homogenates and serum samples collected from all animals. Data are presented as a mean of calcitriol concentration ± SEM of at least 4 measurements. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. main control (Group 1); ^ $p < 0.05$, ^^ $p < 0.01$, and ^^^ $p < 0.001$ vs. control (Group 2). Analyzed using a one-way ANOVA test and Tukey post hoc test.

As presented in Figure 1 and Table S1, the serum concentration of calcitriol recorded in mice on the diet with the standard amount of vitamin D3 was 132.24 pg/mL, while in mice on the diet with a 10-times reduced vitamin D3 level the serum content of calcidiol was significantly lower at 98.61 pg/mL. Murine exposure to exogenous vitamin D3 metabolites altered the serum level of calcitriol. Calcidiol at the concentrations of 100 pg/g and 1000 pg/g increased the calcitriol concentration to 137.29 pg/mL and 147.37 pg/mL,

eliminating the negative impact of a vitamin D3 deficient diet on the examined parameter. The most significant increase in the serum level of calcitriol (recorded concentration 155.39 pg/mL) was noted in response to calcidiol at the concentration of 250 pg/g, while the additional increase in calcidiol doses did not result in a further increase in the concentration of the bioactive form of vitamin D3 in the serum. In the case of calcitriol nebulization, the most significant changes were observed in response to 1,25$(OH)_2$-vitamin D3 at a dose of 5 pg/g, where the calcitriol serum concentration amounted to 136.75 pg/mL, reaching the physiological level. It needs to be highlighted that a further increase in calcitriol doses did not result in a further increase in serum concentrations of this metabolite.

Examination of calcitriol levels in murine lung tissue and serum indicated that one-week exposure to 25(OH)-vitamin D3 at a concentration of 100 pg/g or 1,25$(OH)_2$-vitamin D3 at a concentration of 5 pg/g was able to restore the physiological level of calcitriol in vitamin-D3-deficient mice.

In order to confirm the stability of the observed beneficial changes, the time of murine exposure to the analyzed metabolites of vitamin D3 was extended to 2 and 4 weeks. As presented in Figure 2 and Table S1, murine chronic exposures to investigated compounds in the above-mentioned doses effectively restored the endogenous level of calcitriol in lung tissue samples collected from mice with vitamin D deficiency. The calcitriol level recorded in mice on the diet with 0.05 IU/g cholecalciferol, exposed for 14 and 28 days to 100 pg/g 25(OH)-vitamin D3, elevated from 18.20 pg/mg to 30.29 and 31.83 pg/mg, while in response to 5 pg/g 1,25$(OH)_2$-vitamin D3 they reached the following values: 31.08 and 32.09 pg/mg, respectively. Moreover, serum samples examination (Figure 2 and Table S1) revealed that calcidiol at the concentration of 100 pg/g administered via nebulization for 14 or 28 days to vitamin D3-deficient mice, increased the calcitriol concentrations to 129.18 and 132.12 pg/mL, respectively. Similarly, chronic exposure of mice to exogenous calcitriol at the concentration of 5 pg/g also restored the physiological serum level of this compound; recorded 1,25$(OH)_2$-vitamin D3 concentrations after 14 and 28 days of treatment were 124.03 and 126.82 pg/mL.

A pulmonary function test was conducted as the last step of the study. As presented in Table 2, none of the investigated respiratory parameters depended on the amount of vitamin D in the diet of the mice. Similarly, chronic exposure of the mice to vitamin D3 metabolites did not impact on TV, Ti, Te, frequency of breathing, inspiratory and expiratory flow rates, or duty cycles of inspiration and expiration. On the contrary, mice on the diet with reduced cholecalciferol, exposed for 14 days to calcidiol at the concentration of 100 pg/g, demonstrated a significant increase in MV to the level 109.27 mL/min (Group 5A) from the value of 82.94 mL/min recorded in untreated mice on the diet with standard vitamin D content (Group 1). Disorders in pulmonary function were also observed after comparison data collected from Group 1 and animals that inhaled calcitriol at the concentration of 5 pg/g for 14 days (Group 6A). In the mentioned case, an increase was observed in PIF, PEF, and EF50 from 4.726 mL/s, 3.900 mL/s, and 3.536 mL/s to 6.377 mL/s, 5.307 mL/s, and 4.621 mL/s, respectively. It needs to be highlighted that extending the exposure time of the mice to the tested compounds did not increase the reported differences but, on the contrary, restored the balance disturbed by the 2-week inhalation. Moreover, it is also worth stressing that comparing results from treated and untreated mice fed the same diet did not show any significant changes in the investigated respiratory parameters.

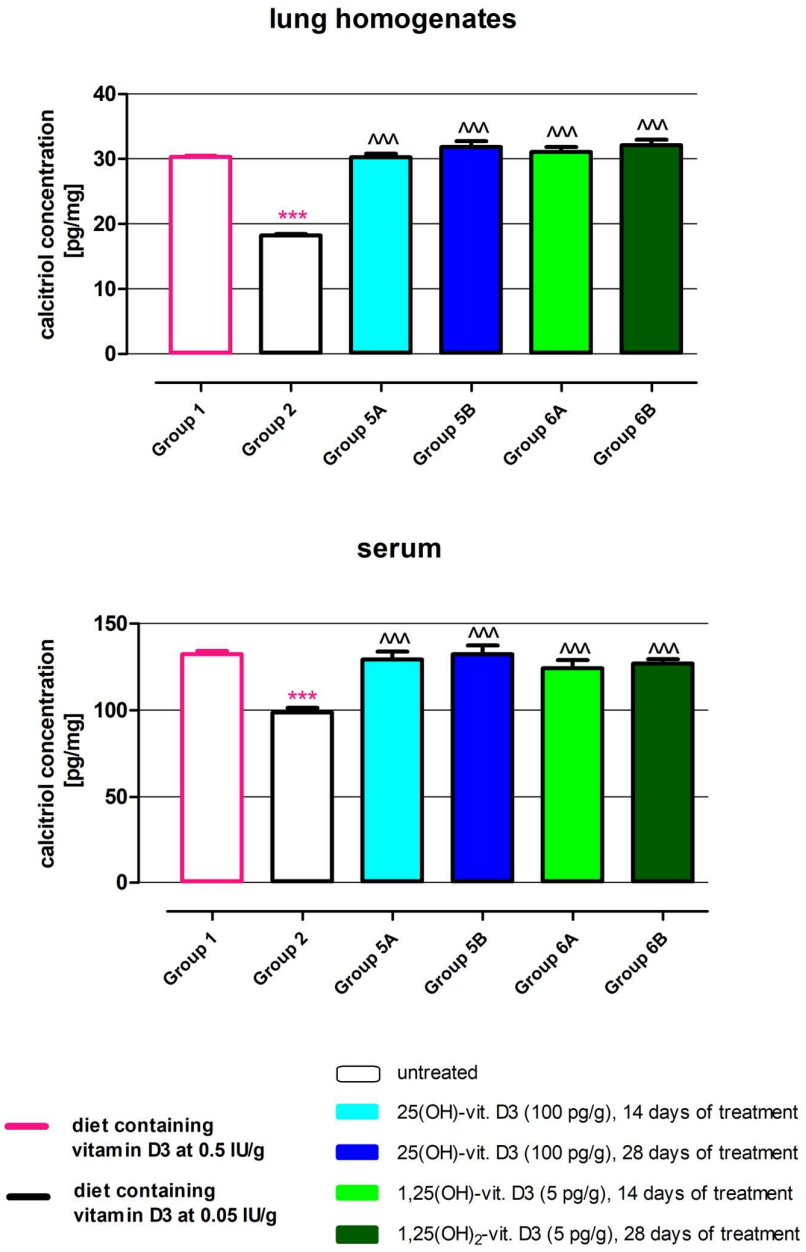

Figure 2. Restoration of calcitriol physiological level in mice with induced vitamin D3 deficiency in response to inhalation with vitamin D3 metabolites. The mice were chronically exposed to calcidiol at a concentration of 100 pg/g or calcitriol at a concentration of 5 pg/g. Inhalations were performed daily for 14 or 28 consecutive days. As the control, untreated mice from Group 1 (main control) and Group 2 were used. Animals from Group 1 were given the diet with a standard cholecalciferol level (0.5 IU/g), while the mice from other research groups were given a diet with a reduced cholecalciferol level (0.05 IU/g). Calcitriol concentration was determined by using the ELISA method in both lung homogenates and serum samples collected from all animals. Data are presented as a mean of calcitriol concentration ± SEM of at least 10 measurements. *** $p < 0.001$ vs. main control (Group 1); ^^^ $p < 0.001$ vs. control (Group 2); Analyzed using a one-way ANOVA test and Tukey post hoc test.

Table 2. Changes in murine pulmonary function in response to inhalations with vitamin D3 metabolites. The mice were chronically exposed to calcidiol at the concentration of 100 pg/g (Group 5) or calcitriol at the concentration of 5 pg/g (Group 6). Inhalations were performed daily for 14 (subgroup A) or 28 (subgroup B) consecutive days. As the control, untreated mice from Group 1 (main control) and Group 2 were used. Animals from Group 1 were given the diet with a standard cholecalciferol level (0.5 IU/g), while the mice from other research groups were given a diet with a reduced cholecalciferol level (0.05 IU/g). Respiratory parameters were investigated using the Non-invasive Airway Mechanics Plethysmograph. The examinations were conducted at the end of every single experiment. Data were collected every 2 s for 10 min. The data are presented as a mean of tested ventilatory parameters ± SD. Statistically significant differences between research groups were determined using a one-way ANOVA with a Tukey post hoc test and marked in the table. * $p < 0.05$; ** $p < 0.01$ vs. main control (Group 1).

Respiratory Parameter	Group 1	Group 2	Group 5		Group 6	
			A	B	A	B
frequency of breathing [breaths/min]	266.72 ± 22.97	291.40 ± 10.79	296.47 ± 22.63	283.97 ± 21.88	289.53 ± 15.28	266.85 ± 15.07
tidal volume [mL]	0.310 ± 0.061	0.330 ± 0.027	0.366 ± 0.039	0.350 ± 0.032	0.364 ± 0.024	0.316 ± 0.041
minute volume [mL/min]	82.94 ± 21.27	95.14 ± 7.44	109.27 ± 13.87 (*)	100.75 ± 16.50	106.19 ± 10.77	85.02 ± 15.43
time of inspiratory [s]	0.104 ± 0.013	0.090 ± 0.004	0.091 ± 0.010	0.094 ± 0.007	0.092 ± 0.007	0.101 ± 0.012
time of expiratory [s]	0.138 ± 0.015	0.129 ± 0.006	0.123 ± 0.011	0.136 ± 0.012	0.140 ± 0.013	0.138 ± 0.008
peak inspiratory flow [mL/s]	4.726 ± 1.071	5.626 ± 0.508	6.066 ± 0.987	5.949 ± 0.855	6.377 ± 0.494 (*)	4.943 ± 0.942
peak expiratory flow [mL/s]	3.900 ± 0.956	4.380 ± 0.246	4.957 ± 0.540	4.829 ± 0.660	5.307 ± 0.401 (**)	4.221 ± 0.625
mid-tidal expiratory flow [mL/s]	3.536 ± 0.880	3.990 ± 0.245	4.375 ± 0.496	4.251 ± 0.570	4.621 ± 0.388 (**)	3.656 ± 0.666
inspiratory flow rate [mL/s]	3.059 ± 0.786	3.660 ± 0.309	4.041 ± 0.552	3.778 ± 0.650	3.999 ± 0.470	3.176 ± 0.645
expiratory flow rate [mL/s]	2.302 ± 0.685	2.564 ± 0.231	2.989 ± 0.457	2.615 ± 0.484	2.632 ± 0.389	2.302 ± 0.331
inspiratory duty cycle [%]	42.91 ± 3.59	41.19 ± 1.12	42.53 ± 3.23	40.89 ± 1.35	39.63 ± 1.42	42.33 ± 3.60
expiratory duty cycle [%]	57.09 ± 3.59	58.81 ± 1.12	57.47 ± 3.23	59.11 ± 1.35	60.37 ± 1.42	57.67 ± 3.60

4. Discussion

According to the best of our knowledge, the presented study is the first to examine the direct delivery of biologically active vitamin D3 (calcitriol), as well as its precursor (calcidiol), to the respiratory tract via inhalation as a potential strategy to neutralize vitamin D deficiencies in lung tissue. The study was performed on murine strain C57BL/6, which is the most common animal model for studying pulmonary disorder with signs of fibrosis [16]. The first challenge for the presented study was the lack of literature data on the physiological and pathological levels of vitamin D3 in C57BL/6 mice. There were also no clear recommendations regarding the standard diet of rodents in the context of vitamin D content (cholecalciferol content in commercial feed for laboratory rodents ranges from 0.5 IU/g to 5 IU/g); even less is known about the method of achieving vitamin D deficiency in the body of small rodents [17,18]. In the presented study, the control mice received a diet with

a cholecalciferol content of 0.5 IU/g, while to induce vitamin D deficiency the mice were given feed with a 10-times lower amount of cholecalciferol. Performed studies revealed that the calcitriol concentration in 3-month-old male mice strain C57BL/6J on the standard diet was 30.31 pg/mg in the lungs and 132.24 pg/mL in the serum. On the other hand, dietary cholecalciferol restriction significantly decreased the calcitriol amount in the investigated mice to the following levels: 18.20 pg/mg (lung tissue) and 98.61 pg/mL (serum). The presented serum results correspond with data collected by Mallya et al. from male and female murine strain FVB after 3 months of feeding with aliments containing 0.5 IU/g or 0.05 IU/g cholecalciferol, which revealed serum concentrations of 1,25$(OH)_2$-vitamin D3, 111 ± 16 pg/mL, and 78 ± 3 pg/mL, respectively [17]. On the contrary, Fleet et al. examined the effect of diets containing cholecalciferol in amounts from 25 to 1000 IU/kg on calcitriol serum levels in 14-week male murine strain C57BL/6, which showed that animals given 50 and 400 IU/kg vitamin D with diet had the following levels of 1,25$(OH)_2$-vitamin D3 in serum: 60 pmol/l (an almost 4-times lower concentration than that obtained in our study) and 160 pmol/l (an almost 2-times lower concentration than that obtained in our study) [18]. Nevertheless, another investigation conducted by Fleet et al. in a 10-week female murine strain C57BL/6, fed with a diet containing cholecalciferol in amounts from 400 IU/kg to 20,000 IU/kg, indicated that animals on the diet with the lowest investigated vitamin D content had calcitriol serum levels around 300 pmol/l, which corresponds to our results. It needs to be highlighted that Fleet et al. reported a negative correlation between vitamin D diet intake (cholecalciferol amount over 100 IU/kg in male mice and over 400 IU/kg in female mice) and calcitriol serum level [18]. Cited research [17,18] confirmed the correctness of the vitamin D doses in animal feed selected by our team to create a physiological and pathological level of calcitriol in the murine body.

The concentrations of vitamin D3 metabolites used in the presented study were inspired by research conducted by Taylor et al. [19], which, according to the best of our knowledge, is the one of three studies that investigated the influence of inhalation of calcidiol and calcitriol on the lungs. Despite the fact that Taylor's research was conducted in a different animal model (neonatal rats with unknown vitamin D3 status) and the aim of the study differed from ours (improve lung maturation), because of the similar concept of the studies (animals chronic exposure to vitamin D3 metabolites administered by nebulization) and in the light of limited knowledge in this area, we decided to use vitamin D3 metabolites in a similar concentration range [19]. In order to achieve the main research goal, mice with vitamin D deficiencies induced by dietary cholecalciferol restriction were inhaled for week with five different doses of calcitriol (1, 5, 10, 25, and 50 pg/g) or its physiologic precursor calcidiol (100, 250, 500, 750, and 1000 pg/g). The effectiveness of inhalations was checked by determining the 1,25$(OH)_2$-vitamin D3 level in lung tissue and serum. Performed studies revealed the lack of a simple linear relationship between the dose of vitamin D3 metabolites and the calcitriol concentration in the lungs and blood of inhaled animals. Changes induced by calcidiol at the lower doses (100, 250, and 500 pg/g) significantly improved the calcitriol status in lung compartments, while doses over 500 pg/g did not cause a further significant increase in the concentration of the tested metabolite. Serum results revealed quite a similar pattern of changes; however, the plateau stage was reached just after administration of calcidiol at a concentration of 250 pg/g. On the contrary, murine exposure to the lowest concentration of calcitriol (1 pg/g) induced only a weak improvement in bioactive vitamin D3 status in both examined compartments, while evident enhancement associated with restoration of physiological calcitriol level was observed in animals exposed to 1,25$(OH)_2$-vitamin D3 at the concentration of 5 pg/g. It has to be noted that calcitriol doses over 5 pg/g provoked diametrically different effects depending on the tested compartments. Calcitriol at the concentration of 10 pg/g additionally increased its endogenous level in lung tissue, while the higher doses of tested compound provoked only discrete improvement. On the contrary, 1,25$(OH)_2$-vitamin D3 doses, starting from 10 pg/g, caused a gradual decrease in serum calcitriol concentration, but the observed changes were not statistically significant compared to untreated mice on the standard diet. The

presented data clearly demonstrated that only the lowest doses of vitamin D3 metabolites (calcidiol at the concentration of 100 pg/g and calcitriol at concentrations of 1 and 5 pg/g) administered via inhalation provoke similar patterns of changes in the pulmonary and serum concentration of calcitriol. Consequently, the calcitriol serum level cannot be used to predict this metabolite concentration in the respiratory system.

Results obtained from lung and serum revealed that restoration of the physiological calcitriol level required 1-week daily inhalation with 25(OH)-vitamin D3 at the concentration 100 pg/g or 1,25$(OH)_2$-vitamin D3 at the concentration of 5 pg/g. In order to verify this data, as well as check the impact of chronic inhalation on pulmonary function, the time of murine exposure to selected doses of mentioned metabolites was extended to 14 and 28 days. Performed studies revealed that tested compounds in the above-mentioned concentrations administered directly to the respiratory system of mice with vitamin D deficiencies allowed neutralization of the effect of dietary cholecalciferol restriction and increased the endogenous calcitriol concentration in both lung tissue and serum to physiological levels. Moreover, results of plethysmography have shown that murine chronic exposure to vitamin D3 metabolites did not affect most of the examined respiratory parameters, including F, TV, Ti, Te, IFR, EFR, as well as duty cycles of inspiration and expiration. Nevertheless, 14 days of vitamin-D-deficient murine treatment with calcidiol at the concentration of 100 pg/g significantly increased MV, compared to untreated mice with physiological calcitriol levels. Similarly, vitamin-D-deficient mice exposed for 2 weeks to 1,25$(OH)_2$-vitamin D3 at the concentration of 5 pg/g demonstrated elevated levels of PIF, PEF, and EF50, compared to healthy untreated mice. Despite the alterations discovered in four out of twelve respiratory parameters, it has to be highlighted that all disorders observed after 14 days of inhalation subsided with continued inhalation—there were no side effects in the lung function of animals exposed to the tested metabolites for 28 days.

Unfortunately, we were not able to compare our results or, more importantly, their interpretation with other scientific data because of the lack of any such study. As mentioned before, there are only three studies that investigated the influence of inhalation of vitamin D3 metabolites on the respiratory system (improvement in development of neonatal rat lungs [19], fighting with *Mycobacterium tuberculosis* [20], and attenuation of LPS-induced acute lung inflammation [21]). Despite obvious differences in our study and that by Taylor [19], such as species of animals (mice vs. rats), age of animals (adults vs. neonates), purpose of the study (restoration of physiological calcitriol levels in the lung tissue of mice with vitamin D3 deficiency vs. stimulation of lung maturation in neonatal rats with unknown vitamin D3 status), and measured parameters (concentration of calcitriol in the lungs and serum and respiratory parameters vs. concentration of calcidiol in serum and markers of lung maturation), it is worth mentioning this research because of a similar therapeutic strategy, i.e., direct administration into the respiratory tract of the biologically active form of vitamin D3 and its precursor in similar concentration ranges (calcidiol doses: 100, 500, and 1000 ng/kg; calcitriol doses: 1, 10, and 50 ng/kg), as well as the approximate duration of the experiment (14 days). The cited study proved that chronic exposure of neonatal rats to calcidiol and calcitriol in the whole range of tested concentrations did not affect the serum levels of both calcium and 25(OH)-vitamin D3 and did not impact on the expression of VDR and PPARγ (peroxisome proliferator-activated receptors) in kidneys, which, according to the authors, proved the low possibility of systemic side effects of the investigated therapeutic strategy. Furthermore, Taylor et al. discovered that neonatal rats' chronic inhalations with vitamin D3 metabolites enhanced lung maturation, which was associated with increased surfactant production, improvement in alveogenesis, as well as stimulation of differentiation of alveolar type II cells, lipofibroblasts and, endothelial cells [19]. Consequently, the mentioned research also supports our observations that the presented therapeutic strategy is effective and safe. It has to be also emphasized that, in the majority of experiments conducted by Taylor's team, vitamin D3 metabolites in the lowest investigated concentrations (calcidiol at 100 ng/kg and calcitriol at 1 ng/kg) revealed the greatest impact on lung maturation [19]. Our research goals were achieved when we used

calcidiol in the same concentration, while calcitriol at a five-times higher dose than Taylor's team [19], but the range of potential therapeutical concentration was still quite similar. Furthermore, our study, as well as the cited research [19], clearly demonstrated that the bioactive form of vitamin D3 could be used in significantly lower doses (1/20 or even 1/100, respectively) than its precursor to induce a similar beneficial effect in pulmonary compartments. Of course, this conclusion is not surprising considering the metabolic changes necessary for calcidiol transformation into an active hormone; however, in our opinion, it is a valuable tip regarding the dosage of vitamin D3 metabolites for inhalations in future studies.

As indicated above, the second study examining the influence of vitamin D3 inhalation on respiratory tracks was conducted by Reddy's team, who focused on the possibility of using calcitriol in the treatment of tuberculosis [20]. The cited studies contain even more differences from our research than the previously discussed study by Taylor's team [19]. The study was conducted on Swiss albino mice infected with *Mycobacterium tuberculosis* (Mtb), with unknown vitamin D3 status; moreover, none of the investigated parameters were common to our examination. Nevertheless, apart from the concept of the therapeutic use of vitamin D3 inhalation, our attention was attracted by the investigation of the chronic (once daily, 5 days a week for 28 days) administration of calcitriol at the concentration of 5 pg/g (potentially therapeutic dose in the light of our studies). Although Mtb-mice chronic exposure to calcitriol did not result in the expected effect, in particular, reduction in bacterial burden and increase in the production of cathelicidin (an immune peptide with bactericidal properties), the performed studies revealed significant improvement in histological parameters, such as inflammation and signs of necrosis and fibrosis, which, according to the authors, suggested a pro-healing impact of the tested compound on lung tissue damaged by infection [20]. Consequently, the presented study also supports the use of vitamin D3 metabolites administered by inhalation in the treatment of lung diseases, as proposed by our team.

Finally, we would also like to mention the studies by Serré et al., who investigated the impact of the single, direct administration into the respiratory tract of calcitriol in the dose of 200 pg/g on LPS-induced acute lung inflammation in mice on standard as well as a vitamin D deficient diets [21]. Studies have been conducted on 8- to 9-week-old male murine strain C57BL/6JolaH. Unfortunately, there is a lack of information about the amount of vitamin D in the applied diets; at the same time, the description of vitamin D status in the research groups was limited to very confusing information about the calcidiol serum level, which in vitamin-D-deficient mice was lower than 2 ng/mL, while in mice on a standard diet was seven- to nine-fold higher. It should also be emphasized that in contrast to previously described studies, including our research, calcitriol was not administered via typical nebulization but by using an aerosolizer syringe, which may explain the placing of mice under mild anesthesia with isoflurane. Unfortunately, apart from the use of the same strain of mice in the research and the concept of administering calcitriol directly to the respiratory tract of mice on diets with different vitamin D3 content, we were not able to find common points with this study, which are necessary to discuss and compare our observations. Nevertheless, it is worth mentioning that Serré's team revealed that murine nebulization with calcitriol reduced acute inflammation caused by LPS, independent of vitamin D3 status and, moreover, in the case of vitamin deficiencies prevented epithelial barrier leakage and lung damage [21]. Thus, the cited study is another argument in favor of the use of vitamin D3 metabolite inhalation in respiratory disorders.

5. Conclusions

In summary, the presented data revealed the usefulness and safety of chronic inhalation with both the bioactive form of vitamin D3 (1,25$(OH)_2$-vitamin D3) as well as its precursor (25(OH)-vitamin D3) in the restoration of the physiological calcitriol level in the lung tissue and serum of mice with vitamin D deficiencies. At the same time, it indicated that the desired effect required the use of a 20-times lower concentration of calcitriol than

its precursor, which provides important advice for future studies. Moreover, the obtained results clearly demonstrated that the calcitriol serum level cannot be used to predict this metabolite concentration in lung tissue. It needs to be highlighted that the presented study is the first one that revealed the physiological concentration of calcitriol in the lung tissue of the mice strain C57BL, which is commonly used in research, in particular, dedicated to pulmonary fibrosis. Furthermore, in the light of the increasing negative health consequences of vitamin D3 deficiencies, the information provided in the paper regarding the induction of this disorder in mice could be useful for scientists focused on this phenomenon.

We hope that the presented results will encourage further research on the use of inhaled vitamin D3 metabolites in the prevention and treatment of lung diseases associated with calcitriol deficiency, especially since in the case of these diseases this route of drug administration is one of the cheapest, fastest, safest, and most convenient, as well the most acceptable methods by patients with respiratory disorders.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app131910672/s1, Table S1: Alterations in calcitriol concentrations in murine lung tissue and serum in response to inhalations with 1,25$(OH)_2$-vitamin D3 or 25(OH)-vitamin D3 as well as diets with standard or reduced amounts of cholecalciferol. Data are presented as a mean of calcitriol concentration ± SEM.

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