

Wide-Range Effects of 1,25(OH)₂D₃ on Group 4A Phospholipases Is Related to Nuclear Factor κ-B and Phospholipase-A2 Activating Protein Activity in Mast Cells

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Keywords

1,25(OH)₂D₃ · Phospholipase-A2 · Nuclear factor κ-B p65 · UBC · Mast cells

Abstract

Introduction: Phospholipases are enzymes that occur in many types of human cells, including mast cells, and play an important role in the molecular background of asthma pathogenesis, and the development of inflammation NF-κB activities that affect numerous biological processes has been reported in many inflammatory diseases including asthma. Vitamin D is a widely studied factor that affects many diseases, including asthma. The aim of this study is to assess the influence of 1,25-(OH)₂D₃ on regulation of chosen phospholipase-A2 (PLA2) expression-selected inflammation mediators. **Methods:** LUVA mast cells were stimulated with 1,25(OH)₂D₃, and inhibitors of NF-κB p65 and ubiquitination. Expression analysis of phospholipases (PLA2G5, PLA2G10, PLA2G12, PLA2G15, PLA2G4A, PLA2G4B, PLA2G4C, PLAA, NF-κB p65, and UBC) was done utilizing real-time PCR and Western blot. Eicosanoid (LTC4, LXA4, 15[S]-HETE, and PGE2) levels and sPLA₂ were also measured. **Results:** We found that 1,25(OH)₂D₃ decreased the expression of PLA2G5, PLA2G15, PLA2G5, UBC, and NF-κB p65 but increased expression of PLAA and PLA2G4C ($p < 0.05$). Moreover, the expression of

PLA2G5 and PLA2G15 decreased after inhibition of NF-κB p65 and UBC. Increased levels of released LXA4 and 15(S)-HETE, decreased levels of LTC4, and sPLA₂s enzymatic activity in response to 1,25(OH)₂D₃ were also observed. Additionally, NF-κB p65 inhibition led to an increase in the LXA4 concentration. **Conclusion:** Future investigations will be needed to further clarify the role of 1,25(OH)₂D₃ in the context of asthma and the inflammatory process; however, these results confirm a variety of effects which can be caused by this vitamin. 1,25(OH)₂D₃-mediated action may result in the development of new therapeutic strategies for asthma treatment.

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Introduction

It has been reported that over 900 genes are regulated by vitamin D [1]. The biologically active form of vitamin D (1,25-dihydroxyvitamin D₃ – 1,25[OH]₂D₃), also known as calcitriol [2], and its signaling predominantly occurs through binding of 1,25(OH)₂D₃ to the vitamin D receptor (VDR), formation of a heterodimer with reti-

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noid X receptor, and subsequent regulation of gene expression by binding of this heterodimer to genomic sequences known as vitamin D response elements (VDRE) [2, 3].

Several in vitro and in vivo studies using animal models have highlighted vitamin D as a potent modulator of the inflammatory response seen in allergic airway inflammation [4–8]. Vitamin D affects structural cells in the airways and innate/adaptive immune system cells and guides transcription of proinflammatory cytokines [9]. Vitamin D deficiency also results in increased expression of the nuclear factor κ -B (NF- κ B) subunits which correspond with increased proinflammatory cytokines [6, 9].

Vitamin D has also been shown to have significant immunomodulatory effects by interacting with multiple immune cells involved in asthma, including mast cells, CD4⁺ T cells of both the TH1 and the TH2 phenotype, monocytes, macrophages and regulatory T cells (Tregs) [10–16].

A growing body of literature confirms an association between serum vitamin D levels and respiratory infections attributed to immunomodulatory properties of vitamin D [17, 18]. This interaction is important to asthmatics, as respiratory infections may influence the frequency, severity, and duration of asthmatic symptoms and exacerbations [2].

In allergic diseases, mast cells – the main effector cells of immune responses – are activated [11]. Inhibitory effects of calcitriol (concentration of 100 nM), dependent on expression and activation of VDR, were observed in mast cells. Calcitriol also inhibits the release of histamine from mast cells [19]. This type of cells is of particular interest in airway studies, as they have been shown to be increased in the airways of asthmatic patients compared with healthy controls [20]. Baroni et al. [11] demonstrated that 1,25(OH)₂D₃ contributes to regulation of the development and function of mast cells. According to their study, 1,25(OH)₂D₃ promotes apoptosis and inhibits maturation of mouse bone marrow-derived mast cell precursors. Moreover, a dose-dependent inhibition of mast cell differentiation was caused by 1,25(OH)₂D₃ at various stages of mast cell development [11].

Mast cells are one of the source of phospholipases in asthma and allergic diseases, apart from monocytes, neutrophils, eosinophils, basophils, lymphocytes, macrophages, bronchial smooth muscle cells, epithelial cells, and endothelial cells [21]. The mechanism of PLA2 action in asthma includes the release of AA, production of lysophospholipids, degradation of surfactant, the release

of cytokins and the influence on inflammatory and immune cells such as leukocytes, dendritic cells, and T cells [22, 23].

Phospholipases are enzymes that occur in many types of human cells, including mast cells, and play an important role in the molecular background of asthma pathogenesis. These enzymes contribute to the release of arachidonic acid – a substrate of lipid proinflammatory mediators such as prostanooids. Therefore, phospholipases play a crucial role in the development of inflammation [24]. In our previous study we demonstrated the influence of cPLA₂ α overexpression and activation on GM-CSF expression in human lung cells [25]. Moreover, we have previously shown that microsatellite fragments (T)_n and (CA)_n in the promoter region of the cPLA₂ α gene (PLA2G4A), though highly polymorphic, are significantly shorter in patients with severe asthma than in healthy subjects [26]. We also evaluated the influence of these microsatellite PLA2G4A promoter fragments on cPLA₂ α mRNA and protein expression and subsequent eicosanoid generation [27] and analyzed the influence of allergens and LPS on cPLA₂ α expression and sPLA₂X secretion in PBMC of asthmatics [28]. Critical evidence for the role of cPLA₂ in asthma and other inflammatory diseases has appeared previously in the literature, showing that cPLA₂ knockout mice (cPLA₂^{-/-} mice) recover faster from allergen-induced bronchoconstriction and have no airway hyperresponsiveness [29]. Additionally, a lack of cPLA₂ results in failure to generate leukotrienes, prostaglandins, and PAF [30, 31]. cPLA₂ is essential for both the immediate and the delayed phases of eicosanoid generation in mast cells [30].

Studies have reported that active vitamin D (1,25(OH)₂D₃) inhibits proinflammatory cytokine expression, promotes anti-inflammatory cytokine production, and regulates immune cell activity. These effects are believed to occur partly via inhibition of the NF- κ B pathway, a transcription factor with a critical role in inflammatory and immune responses, as well as in the pathophysiology of several chronic diseases such as asthma [32–34]. Previous works have suggested that 1,25(OH)₂D₃ directly modulates NF- κ B activity. Among various transcription factors, NF- κ B is the master switch for proinflammatory genes and transactivates arachidonic acid pathway enzymes when activated. Different NF- κ B dimers bind to a specific DNA sequence in gene promoters to regulate the transcription of a wide range of genes, including those involved in immune and inflammatory responses [35]. Szeto et al. [35] found that cells lacking the VDR have reduced I κ B α levels and a

lack of VDR-p65 and therefore exhibit increased NF- κ B activity. Their data suggest that VDR is directly involved in suppression of NF- κ B activation, which may partially explain the VDR-mediated anti-inflammatory 1,25(OH) $_2$ D $_3$ mechanism. Given the crucial role of the NF- κ B in inflammatory response, and the therapeutic potential of vitamin D in the treatment of inflammatory diseases, understanding the relationship and interaction between vitamin D, VDR, and the NF- κ B pathway has broad implications [35]. Moreover, activation of NF- κ B requires ubiquitination – a highly conserved and versatile modification that can regulate cell signaling through both proteasome-dependent and proteasome-independent mechanisms [36]. The exact molecular mechanism underlying 1,25(OH) $_2$ D $_3$ regulation of NF- κ B remains to be defined; however, it has been reported that 1,25(OH) $_2$ D $_3$ arrests p65 nuclear translocation, blocks NF- κ B DNA binding, increases I κ B α levels, or stabilizes I κ B α protein [34, 37–39].

The efforts to establish the role of vitamin D in asthma pathogenesis have resulted in many studies identifying a link between vitamin D deficiency and an overall worse outcome of lung function and symptoms in patients with asthma, yet the role of vitamin D in asthma, especially its pathogenesis, is not yet clear. Therefore, the aim of the study was to analyze the pathways involving the influence of 1,25(OH) $_2$ D $_3$ on phospholipases and inflammatory mediators in the context of NF- κ B and UBC in LUVA mast cells.

Materials and Methods

Cell Culture

The LUVA cell line was obtained as a generous gift of Prof. J.W. Steinke (University of Virginia, Charlottesville, VA, USA). LUVA cells were cultured in StemPro-34 serum-free medium with nutrient supplement, 2 mmol/L L-glutamine, 50 IU/mL Pen/Strep (Gibco, Life Technologies, Carlsbad, CA, USA) with or without 1,25(OH) $_2$ D $_3$ (Sigma-Aldrich, St. Louis, MO, USA), inhibitor of NF- κ B p65 – Hel (Abcam, Cambridge, MA, USA), and inhibitor of ubiquitination – PYR-41 (Sigma-Aldrich) in a humidified incubator (37 °C, 5% CO $_2$). Stock solutions of 1,25(OH) $_2$ D $_3$, Hel, and PYR-41 were prepared in ethanol and dimethyl sulfoxide, respectively. The final concentrations of the tested 1,25(OH) $_2$ D $_3$, Hel, and PYR-41 were obtained by the addition of culture medium. LUVA cells were used between the third and ninth passages.

LUVA cells were incubated for 4 h in the presence of 1,25(OH) $_2$ D $_3$ (1 and 100 nM).

Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from LUVA cells utilizing a Total RNA Mini Kit (A&A Biotechnology, Gdynia, Poland). The quantity of the isolated RNA was measured with a BioDrop

DUO spectrophotometer (BioDrop, Cambridge, UK). Total RNA (1 μ g) was reverse-transcribed to synthesis of cDNA using a high-capacity cDNA kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocols. cDNA was subjected to RT-PCR using assays designed for the selected genes (Hs00173472_m1 for PLA2G5, Hs00358567_m1 for PLA2G10, Hs00261432_m1 for PLA2G12, Hs00952741_m1 for PLA2G15, Hs00233352_m1 for PLA2G4A, Hs00979952_m1 for PLA2G4B, Hs01003743_m1 for PLA2G4C, Hs00972148_m1 for PLAA, Hs01042014_m1 for NF- κ B p65, and Hs00824723_m1 for UBC), and β -actin (ACTB) (Hs99999903_m1) as a qPCR reference (Life Technologies). Each sample was analyzed in duplicate using a TaqMan 7900 analyzer (Life Technologies). The expression levels of target genes were normalized to ACTB (β -actin). The RT-PCR data was analyzed using the comparative C $_t$ ($\Delta\Delta C_t$) value method for relative quantification. The fold change of mRNA expression for each gene was calculated as follows: relative quantity (RQ) = (2 $^{-\Delta\Delta C_t}$).

Immunoblotting

The total protein was extracted from LUVA cells using an RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, and 2 mM EDTA) supplemented with protease inhibitor cocktail (Sigma-Aldrich) according to the manufacturer's protocol. Protein concentrations in isolated samples were determined by Direct Detect (Merck Millipore, Darmstadt, Germany) and analyzed by immunoblotting. Twenty micrograms of total isolated protein per sample was separated by electrophoresis in denaturing polyacrylamide 4–20% NuPage gel (Invitrogen, Carlsbad, CA, USA) at 140 V and 110 mA, for 60 min, and transferred to a nitrocellulose membrane using the eBlot Transfer Protein System (Genscript, Piscataway, NJ, USA). The blocked membranes (5% nonfat milk in Tris-buffered saline [TBST] buffer containing 0.1% Tween 20) were incubated with mouse primary anti-PLA2G5 (sc-18828), anti-PLA2G15 (sc-376078), anti-PLAA(sc-390454), anti-NF- κ B p65 (sc-8008) antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) and primary rabbit anti-PLA2G4C (Sigma-Aldrich), anti-UBC (Gene Tex, Irvine, CA, USA), anti-ACTB (Cell Signaling, Danvers, MA, USA) overnight at 4 °C. The blots were washed 3 times in TBST buffer for 15 min and incubated with anti-mouse secondary antibodies or anti-rabbit secondary antibodies conjugated with alkaline phosphatase (Sigma-Aldrich) for 4 h at 4 °C. After washing in TBST buffer, the bands were visualized with BCIP/NBT alkaline phosphatase substrate (Merck Millipore). Image J 1.34s software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) was used for densitometric analysis of protein levels. ACTB was used as a reference protein standard.

Enzymatic Activity of the cPLA $_2$ s and sPLA $_2$ s Assays

The enzymatic activity of cPLA $_2$ s was measured in lysed LUVA cells, whereas the enzymatic activity of sPLA $_2$ s was measured in LUVA cell culture supernatants using a commercial cPLA $_2$ assay kit and an sPLA $_2$ assay kit (Cayman, Ann Arbor, MI, USA), respectively.

Eicosanoid Measurement

LTC4, LXA4, 15(S)-HETE, and PGE2 levels in LUVA cell culture supernatants were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (PGE2 [R&D Systems,

Minneapolis, MN, USA], 15[S]-HETE, LTC4 [Cayman], and LXA4, [EIAab, Wuhan, China]) according to the manufacturer's protocols.

Statistical Analysis

All of the experiments were repeated 3–5 times with similar results. Statistical analysis was conducted using GraphPad Prism 6.0 (San Diego, CA, USA). An unpaired Student's *t* test or the Mann-Whitney U test was used to assess differences between 2 groups after testing the normality. Differences between 3 or more groups were determined by ANOVA with Bonferroni's correction. Data are presented as means $\pm$ SEM. *p* < 0.05 was considered statistically significant.

Results

1,25(OH)₂D₃ Decreases PLA2G5 and PLA2G15 Expression via Inhibition of NF- κ B p65 and UBC but Increases PLA2G4C and PLAA Expression in LUVA Cells

RT-PCR results demonstrated that 1,25(OH)₂D₃ significantly decreased PLA2G5 (ethanol control [Eth]: 0.86 $\pm$ 0.03 vs. 100 nM 1,25(OH)₂D₃ [100 nM D₃]: 0.39 $\pm$ 0.08; *p* < 0.001), PLA2G15 (Eth: 1.05 $\pm$ 0.1 vs. 100 nM D₃: 0.55 $\pm$ 0.18; *p* < 0.05), UBC (Eth: 1.3 $\pm$ 0.1 vs. 100 nM D₃: 0.54 $\pm$ 0.1; *p* < 0.01), and NF- κ B p65 (Eth: 1.41 $\pm$ 0.24 vs. 100 nM D₃: 0.38 $\pm$ 0.09; *p* < 0.01) mRNA levels but increased PLA2G4C (Eth: 0.91 $\pm$ 0.09 vs. 100 nM D₃: 1.41 $\pm$ 0.17; *p* < 0.05) and PLAA (Eth: 1.03 $\pm$ 0.09 vs. 100 nM D₃: 1.58 $\pm$ 0.14; *p* < 0.05) mRNA levels (Fig. 1a).

No significant changes in the expression of PLA2G10, PLA2G12, PLA2G4A, or PLA2G4B mRNA levels were observed, and thus these results were not verified by Western blot analysis and those genes were not studied furtherly.

Western blot analysis showed that 1 and 100 nM 1,25(OH)₂D₃ significantly decreased PLA2G5 (control [C]: 1.29 $\pm$ 0.13 vs. 1 nM D₃: 0.75 $\pm$ 0.05; *p* < 0.01; Eth: 1.85 $\pm$ 0.18 vs. 100 nM D₃: 0.68 $\pm$ 0.04; *p* < 0.001), PLA2G15 (C: 1.33 $\pm$ 0.13 vs. 1 nM D₃: 0.74 $\pm$ 0.03; *p* < 0.01; Eth: 1.28 $\pm$ 0.05 vs. 100 nM D₃: 0.66 $\pm$ 0.07; *p* < 0.001), UBC (C: 1.42 $\pm$ 0.08 vs. 1 nM D₃: 0.8 $\pm$ 0.05; *p* < 0.001; Eth: 1.52 $\pm$ 0.12 vs. 100 nM D₃: 0.69 $\pm$ 0.04; *p* < 0.001), and NF- κ B p65 (C: 1.36 $\pm$ 0.05 vs. 1 nM D₃: 0.85 $\pm$ 0.03; *p* < 0.001; Eth: 1.44 $\pm$ 0.16 vs. 100 nM D₃: 0.71 $\pm$ 0.03; *p* < 0.01) but increased PLA2G4C (Eth: 1.46 $\pm$ 0.01 vs. 100 nM D₃: 2.27 $\pm$ 0.16; *p* < 0.01) and PLAA (C: 0.77 $\pm$ 0.02 vs. 1 nM D₃: 1.44 $\pm$ 0.12; *p* < 0.001; Eth: 0.93 $\pm$ 0.06 vs. 100 nM D₃: 2.27 $\pm$ 0.12; *p* < 0.001) protein expression (Fig. 1b).

Taking these results into consideration, we hypothesized that 1,25(OH)₂D₃-dependent inhibition of UBC

and NF- κ B p65 might lead to downregulation of PLA2G5 and PLA2G15. To further confirm the involvement of UBC and NF- κ B p65 in 1,25(OH)₂D₃-dependent downregulation of PLA2G5 and PLA2G15 in LUVA cells, we utilized the NF- κ B p65 inhibitor – helenalin (Hel) and/or ubiquitination inhibitor – PYR-41 with or without treatment with 1,25(OH)₂D₃. LUVA cells were incubated with Hel (0.1 and 1 μ M) and/or PYR-41 (5 and 10 μ M) for 1 h to inhibit NF- κ B p65 and UBC following 1,25(OH)₂D₃ was added for 4 h. RT-PCR and Western blot analyses were employed to detect the changes in PLA2G5 and PLA2G15 mRNA and protein expression after inhibition of NF- κ B p65 and UBC, respectively.

RT-PCR results showed a significantly decreased mRNA expression of PLA2G5 in response to 1 μ M Hel alone (0.61 $\pm$ 0.04; *p* < 0.05), 1 μ M Hel with 10 μ M PYR-41 (0.36 $\pm$ 0.05; *p* < 0.01), 0.1 μ M Hel with 10 μ M PYR-41 (0.48 $\pm$ 0.08; *p* < 0.05), 1 μ M Hel with 5 μ M PYR-41 (0.70 $\pm$ 0.11; *p* < 0.05), and PLA2G15 in response to 10 μ M PYR-41 alone (0.60 $\pm$ 0.04; *p* < 0.05) and 10 μ M PYR-41 with 1 nM 1,25(OH)₂D₃ (0.68 $\pm$ 0.03; *p* < 0.01) (Fig. 2a, c).

Western blot analysis demonstrated a significantly decreased PLA2G5 protein expression in response to not only 1 μ M Hel alone (0.53 $\pm$ 0.01 vs. C: 1.64 $\pm$ 0.06; *p* < 0.001), 0.1 μ M Hel alone (0.76 $\pm$ 0.03 vs. C: 1.64 $\pm$ 0.06; *p* < 0.01), 10 μ M PYR-41 alone (0.46 $\pm$ 0.02 vs. C: 1.64 $\pm$ 0.06; *p* < 0.001), 5 μ M PYR-41 alone (0.52 $\pm$ 0.03 vs. C: 1.64 $\pm$ 0.06; *p* < 0.01) but also inhibitors combined with 1,25(OH)₂D₃ as well as with inhibitors together.

The protein expression of PLA2G15 also significantly decreased in response to 0.1 μ M Hel alone (1.19 $\pm$ 0.05 vs. C: 2.30 $\pm$ 0.08; *p* < 0.01) or with 1 nM 1,25(OH)₂D₃ (1.24 $\pm$ 0.06 vs. C: 2.30 $\pm$ 0.08; *p* < 0.001), 1 μ M Hel with 1 nM 1,25(OH)₂D₃ (1.24 $\pm$ 0.07 vs. C: 2.30 $\pm$ 0.08; *p* < 0.05), 5 μ M PYR-41 alone (1.45 $\pm$ 0.07 vs. C: 2.30 $\pm$ 0.08; *p* < 0.05) or with 1 nM 1,25(OH)₂D₃ (1.22 $\pm$ 0.06 vs. C: 2.30 $\pm$ 0.08; *p* < 0.01), 10 μ M PYR-41 with 1 nM 1,25(OH)₂D₃ (1.17 $\pm$ 0.04 vs. C: 2.30 $\pm$ 0.08; *p* < 0.01), and applied inhibitors together (Fig. 2b, d).

RT-PCR results suggest that 1,25(OH)₂D₃ decreases the expression of PLA2G5 via inhibition of NF- κ B p65 and decreases the expression of PLA2G15 via inhibition of UBC. Western blot analysis indicated that 1,25(OH)₂D₃ decreases the expression of both PLA2G5 and PLA2G15 via inhibition of NF- κ B p65 and inhibition of UBC. 1,25(OH)₂D₃ had no effect on PLA2G5 or PLA2G15 mRNA or protein after inhibition of NF- κ B p65 and UBC. These results suggest that 1,25(OH)₂D₃ decreases PLA2G5 and PLA2G15 expression via inhibition of NF- κ B p65 and UBC.

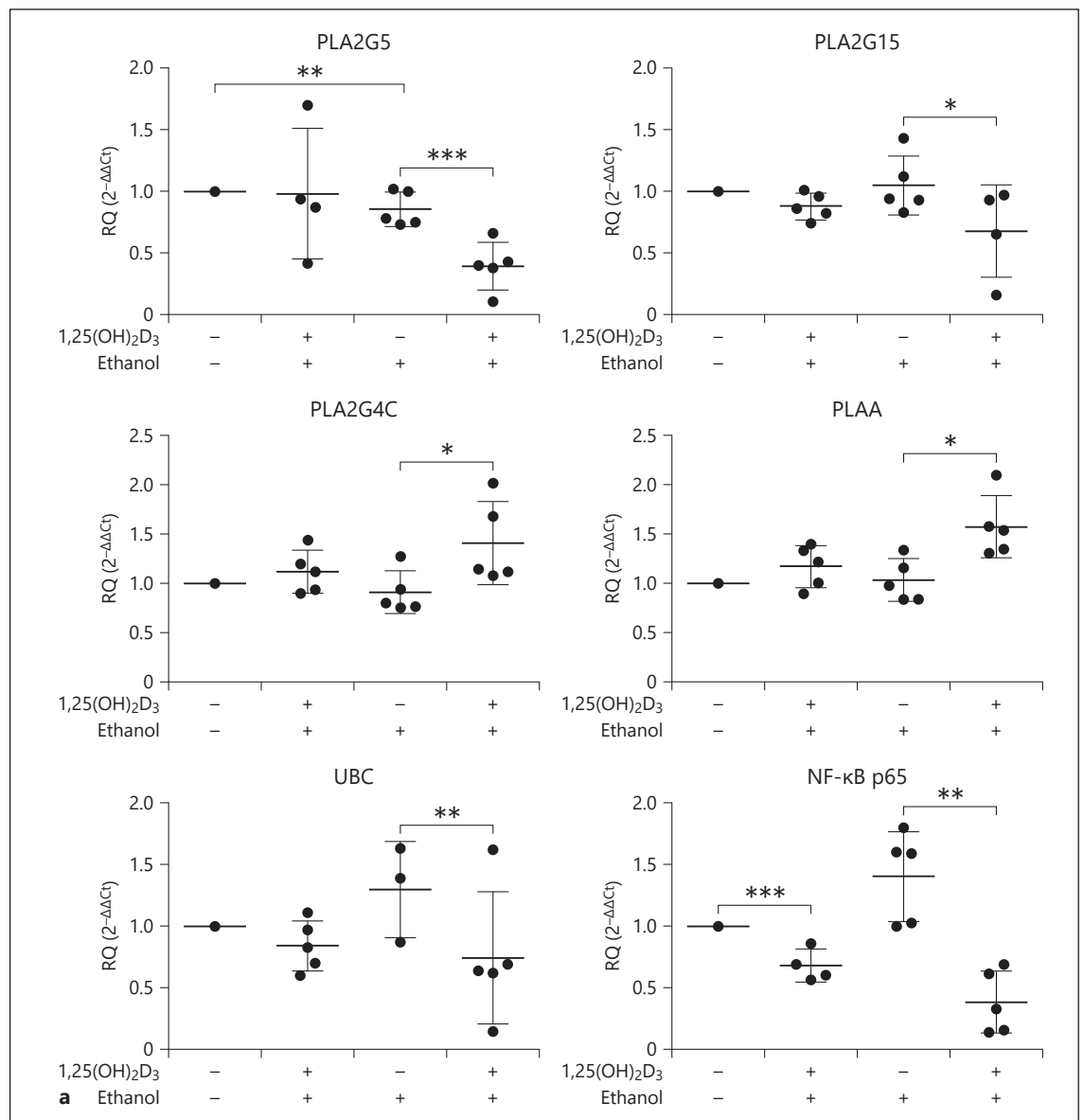
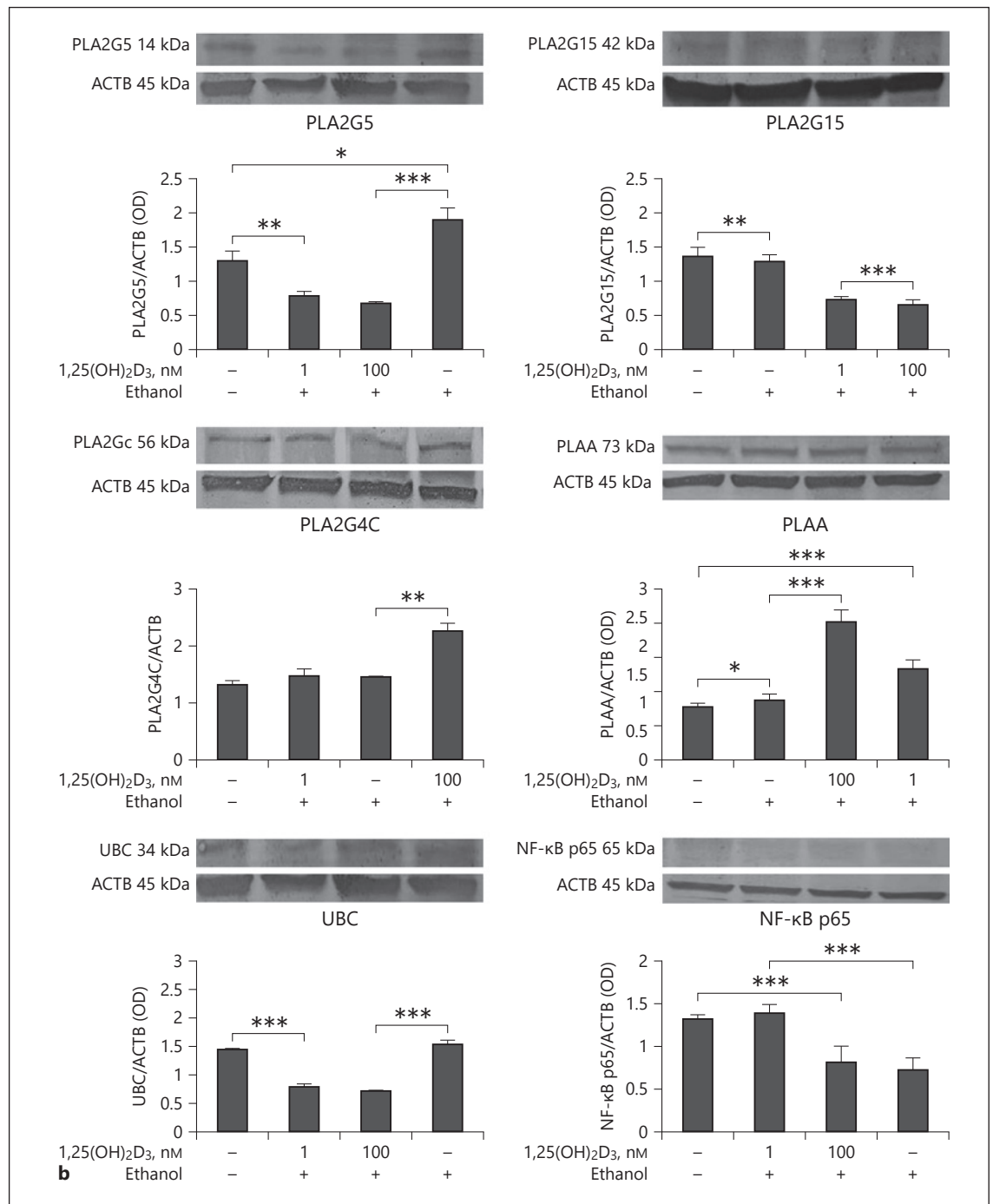


Fig. 1. 1,25(OH)₂D₃ decreased PLA2G5, PLA2G15, UBC, and NF-κB p65 expression but increased PLA2G4C and PLAA expression in LUVA mast cells. LUVA cells were incubated with 1 and 100 nM 1,25(OH)₂D₃ for 4 h, and subsequently PLA2G5, PLA2G15, UBC, NF-κB p65, PLA2G4C, and PLAA mRNA levels were determined by RT-PCR (a). PLA2G5, PLA2G15, UBC, and NF-κB p65 protein levels were determined by Western blot (b). Nontreated cells were used as a control for 1 nM 1,25(OH)₂D₃, whereas ethanol-treated cells were used as a control for 100 nM 1,25(OH)₂D₃. The results of RT-PCR were expressed as a relative expression in relation to ACTB. The fold change of mRNA expression for each gene was calculated as RQ = (2^{-ΔΔCt}). The data are presented as RQ mean ± SEM. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001. The results of immunoblotting are expressed as a percentage of optical density (OD) over the background ratio of target protein expression to ACTB. Data are presented as OD means ± SEM. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001 as compared to controls. (Figure continued on next page.)

1,25(OH)₂D₃ Decreases the Enzymatic Activity of sPLA₂s, but Not cPLA₂s without the Inhibition of UBC and NF-κB p65 in LUVA Cells

We examined the effect of 1,25(OH)₂D₃ on the overall

enzymatic activity of cPLA₂s in lysed LUVA cells and sPLA₂s in LUVA cell culture supernatants. LUVA cells were incubated for 4 h in the presence of 1,25(OH)₂D₃ (1 and nM), and the enzymatic activity of cPLA₂s and sPLA₂s



was determined in lysed cells and cell culture supernatants, respectively. Enzymatic activity evaluation revealed that 100 nM 1,25(OH)₂D₃ significantly decreased the overall enzymatic activity of sPLA₂s (Eth: 0.54 ± 0.02 vs. 100 nM D₃: 0.25 μmol/min/mL ± 0.02; *p* < 0.001) in cell culture supernatants but had no effect on the overall

enzymatic activity of cPLA₂s in lysed LUVA cells (Fig. 3a, c).

Additionally, we determined the effect of inhibition of NF-κB p65 and the UBC on the overall enzymatic activity of cPLA₂s and sPLA₂s. LUVA cells were incubated with Hel (0.1 and 1 μM) and/or PYR-41 (5 μM; 10 μM)

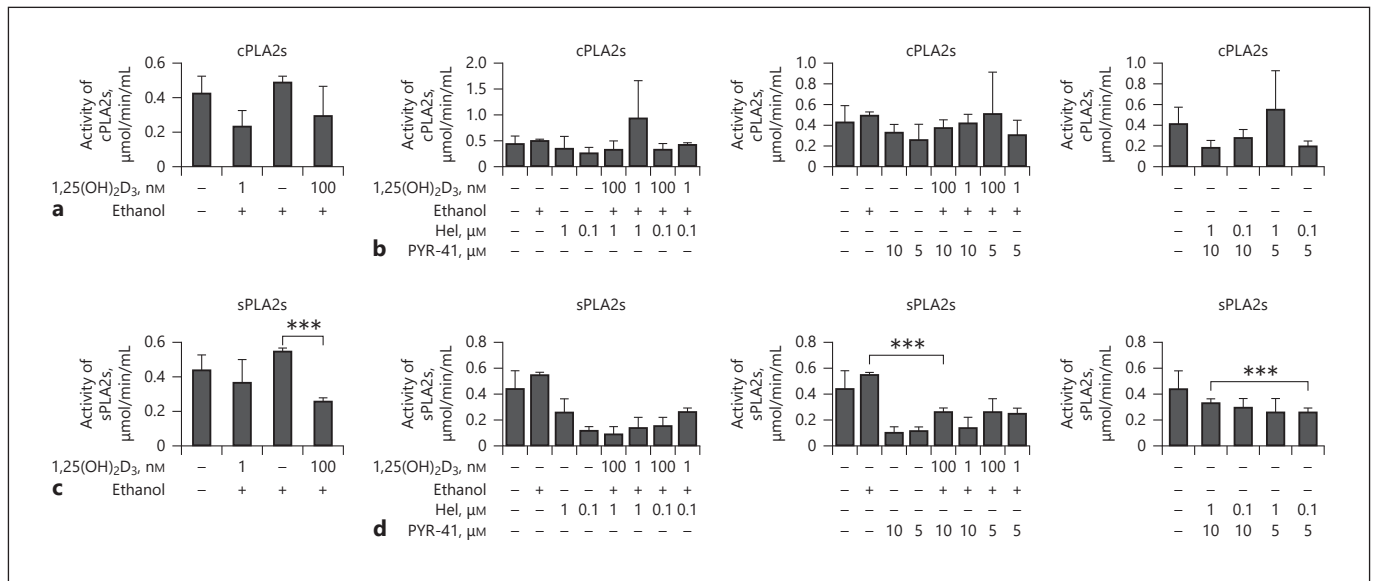


Fig. 3. 1,25(OH)₂D₃ decreased the overall enzymatic activity of sPLA₂s but not cPLA₂s without influence of inhibition of UBC and NF-κB p65 in LUVAs cells. LUVAs cells were incubated with 1 and 100 nM 1,25(OH)₂D₃ for 4 h, and the overall enzymatic activity of cPLA₂s in lysed LUVAs cells and overall enzymatic activity of sPLA₂s in cell-culture supernatants were determined. 1,25(OH)₂D₃ decreased the overall enzymatic activity of sPLA₂s but had no effect on the overall enzymatic activity of cPLA₂s (**a**, **c**). Inhibition of NF-κB p65 and or UBC had no effect on the enzymatic activity of cPLA₂s in lysed LUVAs cells and sPLA₂s in LUVAs cell culture su-

pernatants (**b**, **d**). LUVAs cells were incubated with inhibitor of NF-κB p65 – 0.1 μM; 1 μM Hel and/or inhibitor of ubiquitination – 5 μM; 10 μM PYR-41 with or without 1 nM; 100 nM 1,25(OH)₂D₃, and the overall enzymatic activity of cPLA₂s in lysed LUVAs cells and the overall enzymatic activity of sPLA₂s in cell culture supernatants were determined. Nontreated cells were used as a control for 1 nM 1,25(OH)₂D₃, whereas ethanol-treated cells were used as a control for 100 nM 1,25(OH)₂D₃. Data are presented as means ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

1,25(OH)₂D₃ Increases LXA4 and 15(S)-HETE Levels but Decreases the LTC4 Level and Has No Effect on PGE2 Levels in LUVAs Cells

We also evaluated the effect of 1,25(OH)₂D₃ on the levels of selected eicosanoids, i.e., LTC₄, LXA₄, 15(S)-HETE, and PGE₂, in LUVAs cells. LUVAs cells were incubated for 4 h in the presence of 1,25(OH)₂D₃ (1 and 100 nM), and the levels of LTC₄, LXA₄, 15(S)-HETE, and PGE₂ were determined in cell culture supernatants, respectively.

The results of ELISA tests demonstrated that only 100 nM 1,25(OH)₂D₃ significantly decreased LTC₄ levels (Eth: 1,657 pg/mL ± 129.87 vs. 100 nM D₃: 1,234.5 pg/mL ± 82.41; $p < 0.05$) but increased LXA₄ (Eth: 164.15 pg/mL ± 61.84 vs. 100 nM D₃: 377.38 pg/mL ± 23.19; $p < 0.05$) (Fig. 4a, c). Moreover, 1,25(OH)₂D₃ increased the 15(S)-HETE level in a dose-dependent manner (C: 1,825 pg/mL ± 59.39 vs. 1 nM D₃: 2,997.5 pg/mL ± 285.39; $p < 0.01$; Eth: 2,235 pg/mL ± 346.84 vs. 100 nM D₃: 4,132.5 pg/mL ± 199.55; $p < 0.01$) (Fig. 4e) but had no effect on the PGE₂ level (Fig. 4g).

Additionally, we determined the effect of inhibition of NF-κB p65 and inhibition of UBC on the levels of the selected eicosanoids. LUVAs cells were incubated with Hel (0.1 and 1 μM) and/or PYR-41 (5 μM; 10 μM) for 1 h to inhibit NF-κB p65 and UBC followed by 1,25(OH)₂D₃ exposure for 4 h, respectively.

Inhibition of NF-κB p65 by 1 μM Hel alone significantly increased the LXA₄ level (455.41 ± 50.5 vs. 157.88 pg/mL ± 16.86; $p < 0.05$) (Fig. 4d). Moreover, 15(S)-HETE levels were significantly increased in LUVAs cell culture supernatants in response to 10 μM PYR-41 with 1 nM 1,25(OH)₂D₃ (2,402 ± 83.16 vs. C: 1,850 pg/mL ± 51.06; $p < 0.05$) but decreased in response to 5 μM PYR-41 with 0.1 μM Hel (1,554 pg/mL ± 127.89) vs. 10 μM PYR-41 with 0.1 μM Hel (2,518 pg/mL ± 118.77; $p < 0.05$) and 10 μM PYR-41 with 1 μM Hel (2,652 pg/mL ± 106.64; $p < 0.05$) (Fig. 4f). Thus, NF-κB p65 inhibition increased the level of LXA₄ levels (Fig. 4d).

Inhibition of UBC has no effect on the level of produced 15(S)-HETE because 1 nM 1,25(OH)₂D₃ also significantly increased the 15(S)-HETE level (Fig. 4f). No sig-

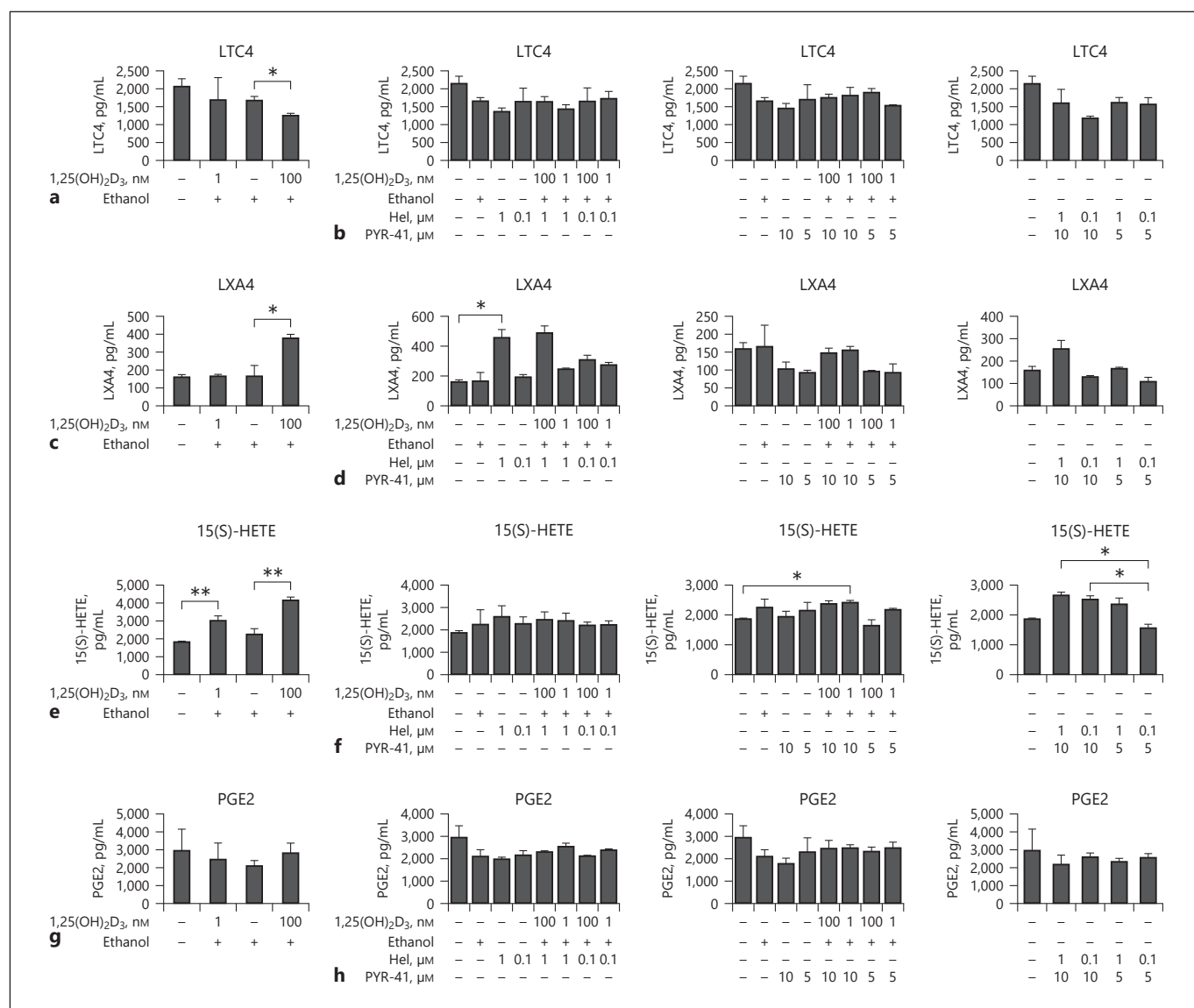


Fig. 4. 1,25(OH)₂D₃ increased the LXA4 and 15(S)-HETE levels but decreased the level of produced LTC4 and had no effect on the PGE2 level in LUVa cells. LUVa cells were incubated with 1 and 100 nM 1,25(OH)₂D₃ for 4 h, and LTC4, LXA₄, 15(S)-HETE and PGE2 levels were determined in cell culture supernatants. Nontreated cells were used as a control for 1 nM 1,25(OH)₂D₃, whereas ethanol-treated cells were used as a control for 100 nM 1,25(OH)₂D₃. 1,25(OH)₂D₃ decreased the levels of produced LTC4 and increased LXA4 and 15(S)-HETE levels but had no effect on the level of

PGE2 in LUVa cells (**a, c, e, g**). Incubation of LUVa cells with inhibitor of NF-κB p65 – 0.1 μM; 1 μM Hel and/or inhibitor of ubiquitination – 5 μM; 10 μM PYR-41 with or without 1 nM; 100 nM 1,25(OH)₂D₃ showed that inhibition of NF-κB p65 decreases the LXA4 level but has no effect on LTC4, 15(S)-HETE, or PGE2 levels in LUVa cells (**b, d, f, h**). Nontreated cells were used as a control for 1 nM 1,25(OH)₂D₃, whereas ethanol-treated cells were used as a control for 100 nM 1,25(OH)₂D₃. Data are presented as means ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

nificant changes in the LTC4 and PGE2 levels were observed after LUVa cell treatment with Hel and/or PYR-41 with or without 1,25(OH)₂D₃ compared to the controls. (Fig. 3b, h). These results suggest that inhibition of ubiquitination and NF-κB p65 have no influence on PGE2, LTC4, and 15(S)-HETE levels (Fig. 3b, f, h).

Discussion

Vitamin D deficiency is associated with increased airway hyperresponsiveness, lower pulmonary functions, worse asthma control, and possibly steroid resistance; therefore, it has been suggested that there might be a

cause-effect relationship of vitamin D deficiency and the increased incidence of asthma [10]. 1,25(OH)₂D₃ has been shown to regulate the NF-κB signaling pathway via binding to VDR and thereby specifically modulate the biological functions of various cell types [40].

In our study, contrary to those results, we demonstrate that 1,25(OH)₂D₃ significantly decreased NF-κB and UBC on mRNA protein levels in human mast cells.

These results are in agreement with previous studies [37, 40–43]. NF-κB is a key player in regulating many pathways in asthma and other inflammatory diseases and may provide a theoretical basis for the clinical treatment of asthma with 1,25-(OH)₂D₃. 1,25(OH)₂D₃ suppression of NF-κB activation has a great biological and pathological relevance. As an example, vitamin D levels are associated with lung function and steroid responses in adult asthma and can modulate T helper type 2-driven asthmatic responses [44]. Moreover, Xystrakis et al. [45] showed that adding vitamin D to cell cultures increased glucocorticoid-induced secretion of IL-10 by Tregs, with similar effects *ex vivo* in patients with steroid-resistant asthma. Studies of adults and children demonstrated that a low vitamin D level is associated with a decreased *in vitro* steroid response, increased steroid use, and impaired lung function [46, 47]. Several clinical and observational studies have suggested a synergistic effect of vitamin D and corticosteroids in asthma outcomes [46, 48, 49]. The results of Litonjua [50] strengthen the notion that vitamin D enhances corticosteroid action and potentially reverses steroid resistance, a crucial topic for asthmatic patients.

Activation of NF-κB involves at least 3 types of ubiquitin chains. They contribute to induction of phosphorylation and activation of IKK, resulting in phosphorylation of the inhibitor of NF-κB (IκB) for subsequent K48-linked polyubiquitylation and degradation, a critical event in releasing active NF-κB for gene regulation [51]. Therefore, ubiquitination of NF-κB and ubiquitin expression seem to be an important point for regulation of the pathways regulated by NF-κB. In our study, UBC expression was decreased by vitamin D; moreover, inhibition of ubiquitin C caused a decrease in PLA2G5 and PLA2G15 expression. These results show an important role of ubiquitin expression that determines the regulatory function NF-κB and influences many mechanisms. They seem to be particularly interesting as phospholipases are involved in the pathogenesis of asthma. The proposed mechanism of action of phospholipases A₂ in inflammatory diseases includes the liberation of arachidonic acid, generation of lysophospholipids, interaction between enzymes belonging to the A₂ superfamily, surfactant degradation, release

of cytokines, and the impact on immunological and inflammatory cells (dendritic cells, T cells, and leukocytes) [52, 53]. On the other hand, in the current study we demonstrated an increase in PLA2G4C expression in LUVa mast cells after 1,25(OH)₂D₃ treatment. These results might show that the regulation of different phospholipases can be differentiated within one group. In our studies, inhibition of NF-κB did not affect the expression of the PLA2G4 gene. It is known, however, that PLA2G4C expression is induced by TNF-α [54] and that this phospholipase elevates the spontaneous release of AA [55]. Together, this may indicate that the potency of proinflammatory action is different among phospholipases, but further studies are necessary to study this phenomenon.

Although the relative contributions of cPLA₂ and sPLA₂ differ between cells and tissues, it is generally accepted that both types take part and often act synergistically in producing proinflammatory lipid mediators. Inhibition of PLA₂ activity has been considered a promising therapeutic strategy in the treatment of inflammatory/allergic diseases, as this can suppress the production of several classes of pathogenic lipid mediators (prostaglandins, leukotrienes, platelet activation factor, and lysophospholipids) [56].

Interestingly, phospholipase A₂ does not merely take part in the general lipid metabolism; it also has important roles in signal transduction. According to Sylvia et al. [57], phospholipase C has been shown to modulate signal transduction through production of DAG and inositol-1,4,5-trisphosphate (IP₃), resulting in rapid Ca²⁺ flux [58]. After exposure to 1,25(OH)₂D₃, arachidonic acid release and Ca²⁺ influx occurred in chondrocytes in their study, suggesting that phospholipase A₂ and phospholipase C may mediate the effect of 1,25(OH)₂D₃ on protein kinase C (PKC) [57]. That means that our results are only a link in the chain, and the effect of 1,25(OH)₂D₃ on phospholipases observed has downstream effects, too. Moreover, activation of PKC in turn mediates the signal transduction pathway which plays a critical role in lymphocyte infiltration and the onset of airway hypersensitivity, airway remodeling, and asthma pathophysiology.

Several *in vitro* and *in vivo* studies support the role of sPLA₂s as proinflammatory mediators [59–62]. This is due to the fact that sPLA₂s induce a multiplicity of cellular responses because of their enzymatic activity and capacity to activate proinflammatory cells by nonenzymatic mechanisms. sPLA₂s are known to act within the cell as well as following secretion in the extracellular environment, thereby contributing to the mobilization of arachi-

donic acid which is converted into eicosanoids (e.g., prostaglandins, thromboxanes, and leukotrienes) [63]. In our study $1,25(\text{OH})_2\text{D}_3$ decreased the level of sPLA₂s enzymatic activity. These results indicate that $1,25(\text{OH})_2\text{D}_3$ may regulate both enzymatic and signal transduction activities of PLA₂s. Elevated secreted phospholipase A₂ contribute in the airways to the pathogenesis of asthma and allergic disease. The enzyme is expressed in epithelial cells, macrophages, and eosinophils in the airways, but it is also elevated in the airway fluid of asthmatics and it is linked to a many asthma features [23, 64–66]. Studies suggests that sPLA₂ can be a key checkpoint which leads to the overproduction of eicosanoids in asthma and can be upregulated by allergen or exercise challenge, as they demonstrated total sPLA₂ activity is elevated in asthmatic airways and that allergen challenge in both the upper and the lower airways leads to an increase in sPLA₂ activity accompanied by AA release and eicosanoids biosynthesis [64, 67, 68]. Therefore the results observed in our study, showing that $1,25(\text{OH})_2\text{D}_3$ decreased the sPLA₂s enzymatic activity, seem to show a promising direction for future therapies of asthma and inflammatory diseases.

There are studies indicating that PLA₂ plays an important role in $1\alpha,25(\text{OH})_2\text{D}_3$ -activated membrane-mediated signaling [69–71]. Phospholipases A₂ activation is stimulated by PLA₂ activating protein (PLAA) [72]. PLAA is a signaling molecule that regulates eicosanoid production and participates in inflammatory responses. Doroudi et al. [69] showed that silencing *Plaa* significantly reduced CaMKII and PLA₂ activity in response to $1\alpha,25(\text{OH})_2\text{D}_3$ treatment [72]. CaMKII in turn, when oxidized, promotes asthma through the activation of mast cells [73]. In our study, PLAA expression was increased by $1\alpha,25(\text{OH})_2\text{D}_3$ in human mast cells. These results might be interesting in the context of the study of Zhang et al. [74], as it demonstrated that overexpression of the *plaa* gene promotes the production of PGE₂, enhanced activation of NF- κ B and cPLA₂, and increased expression of COX-2, induced by TNF- α . Given that induction of PLA₂ and COX-2 results in increased production of PGE₂, these results are contrary to those of our study, as we observed increased but also decreased expression of various phospholipases analyzed after $1\alpha,25(\text{OH})_2\text{D}_3$ stimulation. Moreover, we evaluated PGE₂ but no significant results were noticed. We speculate that this was due to differences in cell lines but also the presence of $1\alpha,25(\text{OH})_2\text{D}_3$ stimulation which might indicate that PLAA can act through different pathways. Induction of PLAA can modulate inflammatory responses, as has been previously shown by Bomalaski et al. [75], and treatment

of human neutrophils with PLAA polypeptide increases neutrophil degranulation and the production of lysosomal enzymes and superoxide anions.

As has been demonstrated, PLAA plays a role in protein ubiquitination and sorting and degradation through its association with VCP [76]. It regulates NF- κ B-mediated inflammatory responses and in particular inducible IL-6. It is probable that PLAA could modulate NF- κ B activity through ubiquitin signaling [74, 77]. These interesting examples show that vitamin D, as the inducer of PLAA expression, also takes part in proinflammatory and oxidative related pathways.

Arachidonic acid released by cytosolic form of phospholipase A₂ – cPLA₂ – in response to cell activation is subsequently metabolized by COX or lipoxygenases to prostaglandins or hydroxyeicosatetraenoic acids (HETE), respectively. Further metabolism of HETE leads to generation of leukotrienes and/ or lipoxins (LX) [78].

15-HETE, a metabolic product of arachidonic acid catalyzed by 15-lipoxygenase (15-LO). Increased amounts of 15-HETE have been found in bronchoalveolar lavage fluid and sputum from asthmatic patients compared to control subjects [79]. Furthermore, 15-HETE levels showed a significant correlation with lung function [80]. In the present study, 15-(S)-HETE was reduced in mast cells by $1,25(\text{OH})_2\text{D}_3$. In turn, LTC₄ was induced in these cells, which is in agreement with the previous study [11]. We speculate that these results might be connected to NF- κ B – it is known that arachidonic acid metabolic products of 5-lipoxygenase and 12-lipoxygenase are also regulated by NF- κ B [81]. In addition, in myotube and human adherent monocytes, 15-LO/15-HETE signaling was observed to induce the activation of NF- κ B [82, 83]. Thus, the relationship between 15-LO/15-HETE and NF- κ B seems to be complicated [81].

Somjen et al. [84] demonstrated that expression of the lipoxygenases is modulated by vitamin D metabolites and analogs. The expression of these enzymes results in the secretion of 15 HETE in bone cells. Moreover, the generation of 15-HETE, which is driven by vitamin D metabolites and analogs, is linked to vitamin D dependence in these cells. It is interesting that 12- and 15-HETE increased local oxidative stress in that study [84]. Nevertheless, as 15-HETE levels are higher in asthma [85], our results confirm the anti-inflammatory nature of $1,25(\text{OH})_2\text{D}_3$, showing that further studies are needed to explore this issue.

Similar data were obtained in our studies concerning LXA₄. LX, including LXA₄, have been approved for their potent anti-inflammatory and immunomodulatory prop-

erties. In addition, the level of LX is decreased in a lot of chronic inflammatory disorders, such as cystic fibrosis, asthma, colonitis, rheumatoid arthritis, etc., and exogenous addition of LX could markedly improve pathogenetic conditions [86–88], suggesting that insufficient LX is an important mediator for these chronic diseases [89].

Furthermore, accumulating evidence indicates that LX might be involved in regulation of the immune response [90–92]. Loss of control of the immune response is the primary cause of inflammatory diseases; especially the infiltrative cells in chronic inflammatory focus were mainly macrophages and lymphocytes [89, 93].

In the present study $1,25(\text{OH})_2\text{D}_3$ increased LXA4 in mast cells, and the same effect was observed after NF- κB inhibition. As the previous study demonstrated that LXA4 inhibits NF- κB [89, 94–96], these data suggest that there might be a feedback in this action. The anti-inflammatory effects of LXA4 may be dependent on suppression of NF- κB activation, as NF- κB activation is the rate-controlling step for inflammatory processes [95].

Conclusion

Despite the increased knowledge gained from research, vitamin D insufficiency and deficiency are still prevalent in many developed countries. A large body of literature suggests that vitamin D acts on cells of the innate and adaptive immune systems as well as structural cells in the airways, with its deficiency promoting inflammation and its supplementation alleviating these effects. Vitamin D levels are associated with lung function and steroid responses in adult asthma and can modulate T helper type 2-driven asthmatic responses. Allergic asthma exhibits a crucial interplay of innate and adaptive immune functions, where NF- κB is considered as the master regulator of both innate and adaptive immune responses. During the pathogenesis of asthma, NF- κB may act at different levels of airway remodeling, such as regulation of ASMC proliferation and their expression of inflammatory cytokines. The exact molecular mechanism underlying $1,25(\text{OH})_2\text{D}_3$ regulation of NF- κB , remains to be defined; nevertheless, its regulation may be a powerful tool, at least as a complement, of classical therapy. Asthma has become one of the most prevalent diseases worldwide, constituting a major public health concern. Among nutritional hypotheses, vitamin D status is of particular interest regarding the controversial beneficial effects in nonskeletal disorders such as asthma but also cardiovascular disease, cancer, schizophrenia, and multiple sclerosis [97–100].

Evidence suggests there may be a cause-effect relationship with vitamin D deficiency and the increased incidence of asthma because vitamin D deficiency is associated with increased airway hyperresponsiveness, lower pulmonary functions, worse asthma control, and possibly steroid resistance [47, 101, 102].

The effect of vitamin D supplementation on patients with bronchial asthma is still controversial, as the literature shows different results, e.g., that vitamin D supplementation was efficacious in the prevention of asthma exacerbation [103] while another study [104] reported inconsistent results.

Nevertheless, a meta-analysis from 14 studies elucidated that vitamin D supplementation played a role in reducing the rate of asthma exacerbation, particularly in patients with a vitamin D insufficiency [105]. This confirms the novelty and importance of the topic and the need for further analyses.

In conclusion, in this study we demonstrated the differential effects of $1,25(\text{OH})_2\text{D}_3$ on chosen phospholipases A_2 and PLAA as well as NF- κB . This study suggests that vitamin D has no one-way effect and its activity in the pathways of inflammatory processes can be unexpected. There is a need to further investigate the exact action of this compound. However, our research suggests that there is a potential for use it in the treatment of asthma and inflammatory diseases.

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Statement of Ethics

No ethical approval was required as the work was done on an LUVA mast cell line.

Disclosure Statement

The authors declare no conflict of interest.

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