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1,25-Dihydroxyvitamin D Enhances Alveolar Fluid Clearance by Upregulating the Expression of Epithelial Sodium Channels

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ABSTRACT

Vitamin D is implicated in the pathogenesis of asthma, acute lung injury, and other respiratory diseases. 1,25-Dihydroxyvitamin D ($1,25(OH)_2D_3$), the hormonal form of vitamin D, has been shown to reduce vascular permeability and ameliorate lung edema. Therefore, we speculate that $1,25(OH)_2D_3$ may regulate alveolar Na^+ transport *via* targeting epithelial Na^+ channels (ENaC), a crucial pathway for alveolar fluid clearance. *In vivo* total alveolar fluid clearance was $39.4 \pm 3.8\%$ in $1,25(OH)_2D_3$-treated mice, significantly greater than vehicle-treated controls (24.7 ± 1.9 %, $n = 10$, $p < 0.05$). $1,25(OH)_2D_3$ increased amiloride-sensitive short-circuit currents in H441 monolayers, and whole-cell patch-clamp data confirmed that ENaC currents in single H441 cell were enhanced in $1,25(OH)_2D_3$-treated cells. Western blot showed that the expression of α-ENaC was significantly elevated in $1,25(OH)_2D_3$-treated mouse lungs and $1,25(OH)_2D_3$-treated H441 cells. These observations suggest that vitamin D augments trans-alveolar fluid clearance, and vitamin D therapy may potentially be used to ameliorate pulmonary edema.

Introduction

In order for gas exchange to occur in an optimum fashion, the alveolar space must remain free of fluid. Pulmonary edema is the abnormal fluid accumulation in the interstitial or alveolar spaces of the lung. Besides its involvement in the well-known calcium and phosphorus metabolism, vitamin D is implicated in the pathogenesis of asthma, acute lung injury, and other respiratory diseases.[1] 1,25-Dihydroxyvitamin D ($1,25(OH)_2D_3$), the hormonal form of vitamin D, has been shown to reduce vascular permeability and ameliorate pulmonary edema,[2-4] but the underlying mechanisms are incompletely understood.

Alveolar salt and fluid reabsorption has been extensively investigated during the last few years. Alveolar fluid clearance (AFC) has been widely used in understanding lung fluid balance, not only under normal but also pathologic conditions, for example, in acute lung injury and acute respiratory distress syndrome. Stimulation of AFC accelerates the resolution of pulmonary edema, which benefits gas exchange across the alveolar epithelium.[5] In contrast, impairment of AFC is often related with worsened survival in acute lung injury and acute respiratory distress syndrome patients,[6,7] which leads to the development of pulmonary edema.[8] Much effort has therefore been focused on identifying the pathogenic mechanisms underlying the relationship of vitamin D and AFC.

Convincing evidence indicates that active sodium transport across the alveolar epithelium *in vivo* contributes to the reabsorption of the fetal fluid and to the maintenance of fluid-free alveolar spaces in adult lungs. Active Na^+ reabsorption across lung epithelia requires the sequential coordination of the entry of Na^+ ions through Na^+-selective, amiloride-sensitive (AS) epithelial Na^+ channels (ENaCs) located at the apical membranes, followed by the extrusion across the basolateral membranes by the energy-consuming Na^+-K^+-ATPase. The entry of Na^+ ions through apical membranes is thought to be the critical step in this process.[9-12] Therefore, we speculate that $1,25(OH)_2D_3$ may regulate alveolar Na^+ transport *via* targeting ENaC, a crucial pathway for AFC.

In this article, we evaluated whether AFC of fluid-instilled mouse lungs is strengthened when BALB/c male mice have been treated with $1,25(OH)_2D_3$ for 2 weeks and measured the ENaC activity in H441 cells, aiming to find the role of strengthened alveolar fluid transport in lung epithelium by vitamin D.

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Materials and Methods

Measurement of In Vivo *Alveolar Fluid Clearance in Mice*

Animals were kept under pathogen-free conditions, and all procedures performed were approved by the Institutional Animal Care and Use Committee of China Medical University. AFC was measured as previously described.[12] In brief, BALB/c male mice (20~30 g) were anesthetized with diazepam (17.5 mg kg^{-1}, intraperitoneally) followed 6 min later by ketamine (450 mg kg^{-1}, intraperitoneally) and were placed on a heating pad. For the 1,25$(OH)_2D_3$-treated group (VD group), mice were treated with vitamin D analogue paricalcitol[13](19-nor-1,25-dihydroxyvitamin D_2, intraperitoneally at 200 ng/kg, dissolved in 70% propylene glycol) or vehicle daily for 2 weeks before the *in vivo* AFC of these mice was measured. The trachea was exposed and cannulated with a trimmed 18-gage intravenous catheter, which was then connected to a mouse respirator (HX-300, Chengdu Taimeng Technology Co. Ltd, Chengdu, China). Mice were ventilated with 100% O_2 with a 200-μL tidal volume (8-10 ml kg^{-1}) at a 160 breaths min^{-1}. Once stable anesthesia was obtained, mice were positioned in the left decubitus position, and 300 μL of isosmolar NaCl containing 5% fatty acid-free bovine serum albumin or 1-mM amiloride (a specific ENaC inhibitor) was instilled *via* the tracheal cannula, followed by 100 μL of room air to clear dead space. After instillation, mice were ventilated for a 30-min period, and then the alveolar fluid was aspirated. All reagents were added to the AFC instillate from stock solutions directly before instillation, in a minimal volume of solvent (1-10 μL mL^{-1}). The protein concentrations in aspirated solutions were measured by Bradford method. We estimated AFC (% AFC_{30}) by the changes of concentration for bovine serum albumin as water was absorbed after 30 min. AFC was calculated as the follow equation: $AFC = [(Vi-Vf)/Vi] \times 100$, where V is the volume of the instilled bovine serum albumin solution (i) and the final alveolar fluid (f). $Vf = Vi \times Pi/Pf$, where P represents the protein concentration in the instilled bovine serum albumin solution (i) and the final alveolar fluid (f).

Cell Culture

H441 cells were obtained from the American Type Culture Collection and grown in Roswell Park Memorial Institute medium (American Type Culture Collection) supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). For ussing chamber studies, cells were seeded on permeable support filters (Costar) at a supraconfluent density (~5×10^6 cells/cm^2) and incubated in a humidified atmosphere of 5% CO_2-95% O_2 at 37°C. Cells reached confluency in the Costar Snapwell culture cups 24 h after plating. At this point, media and nonadherent cells in the apical compartment were removed to adapt the cells to air-liquid interface culture. Culture media in the basolateral compartment was replaced every other day; whereas the apical surface was rinsed with phosphate-buffered saline (PBS). An epithelial tissue voltohmmeter (World Precision Instruments) was used to monitor the transepithelial resistance. Highly polarized tight monolayers with resistance >800 Ω·cm^2 were selected for ussing chamber assays. For patch-clamp studies, cells were seeded in 75-cm^2 flasks and were lifted by 0.25% trypsin and 0.53-mM EDTA (Sigma), then seeded at a density of 1×10^6 cells/mL on round coverslips (8 mm, World Precision Instruments) situated in 24-well culture plates. Cells were grown in the previously mentioned medium supplemented with 200-nM dexamethasone to facilitate sodium channel differentiation, the medium was replaced every other day.

Ussing Chamber Assays

Measurements of short-circuit current (Isc) in H441 monolayers were performed as described previously.[14] Briefly, H441 monolayers treated with vitamin D analogue calcitriol 24 h (VD group, 20 nM) or ethanol vehicle (control group) were mounted in vertical ussing chambers (Physiologic Instruments) and bathed on both sides with solutions containing (in mM) 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.83 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 10 mannitol (apical compartment), and 10 glucose (basolateral compartment). The transepithelial Isc levels were measured with 3-M KCl, 4% agar bridges placed 3 mm on either side of the membrane, which were connected on either side to Ag-AgCl electrodes. The filters were bathed on both sides with the previously mentioned salt solution as designed, bubbled continuously with a 95% O_2-5% CO_2 gas mixture (pH 7.4). The temperature of the bath solution (37°C) was maintained using a waterbath. The transmonolayer potential was short circuited to 0 mV, and Isc level was measured with an epithelial voltage clamp (VCC-MC6, Physiologic Instruments). A 10-mV pulse of 1-s duration was imposed every 10 s to monitor transepithelial resistance. Data were collected using the Acquire and Analyze program, and when Isc level had attained its stable level, 100-μM amiloride was applied to the apical side and AS current component was determined.

Patch-Clamp Recordings

Immediately before each experiment, a coverslip bearing human lung H441 epithelial cells treated with vitamin D analogue calcitriol 24 h (VD group, 20 nM) or vehicle (control group) was removed from the culture dish and put into a recording chamber, which was mounted on the stage of an inverted fluorescent microscope (Leica DM IRB). For the whole-cell mode of patch-clamp recording, cells were perfused continuously with extracellular fluid containing (in mM) 140 NaCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES (pH 7.2).[15] After being exposed to the Na^+ channel blocker amiloride (10 μM), transepithelial transport indicated by AS currents were measured. Pipettes were made from capillary glass electrode with a P-97 micropipette puller (Sutter). They were back-filled with internal solution (in mM) 100 K-gluconate, 40 KCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 2 K_2ATP, 4 egtazic acid, and 10 HEPES (pH 7.2).[16] The pipette resistance varied from 5 to 10 MΩ when filled with this intracellular fluid. Offset potential was corrected before a gigaohm seal formation. Series resistance and capacitance transients were then compensated with an Axopatch 700B amplifier (Molecular Devices). Currents were digitized with a Digidata 1440A converter (Molecular Devices), filtered through an internal 4-pole Bessel filter at 1 kHz, and sampled at 2 kHz. Inward and outward whole-cell currents were elicited by using a step-pulse protocol from −120 mV to +80 mV in 20 mV increments every 10 s for 500 ms duration at a holding potential of −40 mV. Steady state currents were averaged between 100 and 200 ms, and only the cells with stable baseline currents were included in the results.

Western Blot Analysis

Mouse lungs and H441 cells treated or not with 1,25$(OH)_2D_3$ were washed 2 times with PBS and lysed for 15 min under agitation at 4°C in lysis buffer (1% Triton X-100, 150-mM NaCl, 5-mM EDTA, and 50-mM Tris, pH 7.5) supplemented with protease inhibitor and phosphatase inhibitor cocktails (Life Technologies). The cells were subsequently scraped with a rubber policeman, collected, and centrifuged at 12,000 × *g* for 10 min. Protein concentration of the supernatants was evaluated with the Bradford method (Bio-Rad Laboratories, Mississauga, ON). Total proteins (50 μg) were solubilized in sample buffer (62.5 mM Tris·HCl, pH 6.8, 2% SDS, 0.2% bromophenol blue, 10% glycerol, and 7.7% β-mercaptoethanol), subjected to SDS-polyacrylamide gel electrophoresis, and transferred electrophoretically onto polyvinylidene difluoride