Journal of Steroid Biochemistry & Molecular Biology 121 (2010) 212–216

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

24R,25-Dihydroxyvitamin D3 [24R,25(OH)$_2$D$_3$] controls growth plate development by inhibiting apoptosis in the reserve zone and stimulating response to 1α,25(OH)$_2$D$_3$ in hypertrophic cells☆

B.D. Boyan[a,b,*], J. Hurst-Kennedy[b], T.A. Denison[a], Z. Schwartz[a,c]

[a] Wallace H. Coulter Department of Biomedical Engineering at Georgia Tech and Emory University, United States
[b] School of Biology, Georgia Institute of Technology, Atlanta, GA, United States
[c] Department of Periodontics, University of Texas Health Science Center at San Antonio, San Antonio, TX, United States

ARTICLE INFO

Article history:
Received 29 October 2009
Received in revised form 13 March 2010
Accepted 15 March 2010

Keywords:
Vitamin D metabolites
24R,25-Dihydroxyvitamin D3
Growth plate cartilage
Chondrocyte apoptosis

ABSTRACT

Previously we showed that costochondral growth plate resting zone (RC) chondrocytes response primarily to 24R,25(OH)$_2$D$_3$ whereas prehypertrophic and hypertrophic (GC) cells respond to 1α,25(OH)$_2$D$_3$. 24R,25(OH)$_2$D$_3$ increases RC cell proliferation and inhibits activity of matrix processing enzymes, suggesting it stabilizes cells in the reserve zone, possibly by inhibiting the matrix degradation characteristic of apoptotic hypertrophic GC cells. To test this, apoptosis was induced in rat RC cells by treatment with exogenous inorganic phosphate (Pi). 24R,25(OH)$_2$D$_3$ blocked apoptotic effects in a dose-dependent manner. Similarly, apoptosis was induced in ATDC5 cell cultures and 24R,25(OH)$_2$D$_3$ blocked this effect. Further studies indicated that 24R,25(OH)$_2$D$_3$ acts via at least two independent pathways. 24R,25(OH)$_2$D$_3$ increases LPA receptor-1 (LPA R1) expression and production of lysophosphatidic acid (LPA), and subsequent LPA R1/3-dependent signaling, thereby decreasing p53 abundance. LPA also increases the Bcl-2/Bax ratio. In addition, 24R,25(OH)$_2$D$_3$ acts by increasing PKC activity. 24R,25(OH)$_2$D$_3$ stimulates 1-hydroxylase activity, resulting in increased levels of 1,25(OH)$_2$D$_3$, and it increases levels of phospholipase A2 activating protein, which is required for rapid 1α,25(OH)$_2$D$_3$-dependent activation of PKC in GC cells. These results suggest that 24R,25(OH)$_2$D$_3$ modulates growth plate development by controlling the rate and extent of RC chondrocyte transition to a GC chondrocyte phenotype.

1. Introduction

Chondrocytes isolated from the resting zone of rat growth plates respond preferentially to the vitamin D metabolite 24R,25-dihydroxyvitamin D3 [24R,25(OH)$_2$D$_3$], with increased alkaline phosphatase activity and [^{35}S]-incorporation, decreased DNA synthesis, and increased neutral matrix metalloproteinase (MMP) activity [1,2]. Similar observations have been made in avian growth plate chondrocytes [3]. 24R,25(OH)$_2$D$_3$-mediated effects are maintained in mice lacking the nuclear vitamin D receptor (nVDR) [4] and are not inhibited by Ab99, a blocking antibody targeted against the 1,25-dihydroxyvitamin D3 [1α,25(OH)$_2$D$_3$] membrane receptor, protein disulfide isomerase family A, member 3 (PDIA3) [5]. Additionally, the effects of 24R,25(OH)$_2$D$_3$ are rapid, inducing protein kinase C (PKC) activation in as little as 9 min [6]. These observations indicate that 24R,25(OH)$_2$D$_3$ acts through a putative membrane-associated receptor (mVDR$_{24,25}$) that is distinct from the 1α,25(OH)$_2$D$_3$-responsivie PDIA3 and functional nVDRs, but do not rule out the possibility that 24R,25(OH)$_2$D$_3$ is acting via a VDR variant.

2. Rapid actions of 24R,25(OH)$_2$D$_3$ in the resting zone

The actions of 24R,25(OH)$_2$D$_3$ in the resting zone chondrocytes are mediated through rapid activation of PKC. 24R,25(OH)$_2$D$_3$ increases the abundance of diacylglycerol (DAG) [6], an activator of many PKC isoforms. PKC activation by 24R,25(OH)$_2$D$_3$ is maintained in the presence of chemical inhibitors targeted against either phosphatidylcholine (PC)- or phosphatidylinositol (PI)-specific phospholipase C (PLC) [7], indicating that the source of DAG is not due to the action of this enzyme. Inhibition of tyrosine kinase signaling also does not attenuate rapid actions of 24R,25(OH)$_2$D$_3$, eliminating tyrosine kinases as a source of

☆ Special issue selected article from the 14th Vitamin D Workshop held at Brugge, Belgium on October 4–8, 2009.

* Corresponding author at: Department of Biomedical Engineering, Georgia Institute of Technology, 315 Ferst Drive NW, Atlanta, GA 30332-0363, United States. Tel.: +1 404 385 4108; fax: +1 404 894 2291.
E-mail address: barbara.boyan@bme.gatech.edu (B.D. Boyan).

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doi:10.1016/j.jsbmb.2010.03.057

PKC activation [6]. Instead, activation of PKC by 24R,25$(OH)_2D_3$ is dependent upon DAG derived from phosphatidic acid (PA) generated the actions of by phospholipase D (PLD), specifically PLD2 [8,9].

A second phospholipid-dependent mechanism also contributes to the rapid actions of 24R,25$(OH)_2D_3$. Inhibition of phospholipase A2 (PLA_2) enhances PKC activation by 24R,25$(OH)_2D_3$, whereas PLA2 activating protein (PLAA) decreases this [10]. This is in contrast to 1,25$(OH)_2D_3$, which elicits its rapid effects via PLA_2-activation [11]. Inhibition of PLA_2 by 24R,25$(OH)_2D_3$ results in a rapid decrease in arachidonic acid (AA) abundance and cyclooxygenase-1 (COX-1) activity [12,13]. Following the initial decrease in PLA2, 24R,25$(OH)_2D_3$ upregulates arachidonic acid turnover [14,15], altering fluidity of the plasma membrane [16], and increasing the production of prostaglandins E1 and E2 (PGE1, PGE2) to induce protein kinase A (PKA) activity [17]. Inhibition of PKA mitigated 24R,25$(OH)_2D_3$-induced rapid signaling and chondrocyte maturation, demonstrating the importance of this signaling pathway [6,17]. Together, activated PKC and PKA promote MEK and ERK1/2 signaling [1] in response 24R,25$(OH)_2D_3$ to induce changes in gene transcription to promote chondrocyte maturation.

3. The role of lysophosphatidic acid signaling

Recently our group has focused the role of lysophospholipids in 24R,25$(OH)_2D_3$-mediated effects in the growth plate. As previously mentioned, 24R,25$(OH)_2D_3$-induced PLD activity results in the production of DAG, which stimulates PKC. Another consequence of PLD activation is the production of lysophosphatidic acid (LPA) [18,19], a bioactive lysophospholipid that has recently been implicated in the regulation of bone and cartilage [20–23]. These findings implicated LPA as a second messenger in 24R,25$(OH)_2D_3$-directed signaling. We found that 24R,25$(OH)_2D_3$ increased the abundance of extracellular LPA and LPA receptor 1 (LPA1) mRNA. Additionally, inhibition of LPA1 and LPA3 attenuated 24R,25$(OH)_2D_3$-induced chondrocyte maturation and cell survival [21]. Resting zone chondrocytes responded to LPA with increased DNA synthesis, alkaline phosphatase activity, and [^{35}S]-incorporation. Furthermore, LPA protected chondrocytes against inorganic phosphate (Pi)-induced apoptosis by activating the phosphoinositol 3-kinase (PI_3K) and murine double minute 2 (mdm2) signaling, resulting in the degradation of p53 and a decrease in p53-mediated transcription. Interestingly, this is the same mechanism by which LPA enhances cell survival in cancer cells [24]. We have also observed that Gβγ-mediated PLC activation also contributes to the inhibition of Pi-induced apoptosis by 24R,25$(OH)_2D_3$ (Boyan Hurst-Kennedy, et al., unpublished data). The stimulation of the pro-survival actions of LPA by 24R,25$(OH)_2D_3$ establishes an anti-apoptotic function for the metabolite.

4. 24R,25$(OH)_2D_3$ and phosphate-induced apoptosis

Pi induces apoptosis in terminally differentiated chondrocytes, allowing for the invasion of blood vessels and the deposition of new bone [25]. Recently, we have observed that resting zone chondrocytes also undergo apoptosis in response to Pi as evidenced by an increase in DNA fragmentation and caspase-3 activity in response to Pi in male and female resting zone chondrocyte cultures [21,26]. Normally the Pi content of the resting zone cartilage matrix is comparatively low whereas in the hypertrophic cell zone, marked increases in 1α,25$(OH)_2D_3$-dependent alkaline phosphatase result in high Pi content. 24R,25$(OH)_2D_3$ causes a small increase in alkaline phosphatase, which may cause an increase in local Pi. Perhaps more importantly, 24R,25$(OH)_2D_3$ stimulates resting zone cells to produce 1α,25$(OH)_2D_3$ by increasing expression of 1-hydroxylase [27,28]. This suggests that Pi-induced apoptosis is dependent on the chondrocyte microenvironment rather than differentiation state.

We have also observed Pi-induced apoptosis in ATDC5 cells, a mouse chondrogenic cell line that exhibits a resting zone chondrocyte-like phenotype as evidenced by expression of collagens II and X, Sox9, and cartilage oligomeric matrix protein (COMP) [2,29]. ATDC5 cells respond to 24R,25$(OH)_2D_3$ with increased alkaline phosphatase activity and decreased cell number [29]. Treatment with Pi increases DNA fragmentation and caspase-3 activity, both of which are mitigated by 24R,25$(OH)_2D_3$. Furthermore, 24R,25$(OH)_2D_3$ attenuates Pi-induced decreases in DNA synthesis and [^{35}S]-incorporation. Taken together, these results support the hypothesis that 24R,25$(OH)_2D_3$ enhances cell survivability in the presence of Pi. Additionally, these data suggest the existence of an inhibitory feedback loop in the resting zone between Pi and 24R,25$(OH)_2D_3$.

5. Materials and methods

5.1. Cell culture

Chondrocytes were obtained from the resting zone (reserve zone) of costochondral cartilage from 125-g male Sprague-Dawley rats and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% antibiotics, and 50 μg/ml ascorbic acid (GIBCO-BRL, Gaithersburg, MD). Primary cells were cultured until fourth passage prior to experimental analysis. The culture system used in this study has been previously described in detail [30]

ATDC5 cells were cultured in a maintenance medium consisting of a 1:1 ratio of DMEM/F12 media (Cellgro, Manassas, VA) with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT), 10 mg/ml human transferrin (Sigma Chemical Company, St. Louis, MO), 1% penicillin–streptomycin (Invitrogen, Carlsbad, CA), and 3×10^{-8} M sodium selenite (Sigma). After reaching confluence, cells were cultured with differentiation media, which is identical to maintenance media with the addition of 10 mg/ml bovine insulin (Sigma) and 50 μg/ml ascorbic acid (Sigma) [31,32]. At 10 days post-confluence, cells were cultured for 24 h in differentiation media supplemented with Pi (0–20 mM beyond media basal level) and 10% FBS (10% FBS was used to ensure sufficient serum proteins such as fetuin that help regulate pathologic precipitation of calcium phosphate crystals [33,34]).

5.2. Caspase-3 activity

The role of 24R,25$(OH)_2D_3$ in resting zone chondrocyte survival was assessed by examining its ability to reduce caspase-3 activity induced by inorganic phosphate (Pi) [35,36]. Confluent cultures of resting zone chondrocytes were treated for 24 h with 7.5 mM monobasic sodium phosphate to induce apoptosis. At the same time, cells were treated with 24R,25$(OH)_2D_3$ (10^{-7} M). VPC32183(S) (10^{-8} to 10^{-6} M, Avanti Polar Lipids, Alabaster, AL) was used to inhibit LPA1/3 signaling. U73122 (10 μM, Sigma, St. Louis, MO) was used to inhibit PC-PLC. Thapsigargin (3 μM, Sigma, St. Louis, MO) was used to block release of calcium from the endoplasmic reticulum, while wortmannin (10 μM, Calbiochem, Gibbstown, NJ) was used to inhibit PLD and PI_3K signaling. Caspase-3 activity was determined using the colorimetric CaspACE™ Assay System from Promega (Madison, WI). Cells were harvested 24 h post treatment with 200 μl cell lysis buffer followed by two 10 s periods of sonication. After harvest, 2 μl of the caspase-3 selective substrate DEVD-pNA were added to each well containing 100 μl of cell lysate and incubated at 37 °C for 4 h. DEVD-pNA cleavage into the colorimetric product pNA was measured at 405 nm. Caspase-3

activity was normalized to protein content as determined by the Pierce Macro BCA Protein Assay Kit.

5.3. Chondrocyte maturation

Alkaline phosphatase [orthophosphoric monoester phosphohydrolase, alkaline]-specific activity was used as an indication of chondrocyte differentiation. Confluent cultures of ATDC5 cells were treated with vehicle alone (control) or treated with 24R,25$(OH)_2D_3$ (10^{-7} M) and/or 20 mM Pi. Harvested cells were suspended in 0.05% Triton X followed by three freeze–thaw cycles to lyse the cells. Alkaline phosphate activity was measured in cell layer lysates as a function of release of *para*-nitrophenol from *para*-nitrophenylphosphate at pH 10.2. Activity was normalized to the protein concentration of the lysates, determined using the macro-BCA assay (Macro BCA, Pierce Chemical Co., Rockford, IL).

5.4. DNA fragmentation

Regulation of Pi-induced apoptosis by 24R,25$(OH)_2D_3$ in ATDC5 cells was assessed by examining DNA fragmentation. Confluent cultures of ATDC5 cells were treated with vehicle alone (control), 24R,25$(OH)_2D_3$ (10^{-7} M), Pi (20 mM), or a combination of the aforementioned. Cells were labeled with [^{3}H]-thymidine for 4 h prior to treatment. At the end of the treatment period, cell monolayers were washed with DMEM three times to remove unincorporated [^{3}H] and cells were lysed with TE buffer (10 mM Tris–HCl, 1 mM EDTA, 0.2% Triton X-100) for 30 min. Cell lysates were centrifuged at 13,000 × *g* for 15 min to separate intact DNA from fragmented DNA. The amount of incorporated [^{3}H]-thymidine was determined in each fraction to establish the total amount of fragmented DNA.

5.5. Statistical analysis

Each experiment had six independent cultures per variable to ensure sufficient power to detect statistically significant differences. All experiments were conducted multiple times to validate the observations, but data from a single representative experiment are shown in the figures and are expressed as means ± SEM. Statistical analysis was conducted using ANOVA analysis followed by Student's *t*-test with a Bonferroni modification. Differences in means were considered to be statistically significant if the *p* value was less than 0.05.

6. Results

6.1. 24R,25$(OH)_2D_3$ inhibits apoptosis in the resting zone through LPA, PLC, PLD, and calcium signaling

Pi treatment increased caspase-3 activity in the male rat resting zone chondrocytes relative to untreated control (Fig. 1). The addition of 24R,25$(OH)_2D_3$ reduced caspase-3 activity to basal level. The LPA1/3 receptor antagonist VPC32183(S) inhibited 24R,25$(OH)_2D_3$-mediated rescue of Pi-induced apoptosis at a dose of 1 μM. The PC-PLC inhibitor U73122, the intracellular calcium inhibitor thapsigargin, and the PLD/PI_3K inhibitor wortmannin also inhibited the reduction of caspase-3 activity by 24R,25$(OH)_2D_3$ (Fig. 2).

6.2. Pi modulates ATDC5 responsiveness to 24R,25$(OH)_2D_3$

24R,25$(OH)_2D_3$ did not induce an increase in alkaline phosphatase activity in ATDC5 cells that were not pre-treated with Pi (Fig. 3A). However, ATDC5 cells that were pre-treated with Pi did exhibit an increase in alkaline phosphatase activity in response to 24R,25$(OH)_2D_3$. Pi treatment increased DNA fragmentation relative to untreated control (Fig. 3B). 24R,25$(OH)_2D_3$ attenuated the increase in DNA fragmentation levels caused by Pi.

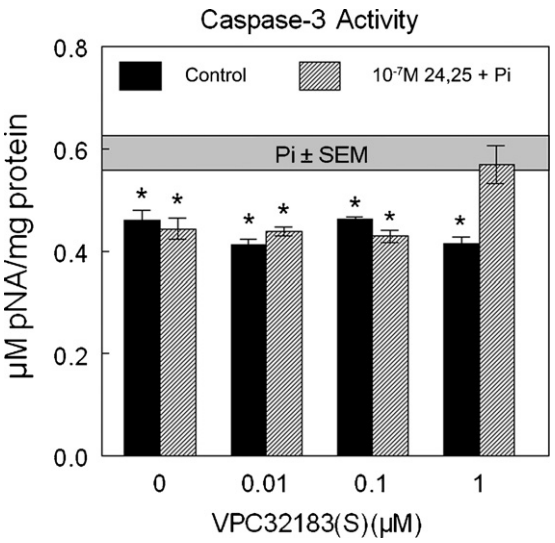

Fig. 1. LPA1/3 signaling is necessary for 24R,25$(OH)_2D_3$-mediated rescue of Pi-induced apoptosis. Confluent cultures of male rat resting zone chondrocytes were treated were mocked-treated (control) or treated with 24R,25$(OH)_2D_3$ (10^{-7} M), Pi (7.5 mM), VPC32183(S) (0.01–1 μM), or a combination of the aforementioned for 24 h. Caspase-3 activity was measured using Promega colorimetric CaspACE™ Assay System and normalized to total protein. Horizontal gray bar = Pi alone treatment ± SEM; $^*p < 0.05$ relative to Pi alone treatment.

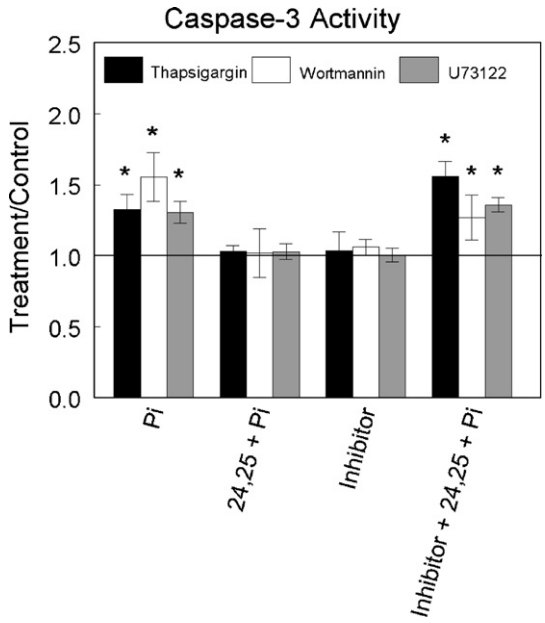

Fig. 2. 24R,25$(OH)_2D_3$ reduces caspase-3 activity through PLD, PLC, and calcium signaling. Confluent cultures of male rat resting zone chondrocytes were treated for 24 h with 7.5 mM monobasic sodium phosphate, 24R,25$(OH)_2D_3$ (10^{-7} M), thapsigargin (3 μM), U73122 (10 μM), wortmannin (10 μM), or a combination of the aforementioned. Caspase-3 activity was measured using Promega colorimetric CaspACE™ Assay System and normalized to total protein. Horizontal black line = untreated control; $^*p < 0.05$ relative to untreated control.

7. Summary

In summary (Fig. 4), 24R,25$(OH)_2D_3$ regulates less mature growth plate chondrocytes through rapid activation of $mVDR_{24,25}$. This results in PLA2 and PLD-mediated phospholipid metabolism and activation of PKC to induce chondrocyte maturation. Our

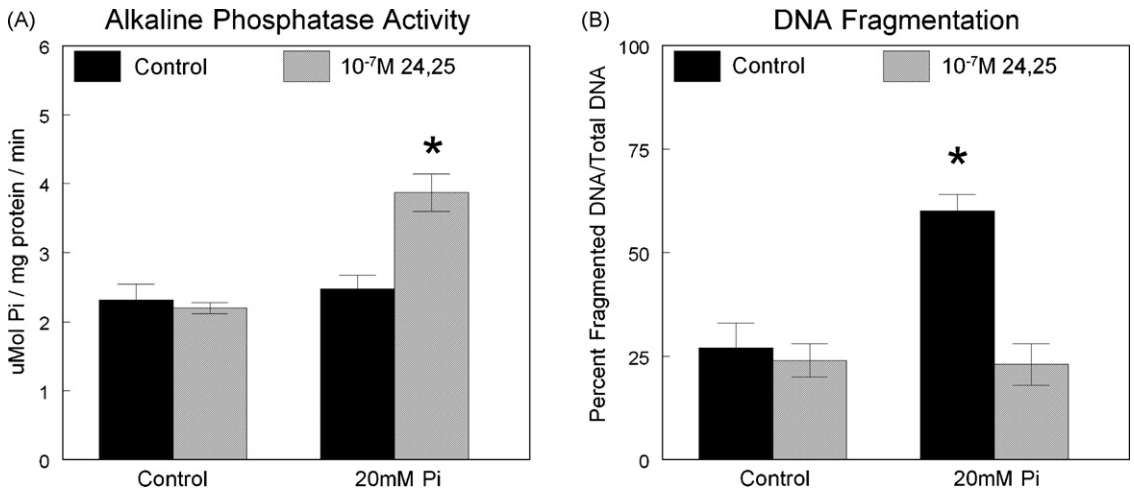

Fig. 3. Pi modulates ATDC5 responsiveness to 24R,25$(OH)_2D_3$. Confluent cultures of ATDC5 cells were treated with vehicle alone (control) or treated with 24R,25$(OH)_2D_3$ (10^{-7} M) and/or 20 mM Pi. (A) Alkaline phosphate activity was measured in cell layer lysates as a function of release of *para*-nitrophenol from *para*-nitrophenylphosphate at pH 10.2. Activity was normalized to the protein concentration of the lysates. (B) DNA fragmentation: cells were labeled with [^{3}H]-thymidine for 4 h prior to treatment. At the end of the treatment period, cell monolayers were washed with DMEM three times to remove unincorporated [^{3}H] and cells were lysed with TE buffer (10 mM Tris–HCl, 1 mM EDTA, 0.2% Triton X-100) for 30 min. Cell lysates were centrifuged at 13,000 × *g* for 15 min to separate intact DNA from fragmented DNA. The amount of incorporated [^{3}H]-thymidine was determined in each fraction to establish the total amount of fragmented DNA. $^*p < 0.05$ relative to untreated control.

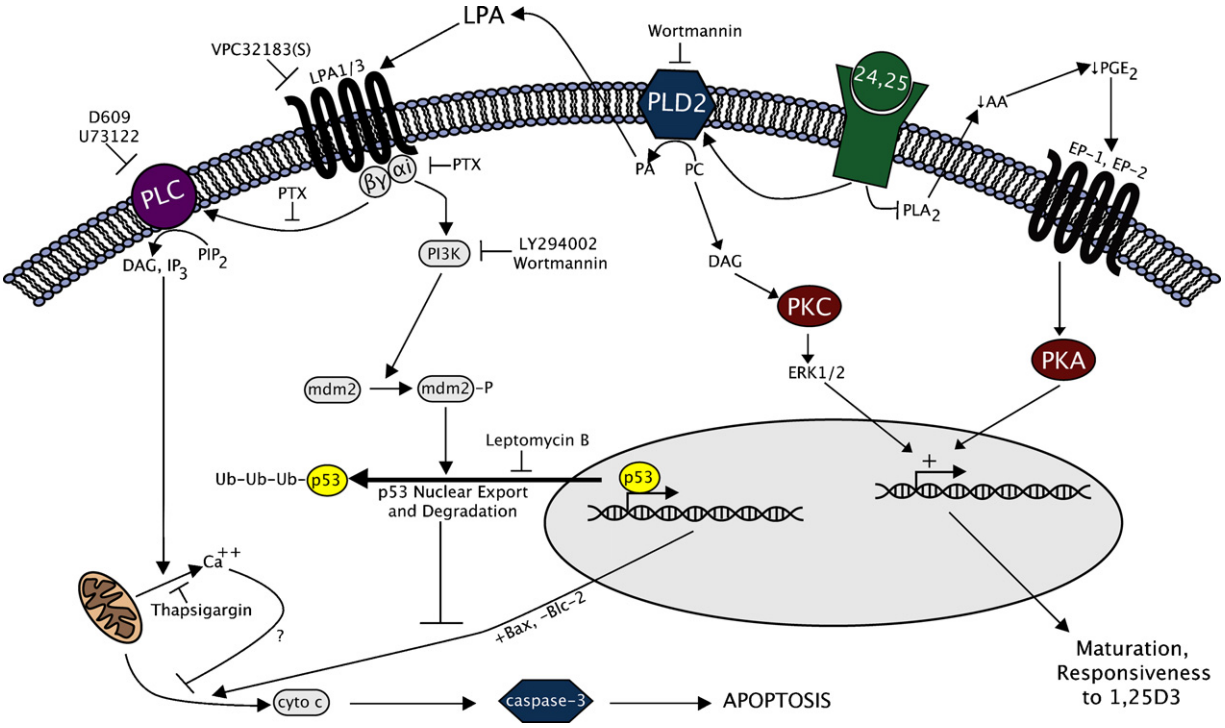

Fig. 4. Mechanisms of 24R,25$(OH)_2D_3$ signaling in resting zone growth plate chondrocytes. 24R,25$(OH)_2D_3$ exerts its effects on resting zone growth plate chondrocytes through activation of a putative membrane-associated vitamin D receptor (mVDR$_{24,25}$). Stimulation of mVDR$_{24,25}$ leads to activation of phospholipase D (PLD), resulting production of LPA and DAG. The latter of these directly activates PKC. LPA stimulates its receptor(s) LPA1 and/or LPA3, resulting in $G_{\alpha i}$-mediated phosphoinositol 3-kinase (PI_3K) activation. A consequence of this signaling is increased activity of murine double minute 2 (mdm2), an E3-ubiquitin ligase that promotes the nuclear export and degradation of the tumor suppressor p53. A decrease in nuclear p53 causes a decrease in p53-mediated transcription. Consequentially, Bcl-2 expression is increased and Bax expression and cytochrome c release from the mitochondria are decreased. Stimulation of LPA1/3 by LPA also initiates Gβγ-mediated PLC signaling, causing increased mitochondrial calcium release. mVDR$_{24,25}$ activation also leads to inhibition of phospholipase A2 (PLA_2). Following this initial decrease, 24R,25$(OH)_2D_3$ increases arachidonic acid (AA) turnover and subsequent prostaglandin production. This results in downstream stimulation of protein kinase A (PKA). Collectively, these signaling events cause resting zone chondrocytes to respond to 24R,25$(OH)_2D_3$ with increased maturation, matrix production, and cell survival.

findings demonstrate that 24R,25$(OH)_2D_3$ also protects chondrocytes from apoptosis induced by Pi in their microenvironment. Collectively, this suggests that 24R,25$(OH)_2D_3$ stabilizes chondrocytes in the resting zone by inhibiting degradation characteristic of apoptotic hypertrophic chondrocytes [37]. This implies that 24R,25$(OH)_2D_3$ modulates growth plate development by controlling the rate and extent of chondrocyte transition from resting zone to growth zone phenotype.

Acknowledgements

This work was supported by Children's Healthcare of Atlanta, The Price Gilbert, Jr. Foundation, and the National Institutes of Health.

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