



24R,25-Dihydroxyvitamin D₃ [24R,25(OH)₂D₃] controls growth plate development by inhibiting apoptosis in the reserve zone and stimulating response to 1 α ,25(OH)₂D₃ in hypertrophic cells[☆]

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ABSTRACT

Previously we showed that costochondral growth plate resting zone (RC) chondrocytes respond primarily to 24R,25(OH)₂D₃ whereas prehypertrophic and hypertrophic (GC) cells respond to 1 α ,25(OH)₂D₃. 24R,25(OH)₂D₃ 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)₂D₃ blocked apoptotic effects in a dose-dependent manner. Similarly, apoptosis was induced in ATDC5 cell cultures and 24R,25(OH)₂D₃ blocked this effect. Further studies indicated that 24R,25(OH)₂D₃ acts via at least two independent pathways. 24R,25(OH)₂D₃ 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)₂D₃ acts by increasing PKC activity. 24R,25(OH)₂D₃ stimulates 1-hydroxylase activity, resulting in increased levels of 1,25(OH)₂D₃, and it increases levels of phospholipase A2 activating protein, which is required for rapid 1 α ,25(OH)₂D₃-dependent activation of PKC in GC cells. These results suggest that 24R,25(OH)₂D₃ modulates growth plate development by controlling the rate and extent of RC chondrocyte transition to a GC chondrocyte phenotype.

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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)₂D₃], with increased alkaline phosphatase activity and [³⁵S]-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)₂D₃-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 [1 α ,25(OH)₂D₃] membrane receptor, protein disulfide isomerase family A, member 3 (PDIA3)

[5]. Additionally, the effects of 24R,25(OH)₂D₃ are rapid, inducing protein kinase C (PKC) activation in as little as 9 min [6]. These observations indicate that 24R,25(OH)₂D₃ acts through a putative membrane-associated receptor (mVDR_{24,25}) that is distinct from the 1 α ,25(OH)₂D₃-responsive PDIA3 and functional nVDRs, but do not rule out the possibility that 24R,25(OH)₂D₃ is acting via a VDR variant.

2. Rapid actions of 24R,25(OH)₂D₃ in the resting zone

The actions of 24R,25(OH)₂D₃ in the resting zone chondrocytes are mediated through rapid activation of PKC. 24R,25(OH)₂D₃ increases the abundance of diacylglycerol (DAG) [6], an activator of many PKC isoforms. PKC activation by 24R,25(OH)₂D₃ 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)₂D₃, eliminating tyrosine kinases as a source of

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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 (PLA₂) 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 PLA₂-activation [11]. Inhibition of PLA₂ 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 [³⁵S]-incorporation. Furthermore, LPA protected chondrocytes against inorganic phosphate (Pi)-induced apoptosis by activating the phosphoinositol 3-kinase (PI3K) 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 $1\alpha,25(OH)_2D_3$ -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 $1\alpha,25(OH)_2D_3$ 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 [³⁵S]-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 PI3K 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 [3H]-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 [3H] 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 $13,000 \times g$ for 15 min to separate intact DNA from fragmented DNA. The amount of incorporated [3H]-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 $\pm$ 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 μ M. 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 rela-

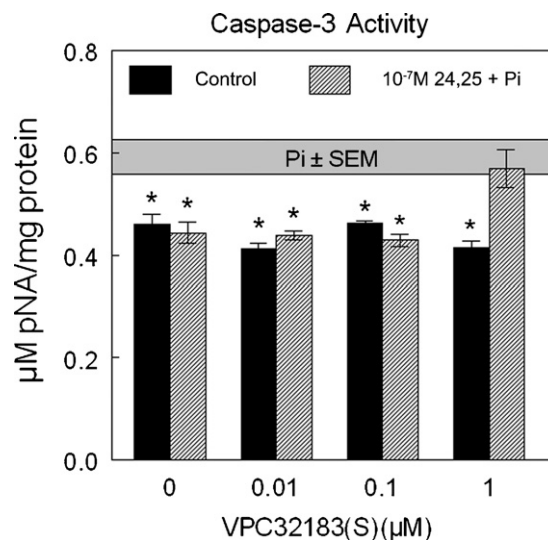


Fig. 1. LPA1/3 signaling is necessary for $24R,25(OH)_2D_3$ -mediated rescue of Pi-induced apoptosis. Confluent cultures of male rat resting zone chondrocytes were treated with vehicle alone (control) or treated with $24R,25(OH)_2D_3$ (10^{-7} M), Pi (7.5 mM), VPC32183(S) (0.01–1 μ M), or a combination 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 $\pm$ SEM; **p* < 0.05 relative to Pi alone treatment.

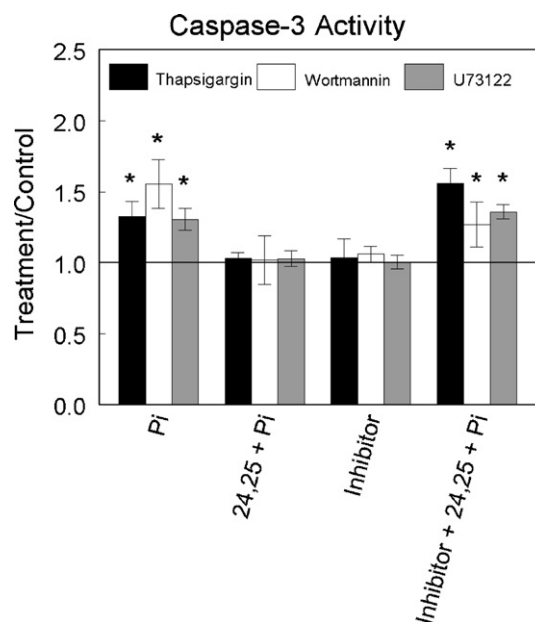


Fig. 2. $24R,25(OH)_2D_3$ reduces caspase-3 activity through PLD, PLC, and calcium signaling. Confluent cultures of male rat resting zone chondrocytes were treated for 24 h with 7.5 mM monobasic sodium phosphate, $24R,25(OH)_2D_3$ (10^{-7} M), thapsigargin (3 μ M), U73122 (10 μ M), wortmannin (10 μ M), 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.

tive to untreated control (Fig. 3B). $24R,25(OH)_2D_3$ attenuated the increase in DNA fragmentation levels caused by Pi.

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

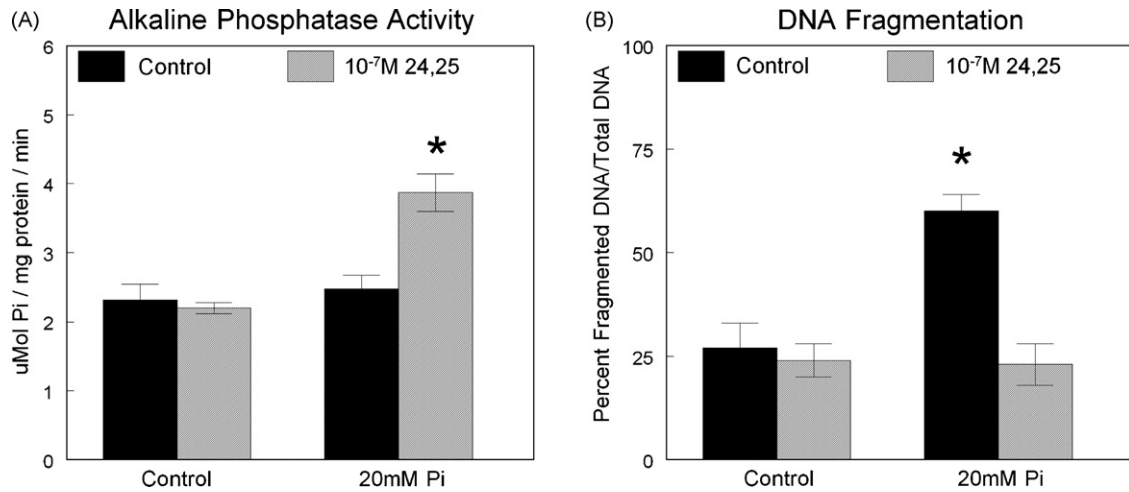


Fig. 3. Pi modulates ATDC5 responsiveness to 24R,25(OH)₂D₃. Confluent cultures of ATDC5 cells were treated with vehicle alone (control) or treated with 24R,25(OH)₂D₃ (10⁻⁷ M) and/or 20 mM Pi. (A) Alkaline phosphate activity was measured in cell layer lysates as a function of release of *para*-nitrophenol from *para*-nitrophenylphosphate at pH 10.2. Activity was normalized to the protein concentration of the lysates. (B) DNA fragmentation: cells were labeled with [³H]-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 [³H] 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 [³H]-thymidine was determined in each fraction to establish the total amount of fragmented DNA. **p* < 0.05 relative to untreated control.

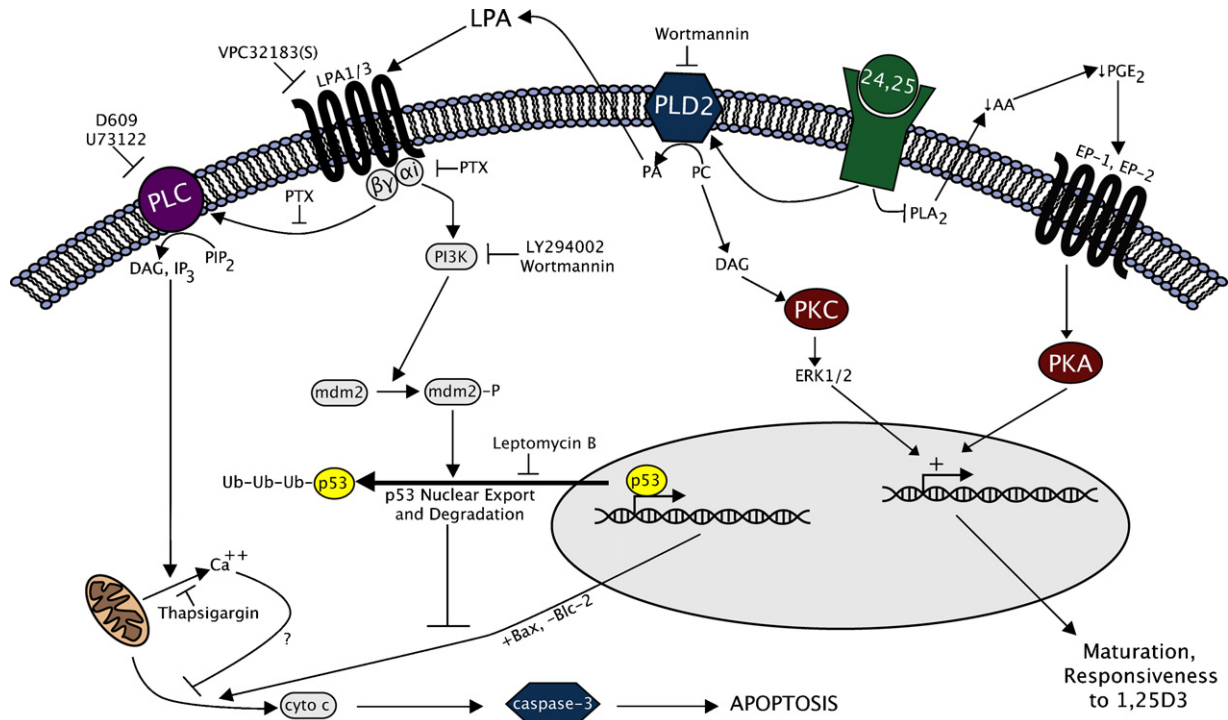


Fig. 4. Mechanisms of 24R,25(OH)₂D₃ signaling in resting zone growth plate chondrocytes. 24R,25(OH)₂D₃ exerts its effects on resting zone growth plate chondrocytes through activation of a putative membrane-associated vitamin D receptor (mVDR_{24,25}). Stimulation of mVDR_{24,25} leads to activation of 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_{αi}-mediated phosphoinositide 3-kinase (PI3K) 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 G_{βγ}-mediated PLC signaling, causing increased mitochondrial calcium release. mVDR_{24,25} activation also leads to inhibition of phospholipase A2 (PLA₂). Following this initial decrease, 24R,25(OH)₂D₃ 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)₂D₃ with increased maturation, matrix production, and cell survival.

findings demonstrate that 24R,25(OH)₂D₃ also protects chondrocytes from apoptosis induced by Pi in their microenvironment. Collectively, this suggests that 24R,25(OH)₂D₃ stabilizes chondrocytes in the resting zone by inhibiting degradation characteristic

of apoptotic hypertrophic chondrocytes [37]. This implies that 24R,25(OH)₂D₃ modulates growth plate development by controlling the rate and extent of chondrocyte transition from resting zone to growth zone phenotype.

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References

- [1] Z. Schwartz, H. Ehland, V.L. Sylvia, D. Larsson, R.R. Hardin, V. Bingham, D. Lopez, D.D. Dean, B.D. Boyan, 1 α ,25-dihydroxyvitamin D(3) and 24R,25-dihydroxyvitamin D(3) modulate growth plate chondrocyte physiology via protein kinase C-dependent phosphorylation of extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase, *Endocrinology* 143 (7) (2002) 2775–2786.
- [2] S. Maeda, D.D. Dean, V.L. Sylvia, B.D. Boyan, Z. Schwartz, Metalloproteinase activity in growth plate chondrocyte cultures is regulated by 1,25-(OH)(2)D(3) and 24,25-(OH)(2)D(3) and mediated through protein kinase C, *Matrix Biol.* 20 (2) (2001) 87–97.
- [3] L.N. Wu, B.R. Genge, Y. Ishikawa, T. Ishikawa, R.E. Wuthier, Effects of 24R,25- and 1 α ,25-dihydroxyvitamin D3 on mineralizing growth plate chondrocytes, *J. Cell. Biochem.* 98 (2) (2006) 309–334.
- [4] B.D. Boyan, V.L. Sylvia, N. McKinney, Z. Schwartz, Membrane actions of vitamin D metabolites 1 α ,25(OH)2D3 and 24R,25(OH)2D3 are retained in growth plate cartilage cells from vitamin D receptor knockout mice, *J. Cell. Biochem.* 90 (6) (2003) 1207–1223.
- [5] B.D. Boyan, L.F. Bonewald, V.L. Sylvia, I. Nemere, D. Larsson, A.W. Norman, J. Rosser, D.D. Dean, Z. Schwartz, Evidence for distinct membrane receptors for 1 α ,25-(OH)(2)D(3) and 24R,25-(OH)(2)D(3) in osteoblasts, *Steroids* 67 (3–4) (2002) 235–246.
- [6] S. Helm, V.L. Sylvia, T. Harmon, D.D. Dean, B.D. Boyan, Z. Schwartz, 24,25-(OH)2D3 regulates protein kinase C through two distinct phospholipid-dependent mechanisms, *J. Cell Physiol.* 169 (3) (1996) 509–521.
- [7] B.D. Boyan, V.L. Sylvia, D.D. Dean, F. Del Toro, Z. Schwartz, Differential regulation of growth plate chondrocytes by 1 α ,25-(OH)2D3 and 24R,25-(OH)2D3 involves cell-maturation-specific membrane-receptor-activated phospholipid metabolism, *Crit. Rev. Oral Biol. Med.* 13 (2) (2002) 143–154.
- [8] Z. Schwartz, V.L. Sylvia, M.H. Luna, P. DeVeau, R. Whetstone, D.D. Dean, B.D. Boyan, The effect of 24R,25-(OH)(2)D(3) on protein kinase C activity in chondrocytes is mediated by phospholipase D whereas the effect of 1 α ,25-(OH)(2)D(3) is mediated by phospholipase C, *Steroids* 66 (9) (2001) 683–694.
- [9] V.L. Sylvia, Z. Schwartz, F. Del Toro, P. DeVeau, R. Whetstone, R.R. Hardin, D.D. Dean, B.D. Boyan, Regulation of phospholipase D (PLD) in growth plate chondrocytes by 24R,25-(OH)2D3 is dependent on cell maturation state (resting zone cells) and is specific to the PLD2 isoform, *Biochim. Biophys. Acta* 1499 (3) (2001) 209–221.
- [10] B.D. Boyan, E.G. Jennings, L. Wang, Z. Schwartz, Mechanisms regulating differential activation of membrane-mediated signaling by 1 α ,25(OH)2D3 and 24R,25(OH)2D3, *J. Steroid Biochem. Mol. Biol.* 89–90 (1–5) (2004) 309–315.
- [11] Z. Schwartz, E.J. Graham, L. Wang, S. Lossdorfer, I. Gay, T.L. Johnson-Pais, D.L. Carnes, V.L. Sylvia, B.D. Boyan, Phospholipase A2 activating protein (PLAA) is required for 1 α ,25(OH)2D3 signaling in growth plate chondrocytes, *J. Cell. Physiol.* 203 (1) (2005) 54–70.
- [12] B.D. Boyan, V.L. Sylvia, D. Curry, Z. Chang, D.D. Dean, Z. Schwartz, Arachidonic acid is an autocrine mediator of the differential action of 1,25-(OH)2D3 and 24,25-(OH)2D3 on growth plate chondrocytes, *J. Cell. Physiol.* 176 (3) (1998) 516–524.
- [13] Z. Schwartz, V.L. Sylvia, F. Del Toro, R.R. Hardin, D.D. Dean, B.D. Boyan, 24R,25-(OH)(2)D(3) mediates its membrane receptor-dependent effects on protein kinase C and alkaline phosphatase via phospholipase A(2) and cyclooxygenase-1 but not cyclooxygenase-2 in growth plate chondrocytes, *J. Cell. Physiol.* 182 (3) (2000) 390–401.
- [14] L.D. Swain, Z. Schwartz, B.D. Boyan, 1,25-(OH)2D3 and 24,25-(OH)2D3 regulation of arachidonic acid turnover in chondrocyte cultures is cell maturation-specific and may involve direct effects on phospholipase A2, *Biochim. Biophys. Acta* 1136 (1) (1992) 45–51.
- [15] Z. Schwartz, L.D. Swain, V. Ramirez, B.D. Boyan, Regulation of arachidonic acid turnover by 1,25-(OH)2D3 and 24,25-(OH)2D3 in growth zone and resting zone chondrocyte cultures, *Biochim. Biophys. Acta* 1027 (3) (1990) 278–286.
- [16] L.D. Swain, Z. Schwartz, K. Caulfield, B.P. Brooks, B.D. Boyan, Nongenomic regulation of chondrocyte membrane fluidity by 1,25-(OH)2D3 and 24,25-(OH)2D3 is dependent on cell maturation, *Bone* 14 (4) (1993) 609–617.
- [17] Z. Schwartz, V.L. Sylvia, D. Curry, M.H. Luna, D.D. Dean, B.D. Boyan, Arachidonic acid directly mediates the rapid effects of 24,25-dihydroxyvitamin D3 via protein kinase C and indirectly through prostaglandin production in resting zone chondrocytes, *Endocrinology* 140 (7) (1999) 2991–3002.
- [18] J. Aoki, Mechanisms of lysophosphatidic acid production, *Semin. Cell Dev. Biol.* 15 (5) (2004) 477–489.
- [19] C. Luquain, A. Singh, L. Wang, V. Natarajan, A.J. Morris, Role of phospholipase D in agonist-stimulated lysophosphatidic acid synthesis by ovarian cancer cells, *J. Lipid Res.* 44 (10) (2003) 1963–1975.
- [20] M.K. Kim, H.Y. Lee, K.S. Park, E.H. Shin, S.H. Jo, J. Yun, S.W. Lee, Y.H. Yoo, Y.S. Lee, S.H. Baek, Y.S. Bae, Lysophosphatidic acid stimulates cell proliferation in rat chondrocytes, *Biochem. Pharmacol.* 70 (12) (2005) 1764–1771.
- [21] J. Hurst-Kennedy, B.D. Boyan, Z. Schwartz, Lysophosphatidic acid signaling promotes proliferation, differentiation, and cell survival in rat growth plate chondrocytes, *Biochim. Biophys. Acta* (2009).
- [22] Y. Aki, A. Kondo, H. Nakamura, A. Togari, Lysophosphatidic acid-stimulated interleukin-6 and -8 synthesis through LPA1 receptors on human osteoblasts, *Arch. Oral Biol.* 53 (3) (2008) 207–213.
- [23] J. Gidley, S. Openshaw, E.T. Pring, S. Sale, J.P. Mansell, Lysophosphatidic acid cooperates with 1 α ,25(OH)2D3 in stimulating human MG63 osteoblast maturation, *Prostaglandins Other Lipid Mediat.* 80 (1–2) (2006) 46–61.
- [24] M.M. Murph, J. Hurst-Kennedy, V. Newton, D.N. Brindley, H. Radhakrishna, Lysophosphatidic acid decreases the nuclear localization and cellular abundance of the p53 tumor suppressor in A549 lung carcinoma cells, *Mol. Cancer Res.* 5 (11) (2007) 1201–1211.
- [25] B. Pucci, C.S. Adams, J. Fertala, B.C. Snyder, K.D. Mansfield, M. Tafani, T. Freeman, I.M. Shapiro, Development of the terminally differentiated state sensitizes epiphyseal chondrocytes to apoptosis through caspase-3 activation, *J. Cell. Physiol.* 210 (3) (2007) 609–615.
- [26] M. Zhong, D.H. Carney, J.T. Ryaby, Z. Schwartz, B.D. Boyan, Inhibition of phosphate-induced apoptosis in resting zone chondrocytes by thrombin peptide 508, *Cells Tissues Organs* 189 (1–4) (2009) 56–59.
- [27] H.A. Pedrozo, B.D. Boyan, J. Mazock, D.D. Dean, R. Gomez, Z. Schwartz, TGF β 1 regulates 25-hydroxyvitamin D3 1 α - and 24-hydroxylase activity in cultured growth plate chondrocytes in a maturation-dependent manner, *Calcif. Tissue Int.* 64 (1) (1999) 50–56.
- [28] Z. Schwartz, B. Brooks, L. Swain, F. Del Toro, A. Norman, B. Boyan, Production of 1,25-dihydroxyvitamin D3 and 24,25-dihydroxyvitamin D3 by growth zone and resting zone chondrocytes is dependent on cell maturation and is regulated by hormones and growth factors, *Endocrinology* 130 (5) (1992) 2495–2504.
- [29] T.A. Denison, C.F. Koch, I.M. Shapiro, Z. Schwartz, B.D. Boyan, Inorganic phosphate modulates responsiveness to 24,25(OH)2D3 in chondrogenic ATDC5 cells, *J. Cell. Biochem.* 107 (1) (2009) 155–162.
- [30] B.D. Boyan, Z. Schwartz, L.D. Swain, D.L. Carnes Jr., T. Zislis, Differential expression of phenotype by resting zone and growth region costochondral chondrocytes in vitro, *Bone* 9 (3) (1988) 185–194.
- [31] T. Atsumi, Y. Miwa, K. Kimata, Y. Ikawa, A chondrogenic cell line derived from a differentiating culture of AT805 teratocarcinoma cells, *Cell Differ. Dev.* 30 (2) (1990) 109–116.
- [32] F.M. Altaf, T.M. Hering, N.H. Kazmi, J.U. Yoo, B. Johnstone, Ascorbate-enhanced chondrogenesis of ATDC5 cells, in: 64-L 69, *Eur. Cell. Mater.* 12 (2006), discussion 69–70.
- [33] T. Schinke, C. Amendt, A. Trindl, O. Poschke, W. Muller-Esterl, W. Jähnen-Dechent, The serum protein α 2-HS glycoprotein/fetuin inhibits apatite formation in vitro and in mineralizing calvaria cells. A possible role in mineralization and calcium homeostasis, *J. Biol. Chem.* 271 (34) (1996) 20789–20796.
- [34] P.A. Price, T.M. Nguyen, M.K. Williamson, Biochemical characterization of the serum fetuin-mineral complex, *J. Biol. Chem.* 278 (24) (2003) 22153–22160.
- [35] C.C. Teixeira, K. Mansfield, C. Hertkorn, H. Ischiropoulos, I.M. Shapiro, Phosphate-induced chondrocyte apoptosis is linked to nitric oxide generation, *Am. J. Physiol. Cell Physiol.* 281 (3) (2001) C833–C839.
- [36] M. Zhong, D.H. Carney, J.T. Ryaby, Z. Schwartz, B.D. Boyan, Inhibition of phosphate-induced apoptosis in resting zone chondrocytes by thrombin peptide 508, *Cells Tissues Organs* (2008).
- [37] V.L. Sylvia, Z. Schwartz, S.C. Holmes, D.D. Dean, B.D. Boyan, 24,25-(OH)2D3 regulation of matrix vesicle protein kinase C occurs both during biosynthesis and in the extracellular matrix, *Calcif. Tissue Int.* 61 (4) (1997) 313–321.