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Induction of CFTR gene expression by 1,25$(OH)_2$ vitamin D_3, 25OH vitamin D_3, and vitamin D_3 in cultured human airway epithelial cells and in mouse airways

Kristina M. DiFranco[a], Jennifer K. Mulligan[b], Aman S. Sumal[b], Gill Diamond[a,*]

[a] Department of Oral Biology, University of Florida, Gainesville, FL 32610, United States, United States
[b] Department of Otolaryngology, Head and Neck Surgery, Medical University of South Carolina, Charleston, SC 29425, United States

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ABSTRACT

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which often leads to protein misfolding and no CFTR surface localization. This then leads to chronic airway infections, inflammation, and tissue damage. Although vitamin D has been explored as a therapy to treat CF due to its antimicrobial-inducing and anti-inflammatory properties, the effect of 1,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) on CFTR directly has not been studied. We treated cultured healthy and diseased bronchial epithelial cells (BEC) with 10 nM 1α,25$(OH)_2D_3$ for 6 and 24 h and found that 1α,25$(OH)_2D_3$ increases both mRNA and protein CFTR levels using RT-qPCR, flow cytometry and fluorescence immunohistochemistry. Treatment of CF cells with 10 nM 1α,25$(OH)_2D_3$ led to an increase in both total and surface CFTR expression, suggesting 1α,25$(OH)_2D_3$ could be used to increase properly localized CFTR in airway cells. To determine if BEC could convert the more clinically relevant cholecalciferol to 25OHD_3, cultured non-CF and CF BECs were treated with a range of cholecalciferol concentrations, and 25OHD_3 levels were quantified by ELISA. We found that 25OHD_3 levels increased in a concentration-dependent manner. Treatment of BEC with 10 μM cholecalciferol led to increases in both CYP24A1 and CFTR mRNA levels, even when added to the apical surface of cells grown in an air-liquid interface, suggesting that topical administration of vitamin D could be used therapeutically. To demonstrate this *in vivo*, we intranasally delivered 1 μM 1α,25$(OH)_2D_3$ into mice. After 6 h, we observed induction of both Cyp24A1 and CFTR expression in the tracheas of treated mice. The major findings of this study are that vitamin D can be converted to the active form when topically administered to the airway, and this could be used to increase CFTR levels in patients with CF. This could potentially be useful as an adjunctive therapy, together with newly developed CF treatments.

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a chloride ion channel. CFTR is expressed in many organs, including the lungs, pancreas, salivary glands, kidneys, liver, sweat glands, and reproductive tract [1]. There are over 2000 CFTR mutations that, depending on the specific mutation, affect either trafficking, synthesis, or function of CFTR [2]. The most common mutation in Caucasians is a phenylalanine deletion at position 508 (F508del). The loss of this amino acid causes misfolding of the protein in the endoplasmic reticulum (ER), where it is then subsequently degraded prior to reaching the plasma membrane [3–5].

Improper chloride transport from defective CFTR leads to thickened mucous secretions, persistent bacterial infections, lung tissue damage and malnutrition due to poor vitamin absorption [6–10]. The lungs are the organ most commonly studied in CF because of the severity of the effects at that site. Itself, the thickened mucus in the lungs provides an environment favorable for microbial growth [11]. The most common bacterial species found in CF is *Pseudomonas aeruginosa*, with 80% of adult CF patients colonized by the bacteria [12]. Chronic bacterial infection leads to immune system activation and release of neutrophilic proteases. However, instead of clearing the bacteria, tissue damage

* Corresponding author at: Department of Oral Biology, University of Florida, Box 100424, Gainesville, FL, 32610, United States.
E-mail address: gdiamond@dental.ufl.edu (G. Diamond).

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ensues and a lung transplant becomes necessary. In fact, 80% of CF patients' deaths are due to lung infection [1].

Besides the lungs, other organs are affected in CF due to the widespread expression of CFTR. For example, malnutrition is common because mucus clogs the pancreas and digestive enzymes can't be released, resulting in poor vitamin absorption, especially fat-soluble vitamins A, D, E, and K [8,13,14]. Vitamin D is best known to be a principal factor that maintains calcium homeostasis and is required for bone development and maintenance [15]. Early vitamin D deficiency could lead to rickets while adult deficiency leads to osteoporosis and increases in fractures in the elderly (reviewed in [16]). Additionally, vitamin D deficiency is also associated with a number of non-bone related disorders including multiple sclerosis, diabetes and Crohn's disease, as well as a variety of cancers [17–21]. Vitamin D is of particular interest for CF patients because of its diverse functions and ability to act on a wide variety of tissues. Up to 90% of CF patients are deficient in vitamin D, even with supplementation, and this deficiency is associated with increased pulmonary exacerbations, increased bacterial infection and inflammation[22,23].

Vitamin D undergoes a multistep, multiorgan conversion process that ultimately leads to its final hydroxylation and activation to 1,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) (reviewed in [24]). Initially, vitamin D_3 is either taken as an oral supplement or converted by the skin after sunlight exposure and subsequently hydroxylated in the liver to $25OHD_3$. Multiple 25-hydroxylases are reported to convert vitamin D_3 to $25OHD_3$, with the best-studied being Cyp27A1 and Cyp2R1 [25,26]. $25OHD_3$ is then transported to the kidneys where it undergoes a second hydroxylation step by the 1-alpha-hydroxylase Cyp27B1 to be converted to the active $1\alpha,25(OH)_2D_3$ [24,27]. Recently, the conversion of $25OHD_3$ to $1\alpha,25(OH)_2D_3$ has been reported in extrarenal tissues including macrophages, colon, keratinocytes, osteoclasts and lung [28–30]. The actions of $1\alpha,25(OH)_2D_3$ are mediated by binding to the vitamin D receptor (VDR), where it heterodimerizes with retinoid X receptor and interacts with a specific consensus sequence called a vitamin D response element (VDRE) [31,32]. This then leads to the activation and transcription of target genes. While the VDR/$1\alpha,25(OH)_2D_3$ transcription complex can take several hours to result in an observable change in cellular activities, $1\alpha,25(OH)_2D_3$ has also been reported to rapidly affect intracellular processes by binding membrane VDR or $1,25D_3$-membrane-associated, rapid response steroid-binding protein ($1,25D_3$-MARRS) receptor to turn on a number of kinases, such as protein kinases A and C [33–41].

Currently, the greatest appeal of vitamin D lies in its antimicrobial and anti-inflammatory properties (reviewed in [9]). Vitamin D is known to downregulate the expression of inflammatory cytokines IL-6 and IL-8, which have been reported to be upregulated in CF patients [42,43]. Ideally, these anti-inflammatory properties would prevent recruitment of neutrophils, which contribute to the lung tissue damage. This was observed in an *in vivo* hamster model where LPS inhalation followed by $1\alpha,25(OH)_2D_3$ administration decreased neutrophil recruitment [44]. $1\alpha,25(OH)_2D_3$ treatment was also found to upregulate gene expression of the antimicrobial peptides LL-37 and β-defensins, as well as other innate immune mediators in CF and normal airway epithelial cells, which would be beneficial to combat opportunistic infections [45–47]. *P. aeruginosa* viability was decreased when exposed to supernatants of bronchial epithelial cells treated with $1\alpha,25(OH)_2D_3$ [48].

While examining the effect of vitamin D treatment on airway epithelial cells, we observed an unexpected induction of CFTR mRNA, suggesting a potential therapeutic role for vitamin D in CF treatment. Currently, a major obstacle for using oral vitamin D to treat cystic fibrosis is that vitamin D_3 must undergo two hydroxylation steps in the liver and kidney, resulting in 25 $(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$, respectively. This multiorgan activation combined with poor vitamin D absorption in CF patients would most likely lead to insufficient levels of activated vitamin D reaching the lungs and ultimately no change in CFTR. While treating patients with either $25OHD_3$ or $1\alpha,25(OH)_2D_3$ would avoid the need for multiple hydroxylations, treating with either metabolite is not feasible because of their short half-lives of several hours when ingested and the dangerous side effect of hypercalcemia [49–53]. As of now, these two forms are only used to raise calcium levels in patients on long-term renal dialysis. In this study, we investigated the effect of $1\alpha,25(OH)_2D_3$ on CFTR both *in vitro* and *in vivo*, the conversion of vitamin D_3 to active $1\alpha,25(OH)_2D_3$ in lung epithelial cells, and the feasibility of topical administration of vitamin D to the airways.

2. Methods and materials

2.1. Cell culture

Human bronchial epithelial (HBE) cells were from two sources. Normal HBE cells (NHBE) were purchased from Lonza (Walkersville, MD) and grown in bronchial epithelial growth medium (BEGM) from the same company. CF HBE cells were obtained from lungs explanted during transplantation under IRB-approved protocols and were cultures at an air liquid interface (ALI) until well differentiated using well described protocols ([54]). UNCN3T (non-CF) and UNCCF1T (CF) cells were a gift from Scott Randell (University of North Carolina), and have been described previously [55]. BEAS-2B cells were obtained from the American Type Culture Collections (Rockville, MD) and grown in BEGM medium. HEK293 cells were a generous gift from Dr. Edward Chan (University of Florida) and were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin (Thermo Scientific, Rockford, IL). All cells were grown at 37 °C in 5% CO_2. Submerged cells were grown in 12-well plates and cells grown at an ALI were cultured in Transwell® inserts (Fisher Scientific, Pittsburgh, PA).

2.2. RNA isolation, RT-PCR, and PCR

After cells were treated with $1\alpha,25(OH)_2D_3$ (Sigma-Aldrich, St. Louis, MO) or vitamin D_3, total RNA was isolated using RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, and was reverse transcribed using iScipt cDNA synthesis kit (Bio-Rad, Hercules, CA) using the T100 ThermoCycler (Bio-Rad). RT-PCR was performed using SoAdvanced Universal SYBR Green Supermix (Bio-Rad) using the CFX96 Real Time PCR Detection System (Bio-Rad). Data was analyzed with CFX Manager Software (Bio-Rad). For PCR, cDNA was amplified using iProof HF Master (Bio-Rad) and fragments were visualized on a 2% agarose gel. Bands were cut out and sent to UF Interdisciplinary Center for Biotechnology Research for Sanger sequencing to confirm identity of the bands. β-2–macroglobulin (B2M) was used as the reference gene and primers for both RT-PCR and PCR are listed in Table 1 and were designed and purchased from Integrated DNA Technologies (Coralville, IA).

2.3. Western blot

Cells were lysed using RIPA buffer. Samples were mixed with LDS sample buffer (Thermo Scientific) and run on a 10% NuPAGE Tris-Acetate gel (Thermo Scientific). Proteins were transferred using the iBlot (Thermo Fisher) transfer apparatus to nitrocellulose membranes. Membranes were blocked in 5% milk in Tris-buffered saline with Tween 20 (TBS-T) and incubated with either anti-Cyp2R1 (Abcam Inc., Boston, MA), anti-Cyp27A1 EPR7529

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