Journal of Steroid Biochemistry & Molecular Biology 122 (2010) 264–271

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

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

24R,25-Dihydroxyvitamin D3, lysophosphatidic acid, and p53: A signaling axis in the inhibition of phosphate-induced chondrocyte apoptosis

J. Hurst-Kennedy[a], M. Zhong[b], V. Gupta[b], B.D. Boyan[a,b,*], Z. Schwartz[b,c]

[a] School of Biology, Georgia Institute of Technology, Atlanta, GA, United States
[b] Department of Biomedical Engineering, 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 4 January 2010
Received in revised form 23 May 2010
Accepted 27 May 2010

Keywords:
Vitamin D metabolites
24R,25-Dihydroxyvitamin D3
Lysophosphatidic acid
Growth plate cartilage
Chondrocyte apoptosis
p53

ABSTRACT

Maintenance of the pool of chondrocytes in the resting zone of the growth plate in the presence of the physiological apoptogen inorganic phosphate (Pi) is crucial for skeletal development. Costochondral resting zone chondrocytes are regulated by the vitamin D metabolite 24R,25-dihydroxyvitamin D3 [24R,25$(OH)_2D_3$], with increased production of sulfated glycosaminoglycan-rich extracellular matrix, and reduced matrix metalloproteinase activity. The effects of 24R,25$(OH)_2D_3$ are mediated by activation of phospholipase D (PLD), resulting in increased production of lysophosphatidic acid (LPA) and LPA-mediated proliferation, maturation, inhibition of Pi-induced apoptosis, and reduction of p53. However, the exact mechanism by which 24R,25$(OH)_2D_3$ and LPA exert their effects is not fully understood. It was found that both 24R,25$(OH)_2D_3$ and LPA attenuate Pi-induced caspase-3 activity. The actions of 24R,25$(OH)_2D_3$ and LPA were dependent upon $G_{\alpha i}$, LPA receptor(s) 1 and/or 3, PLD, phospholipase C (PLC), and intracellular calcium, phosphoinositide 3-kinase (PI_3K) signaling, and nuclear export. 24R,25$(OH)_2D_3$ decreased both p53 abundance and p53-medaited transcription and inhibited Pi-induced cytochrome *c* translocation. Moreover, LPA induced increased mdm2 phosphorylation, a negative regulator of p53. Taken together, these data show that 24R,25$(OH)_2D_3$ inhibits Pi-induced apoptosis through Ca^{2+}, PLD, and PLC signaling and through LPA-LPA1/3-$G_{\alpha i}$-PI_3K-mdm2-mediated p53 degradation, resulting in decreased cytochrome *c* translocation and caspase-3 activity.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Longitudinal bone growth in children and adolescents is mediated by growth plate cartilage, which is divided into four zones of maturation: the resting zone, the growth zone, the hypertrophic zone, and the calcifying cell zone. Inorganic phosphate (Pi), a physiological apoptogen, induces apoptosis in terminal growth plate chondrocytes at the epiphyseal–metaphyseal junction [1–3], allowing for the invasion of blood vessels and deposition of new bone. The resting zone of the growth plate supplies a pool of chondrocytes for the remainder of the growth plate. In response to growth stimuli, these cells undergo proliferation, terminal differentiation, hypertrophy, and apoptosis as they mature.

Studies using resting zone chondrocytes from the costochondral cartilage show that they respond preferentially to the vitamin D metabolite 24R,25-dihydroxyvitamin D3 [24R,25$(OH)_2D_3$], whereas hypertrophic chondrocytes respond preferentially to 1α,25$(OH)_2D_3$. When resting zone chondrocyte cultures are treated with 24R,25$(OH)_2D_3$, protein kinase C alpha (PKCα) activity is increased and there is an increase in extracellular matrix production, maturation and cell survival [4–7]. 24R,25(OH)2D3 exerts its effects through a membrane-associated vitamin D receptor [8], resulting in activation of phospholipase D (PLD) and production of lysophosphatidic acid (LPA 18:1; 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate) [9,10].

LPA is the most simple of the glycerol lipids consisting of a single fatty acyl chain, a glycerol backbone, and a phosphate head group. It exerts its effects on cells through activation of cell surface G-protein coupled receptors (GPCRs) LPA1/Edg2, LPA2/Edg4, LPA3/Edg7, LPA4/GPR23, and LPA5/GPR92 and through the nuclear receptor peroxisome-proliferation-activation-receptor gamma (PPARγ) [11–15]. Consequences of LPA signaling are broad and include neurite retraction, tumorigenesis, wound healing, proliferation, migration, and cell survival [16–22]. Several recent studies have demonstrated that chondrocytes, osteoblasts, and osteocytes are sensitive to LPA, establishing LPA as a regulator of bone and cartilage [23–27].

Recently, it was observed that 24R,25$(OH)_2D_3$ protects the prechondrocyte-like ATDC5 cell line from Pi-induced apoptosis [28]. Additionally, it has reported that LPA is produced by resting zone

* Corresponding author at: Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology, 315 Ferst Drive NW, IBB Building, 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).

0960-0760/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2010.05.010

chondrocytes in response to 24R,25$(OH)_2D_3$ and that the lipid mediator acts as an autocrine regulator to promote proliferation, cell survival, and maturation [10]. LPA exerts these effects through activation of LPA1 and/or LPA3 (LPA1/3) and by modulating the abundance and transcriptional activity of the tumor suppressor p53. In cancer cells, LPA has been shown to regulate p53 in a similar manner through activation of the phosphoinositol 3-kinase (PI_3K) signaling cascade to promote nuclear export and subsequent degradation of p53 [29].

Collectively, these findings have led us to hypothesize that 24R,25$(OH)_2D_3$ protects resting zone chondrocytes from Pi-induced apoptosis through LPA-mediated inhibition of p53. To test this hypothesis, rat costochondral resting zone chondrocytes were treated with Pi and 24R,25$(OH)_2D_3$ and assessed the roles of $G_{\alpha i}$, $G_{\alpha s}$, PLD, PLC, PI_3K, and intracellular Ca^{2+} in modulating Pi-induced apoptosis. In addition, p53 abundance was examined to determine whether nuclear export of p53 or mdm2 signaling was involved.

2. Materials and methods

2.1. Reagents

18:1 LPA (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate) was purchased from Avanti Polar Lipids (Alabaster, AL) and was reconstituted in 1% charcoal-stripped bovine serum albumin (BSA) prior to treatment of cells. Unless otherwise stated, all other reagents were acquired from VWR International (West Chester, PA).

2.2. Cell culture

The culture system used in this study has been previously described in detail [30]. Chondrocytes were obtained from the resting zone (reserve zone) of costochondral cartilage from the ribs of 125 g male Sprague–Dawley rats and cultured in Dulbecco's modified Eagle's 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. These cells continue to express type II collagen, aggrecan, and cartilage oligomeric matrix protein.

2.3. Regulation of LPA receptor expression

Confluent cultures of resting zone chondrocytes were treated for 6 h with vehicle alone (control), 7.5 mM sodium phosphate, 10^{-6} M LPA, or 10^{-7} M 24R,25$(OH)_2D_3$. After treatment, mRNA was harvested using Trizol (Invitrogen, Carlsburg, CA). Quantitative real-time polymerase chain reaction (QRT-PCR) was used to identify the presence of the LPA receptors LPA1-5 and PPAR-γ. The following sequence specific primers were used: LPA1 sense: 5′-GGTTCTCTACGCTCACATC-3′, LPA1 antisense 5′-GCAGTAGCAAGACCAATCC-3′, LPA2 sense: 5′-CACCACCTCACAGCCATCC-3′, LPA2 antisense: 5′-AGACATCCACAGCACTCAGC-3′, LPA3 sense: 5′-CTACAACAGGAGCAACAC-3′, LPA3 antisense: 5′-CCAGCAGGTAGTAGAAGG-3′, LPA4 sense: 5′-ACAACTTTAACCGCCACTGG-3′, LPA4 antisense: 5′-ATTCCTCCTGGTC CTGATGG-3′, LPA5 sense: 5′-ACCTTGGTGTTCCCTATAATGC-3′, LPA5 antisense: 5′-AGCCAGAGCGTTGAGAGG-3′, PPAR-γ sense: 5′-CCGAAGAACCATCCGATTGAAG-3′, and PPAR-γ antisense: 5′-CTCCGCCAACAGCTTCTCC-3′. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was amplified as a control in each experiment: GAPDH sense: 5′-ATGCAGGGATGATGTTC-3′, GAPDH antisense: 5′-TGCACCA CCAACTGCTTAG-3′.

2.4. Cell viability

The LPA1/3-selective antagonist (S)-phosphoric acid mono-(2-octadec-9-enoylamino-3-[4-(pyridine-2-ylmethoxy)-phenyl]-propyl) ester (VPC32183(S), Avanti Polar Lipids, Alabaster, AL) [31] was used to assess the role of LPA receptor signaling in 24R,25$(OH)_2D_3$-mediated rescue of Pi-induced apoptosis. Confluent cultures of the resting zone chondrocytes were treated with 7.5 mM monobasic sodium phosphate, 10^{-7} M 24R,25$(OH)_2D_3$, VPC32183(S) (10^{-8}–10^{-6} M), or a combination of the afore mentioned. Cell viability was assessed using the MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, MO) dye. The conversion of tetrazolium salt to formazan salt was measured at 570 nm.

2.5. DNA fragmentation

Confluent cultures of resting zone chondrocytes were labeled with [^{3}H]thymidine for 4 h prior to treatment with vehicle alone, 10^{-6} M LPA, 7.5 mM Pi, and/or 10^{-5} M chelerythrine (CHEL). 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.

2.6. Caspase-3 activity

The roles of 24R,25$(OH)_2D_3$ and LPA in chondrocyte survival were assessed by examining their ability to reduce caspase-3 activity induced by Pi [2,3]. 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) or LPA (10^{-6} M). To determine the signaling pathways involved in caspase-3 regulation by 24R,25$(OH)_2D_3$ or LPA, one half of the cultures in each experiment were treated with one of the following compounds: VPC32183(S) (10^{-8}–10^{-6} M, Avanti Polar Lipids) was used to inhibit LPA1/3 signaling; pertussis toxin (PTX, 0.25–25 ng/ml, Sigma, St. Louis, MO) was used to inhibit $G_{\alpha i}$ signaling; cholera toxin (CTX, 1–100 ng/ml, Calbiochem, Gibbstown, NJ) was utilized to stimulate $G_{\alpha s}$ signaling; LY294002 (LY, 10^{-7}–10^{-5} M, Cayman Chemicals, Ann Arbor, MI) was used to inhibit PI_3K; D609 (50 μM, Calbiochem); and U73122 (10 μM, Sigma, St. Louis, MO) were used to inhibit phosphatidylcholine-dependent PLC (PC-PLC) and phosphatidylinositol-dependent PLC (PI-PLC), respectively; thapsigargin (3 μM, Sigma) was used to block release of Ca^{2+} ions from the endoplasmic reticulum; and wortmannin (10 μM, Calbiochem) was used to inhibit PLD signaling. The concentrations selected for each inhibitor were based on previously published dose–response studies assessing the signaling pathways stimulated by 24R,25$(OH)_2D_3$ [4,32]. Leptomycin B (LMB, 0.5–50 ng/ml, Biomol, Plymouth Meeting, PA) was used to block nuclear export. 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 was 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 total protein content as determined by the Pierce Macro BCA Protein Assay Kit (Rockford, IL).

2.7. Cytochrome c translocation

Confluent cultures of resting zone chondrocytes were mock-treated (control) or treated with 7.5 mM Pi, 10^{-7} M 24R,25$(OH)_2D_3$, or a combination of the afore mentioned for 24 h. At harvest,

cell lysates were fractionated into mitochondrial and cytoplasmic fractions using the Cytochrome C Apoptosis Assay Kit (BioVision, Mountain View, CA). Immunoblotting was conducted as described previously using a mouse monoclonal anti-cytochrome *c* antibody (BioVision).

2.8. Modulation of p53 abundance and p53-mediated transcription

Confluent cultures of resting zone chondrocytes were treated for 6 h with vehicle alone, 24R,25$(OH)_2D_3$ (10^{-9}–10^{-7} M), 10^{-6} M LPA, or a combination of the afore mentioned. Alternatively, chondrocytes were treated with 0, 2.5, 5 or 7.5 mM Pi for 24 h. After treatment, the cell monolayer was washed twice with PBS and harvested with 300 μL RIPA buffer. Whole cell lysates were centrifuged at 13,000 rpm for 10 min and the supernatant was collected (cytoplasmic fraction). The remaining pellet (nuclear fraction) was resuspended with 300 μL RIPA. The abundance of p53 protein in both the nuclear and cytoplasmic fractions was determined by ELISA (p53 pan ELISA, Roche) and normalized to total cellular or total fraction protein as determined by Pierce Macro BCA Protein Assay Kit.

To assess changes in p53-mediated transcription, luciferase reporter gene assays were conducted as previously described [33]. Cells were transfected with two plasmids: one containing p53-controlled firefly luciferase (pp53_TA-Luc, Clonetech, Mountain View, CA) and the other carrying constitutively expressed *Renilla* luciferase (pLR-TK, Promega, Madison, WI). 24 h after transfection, cells were treated with 0, 10^{-9}, 10^{-8}, or 10^{-7} M 24R,25$(OH)_2D_3$ for 16 h and luciferase activity was measured using the Dual Luciferase Reporter Assay kit (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity to determine p53-mediated transcription.

2.9. Abundance of mdm2 and Phospho-mdm2 protein

Western blots were performed to determine the effects of 24R,25$(OH)_2D_3$ (10^{-9}–10^{-7} M) and LPA (10^{-8}–10^{-6} M) on the protein abundance of mdm2 and phosphorylated mdm2. Cell culture lysates were prepared from confluent resting zone cells and were resolved on 10% SDS-polyacrylamide gels. Blots of the gels were probed with monoclonal antibodies against mdm2 (AbCam, Cambridge, MA), Phospho-mdm2 (Ser 166, Cell Signaling, Boston, MA), or GAPDH (MAB374, Chemicon, Billerica, MA). Immunoreactive bands were detected using 1:5000 dilutions of horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA), and visualized using enhanced chemiluminescence (Super-Signal WestPico Chemiluminescent Substrate, Pierce). Densitometry measurements were collected using Quantity One image analysis software (BioRad, Hercules, CA). Total and phosphorylated mdm2 densitometry values were normalized to those of GAPDH.

2.10. 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.

3. Results

3.1. Regulation of LPA receptor expression

Resting zone chondrocytes express mRNAs for the LPA receptors LPA1–5 and PPAR-γ (Fig. 1A). Both 10^{-7} M 24R,25$(OH)_2D_3$ and 10^{-6} M LPA increased the mRNA expression of LPA1 (Fig. 1A and B). Expression of LPA3, LPA4, and LPA5 was decreased after treatment with 7.5 mM phosphate (Fig. 1B). LPA5 and PPAR-γ mRNAs were decreased by LPA.

3.2. LPA1/3 signaling in 24R,25$(OH)_2D_3$-mediated inhibition of apoptosis

24R,25$(OH)_2D_3$ inhibited Pi-induced apoptosis as assessed by MTT cell viability assay (Fig. 2A) and caspase-3 activity assay (Fig. 2B). The LPA1/3-selective antagonist VPC32183(S) abolished the ability of 24R,25$(OH)_2D_3$ to reduce the increase in caspase-3 activity caused by Pi.

3.3. The roles of PLD, calcium, $G_{\alpha s}$, $G_{\alpha i}$, PI_3K, PLC, and nuclear export

24R,25$(OH)_2D_3$ reduced Pi-induced caspase-3 activity via a G-protein dependent mechanism (Fig. 3). The $G_{\alpha i}$ inhibitor pertussis toxin (PTX) (Fig. 3A) attenuated 24R,25$(OH)_2D_3$-mediated rescue, whereas the $G_{\alpha s}$ stimulator cholera toxin (CTX) (Fig. 3B), the PI_3K inhibitor LY294002 (Fig. 3C), and the PC-PLC inhibitor D609 (Fig. 3D) did not. The PI-PLC inhibitor U73122, the calcium ATPase inhibitor thapsigargin, and the PLD inhibitor wortmannin also inhibited this effect (Fig. 3E–G).

LPA treatment also attenuated the increases in DNA fragmentation caused by Pi and CHEL (Table 2) and increases in caspase-3

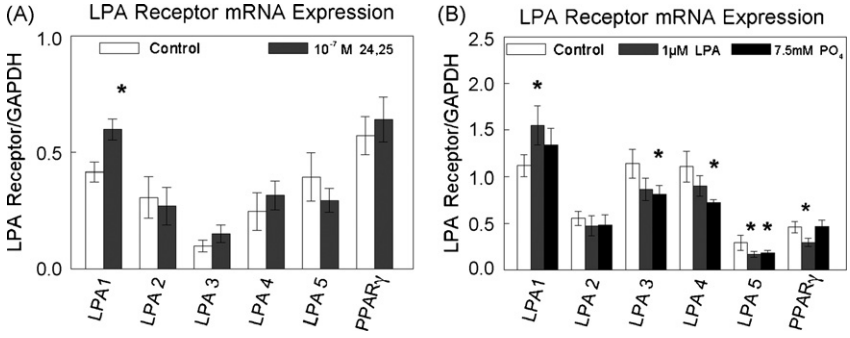

Fig. 1. LPA receptor expression in the presence of 24R,25$(OH)_2D_3$, LPA, and phosphate. LPA receptor mRNA levels from cultures of mock-treated resting zone chondrocytes (Control A and B) or cultures treated with 24R,25$(OH)_2D_3$ (A), Pi (B), or LPA (B) (*significant relative to untreated control).

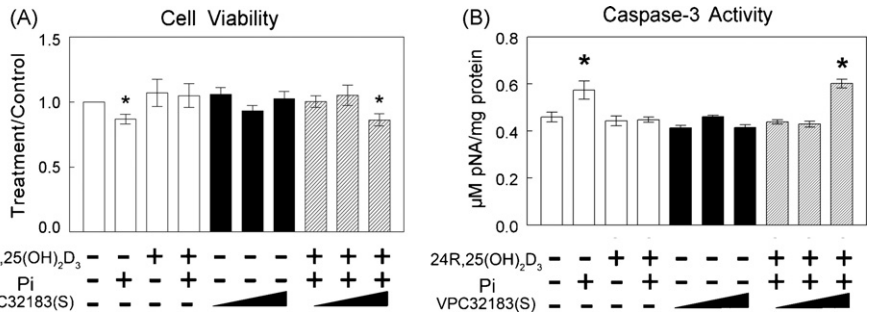

Fig. 2. The effect of VPC32183(S) on 24R,25(OH)$_2$D$_3$-mediated inhibition of apoptosis. MTT metabolism (A) and caspase-3 activity (B) in resting zone chondrocytes treated with 24R,25(OH)$_2$D$_3$, Pi, VPC32183(S), or a combination of the afore mentioned (*significant relative to untreated control).

activity induced by Pi (Fig. 4). This rescue of Pi-induced apoptosis by LPA was inhibited by PTX, but not by CTX (Fig. 4A and B). LY294002 and the nuclear export inhibitor LMB also blocked LPA-mediated inhibition of Pi cell death (Fig. 4C and D).

3.4. Modulation of p53 levels by 24R,25(OH)$_2$D$_3$

Pi treatment increased total p53 abundance in a dose-dependant manner as assessed by ELISA (Table 1). 24R,25(OH)$_2$D$_3$ and LPA treatment resulted in a decrease in nuclear p53 abundance (Fig. 5A and B). Additionally, 24R,25(OH)$_2$D$_3$ reduced p53-mediated transcription (Fig. 5C) in a dose-dependent manner.

Table 1
DNA fragmentation in response to LPA, Pi, and CHEL. Percent fragmented DNA in resting zone chondrocytes after treatment with LPA, Pi, and/or chelerythrine (CHEL).

Treatment	Percent total fragmented DNA
Control	7.6 ± 0.8
7.5 mM Pi	37.3 ± 12.0*
10^{-5} M CHEL	42.7 ± 3.2*
1 μM LPA	10.4 ± 0.8
1 μM LPA + 7.5 mM Pi	10.8 ± 2.4
1 μM LPA + 10^{-5} M CHEL	29.7 ± 1.9#

* Significant relative to untreated control.
\# Significant relative to CHEL alone.

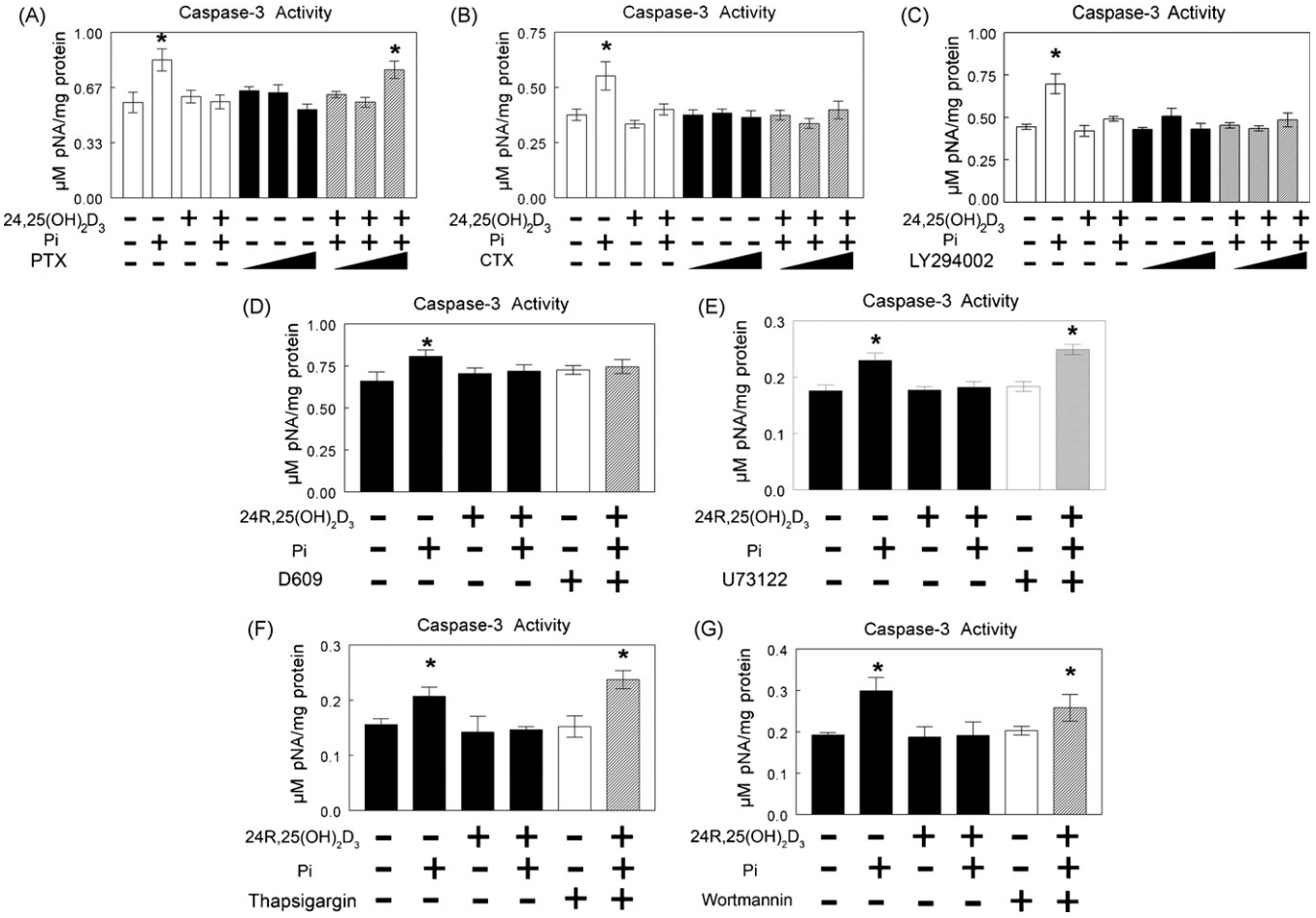

Fig. 3. The roles of $G_{\alpha i}$, PLD, and Ca^{2+} in the inhibition of Pi-induced apoptosis by 24R,25(OH)$_2$D$_3$. Caspase-3 activity in resting zone chondrocytes treated with Pi, 24R,25(OH)$_2$D$_3$, pertussis toxin (A, PTX), cholera toxin (B, CTX), LY294002 (C), D609 (D), U73122 (E), thapsigarin (F), wortmannin (G) or a combination of the afore mentioned (*significant relative to untreated control).

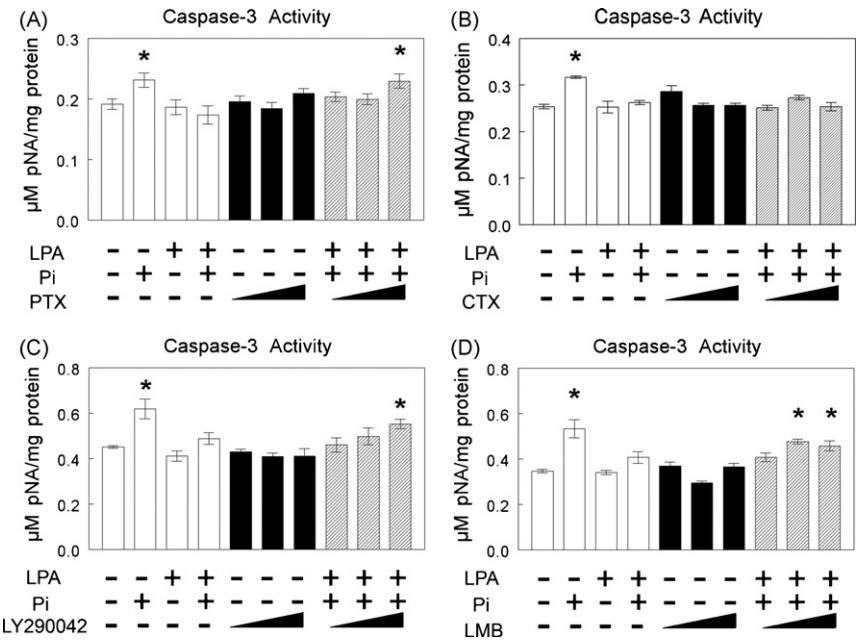

Fig. 4. The roles of $G_{\alpha i}$, PI_3K, and nuclear export-dependent signaling in LPA-mediated inhibition of apoptosis. Caspase-3 activity in resting zone chondrocytes treated with Pi, LPA, pertussis toxin (A, PTX), cholera toxin (B, CTX), LY294002 (C), leptomycin B (D, LMB), or a combination of the afore mentioned (*significant relative to untreated control).

24R,25$(OH)_2D_3$ did not alter the abundance of active, phosphorylated or total mdm2, an E3-ubiquitin ligase that negatively regulates p53 (Fig. 6A and B). LPA, however, did increase the phosphorylation of mdm2, while having no effect on total mdm2 (Fig. 6C and D).

3.5. Pi-induced cytochrome c translocation

Cytochrome *c* is primarily localized in the mitochondria in control and 24R,25$(OH)_2D_3$ treated lysates (Fig. 7). Pi treatment induced an increase in the abundance of cytochrome *c* in the cyto-

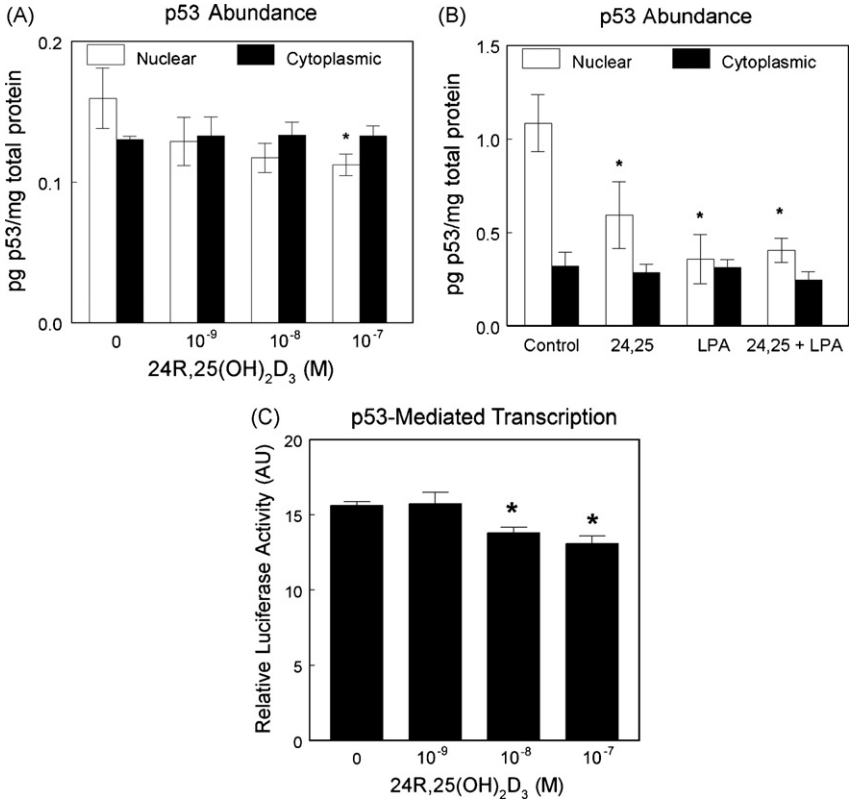

Fig. 5. . p53 abundance and p53-mediated transcription in the presence of 24R,25$(OH)_2D_3$. p53 protein levels (A and B) and p53-mediated transcription (C) in resting zone chondrocytes in response to treatment with vehicle alone, 24R,25$(OH)_2D_3$, LPA, or a combination of the afore mentioned (*significant relative to untreated control).

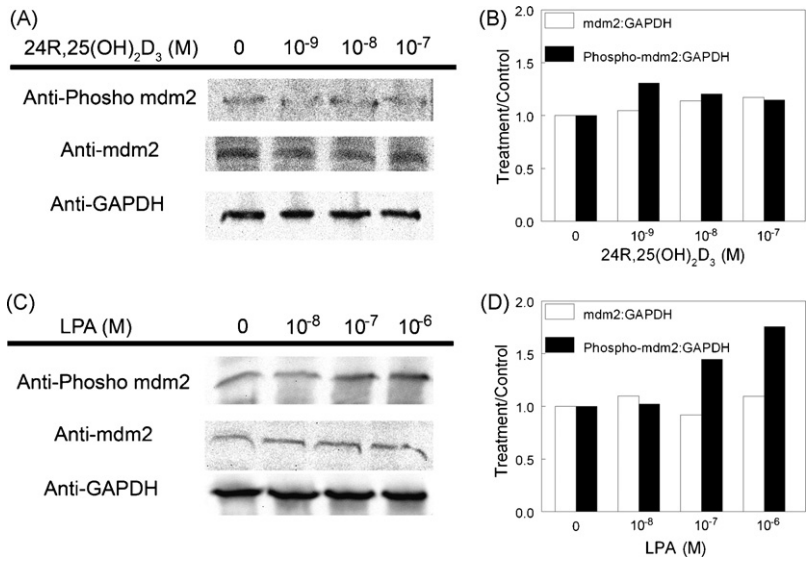

Fig. 6. Levels of total and phosphorylated mdm2 in the presence of LPA and 24R,25$(OH)_2D_3$. Total and phospho-mdm2 abundance in resting zone chondrocytes treated with 24R,25$(OH)_2D_3$ (A and B) or LPA (C and D).

Table 2
p53 abundance in Pi-treated resting zone chondrocytes.

Pi (mM)	pg p53/mg total protein
0	0.56 ± 0.08
2.5	0.54 ± 0.04
5	1.39 ± 0.11*
7.5	1.52 ± 0.11*

* Significant relative to untreated control.

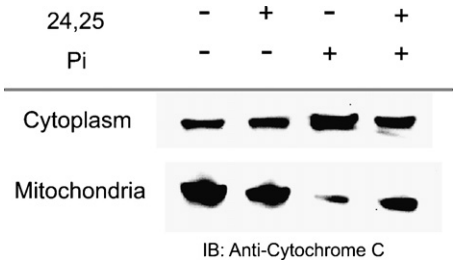

Fig. 7. Cytochrome *c* subcellular localization in response to Pi. The abundance of cytochrome *c* in mitochondrial and cytoplasmic fraction of resting zone chondrocytes whole cell lysates after treatment with Pi or 24R,25$(OH)_2D_3$.

plasmic fraction, while decreasing it in the mitochondrial fraction. This was attenuated with the addition of 24R,25$(OH)_2D_3$.

4. Discussion

The presented data demonstrate that 24R,25$(OH)_2D_3$ inhibits Pi-induced apoptosis in resting zone chondrocytes through LPA signaling. Previously it has been shown that 24R,25$(OH)_2D_3$ increases the abundance of extracellular LPA and that 24R,25$(OH)_2D_3$-stimulated alkaline phosphatase activity is dependent upon LPA1/3 receptor signaling [10]. In this study, it is shown that 24R,25$(OH)_2D_3$ also increases the mRNA expression of LPA1 and that 24R,25$(OH)_2D_3$-mediated inhibition of Pi-induced apoptosis is sensitive to the LPA1/3-selective antagonist VPC32183(S). Collectively, these observations implicate LPA as a second messenger during the promotion of cell maturation and survival in chondrocytes by 24R,25$(OH)_2D_3$. Recently, a single nucleotide polymorphism (SNP) in LPA1 has been identified in osteoarthritic patients [34]. Moreover, the LPA1−/− mouse exhibits a smaller skeleton relative to wild-type mice [35]. These findings, in conjunction with those demonstrating that 24R,25$(OH)_2D_3$ and LPA [10] exert their effects in chondrocytes via LPA1 and/or LPA3, indicate that LPA1 is significant in the regulation of cartilage.

In this study, LPA-mediated rescue of Pi-induced apoptosis is sensitive to pertussis toxin and LY294002, but not cholera toxin. This indicates that LPA exerts its anti-apoptotic effects through $G_{\alpha i}$ and PI_3K signaling. A consequence of activation of this cascade is the activation of Akt/protein kinase B (PKB) [36]. Akt phosphorylates mdm2, inducing its translocation into the nucleus where it mono- and poly-ubiquitinates p53 [37,38]. This results in the nuclear export and subsequent proteasomal degradation of p53. The data show that the reduction of caspase-3 by LPA is sensitive to the nuclear export inhibitor leptomycin B and that LPA increases the phosphorylation of mdm2, supporting the claim that LPA is modulating p53 through this pathway. Interestingly, this is the mechanism by which LPA inhibits p53 signaling in A549 lung carcinoma cells [29], suggesting that this pathway is conserved among different cell types.

In this study, 24R,25$(OH)_2D_3$-mediated inhibition of Pi-induced caspase-3 activity was not inhibited by the PI_3K inhibitor LY294002. Furthermore, 24R,25$(OH)_2D_3$ did not increase the abundance of phosphorylated mdm2 at 6 h post-treatment. This suggests that 24R,25$(OH)_2D_3$ is stimulating one or more other pathways to inhibit apoptosis in addition to LPA-mediated inhibition of p53. Resting zone chondrocytes mature in response to 24R,25$(OH)_2D_3$ through increased PKC and PKA activity, resulting in MEK1/2 signaling [4]. Further support for this is the inhibition of 24R,25$(OH)_2D_3$-dependent rescue of Pi-induced caspase-3 by wortmannin. It has been previously shown that wortmannin inhibits PLD and PLD-dependent PKC in resting zone chondrocytes treated with 24R,25$(OH)_2D_3$ [9]. Wortmannin has also been shown in other cell types to inhibit PI_3K [39]. The failure of the PI_3K specific inhibitor LY294002 to block the effects of 24R,25$(OH)_2D_3$ supports the conclusion that PLD was the target of wortmannin. Interestingly, PLD mediates the effects of 24R,25$(OH)_2D_3$ on PKC [9] and PKC is associated with cell proliferation in many cell types [40].

Another possible pathway is the PLC signaling cascade. 24R,25$(OH)_2D_3$-mediated inhibition of apoptosis was attenuated by pertussis toxin, a $G_{\alpha i}$ inhibitor. Pertussis toxin also inhibits $G_{\beta\gamma}$ signaling, which can stimulate PLC activation [41,42]. The data in this study show that LPA1/3, PI-PLC, and release of intracel-

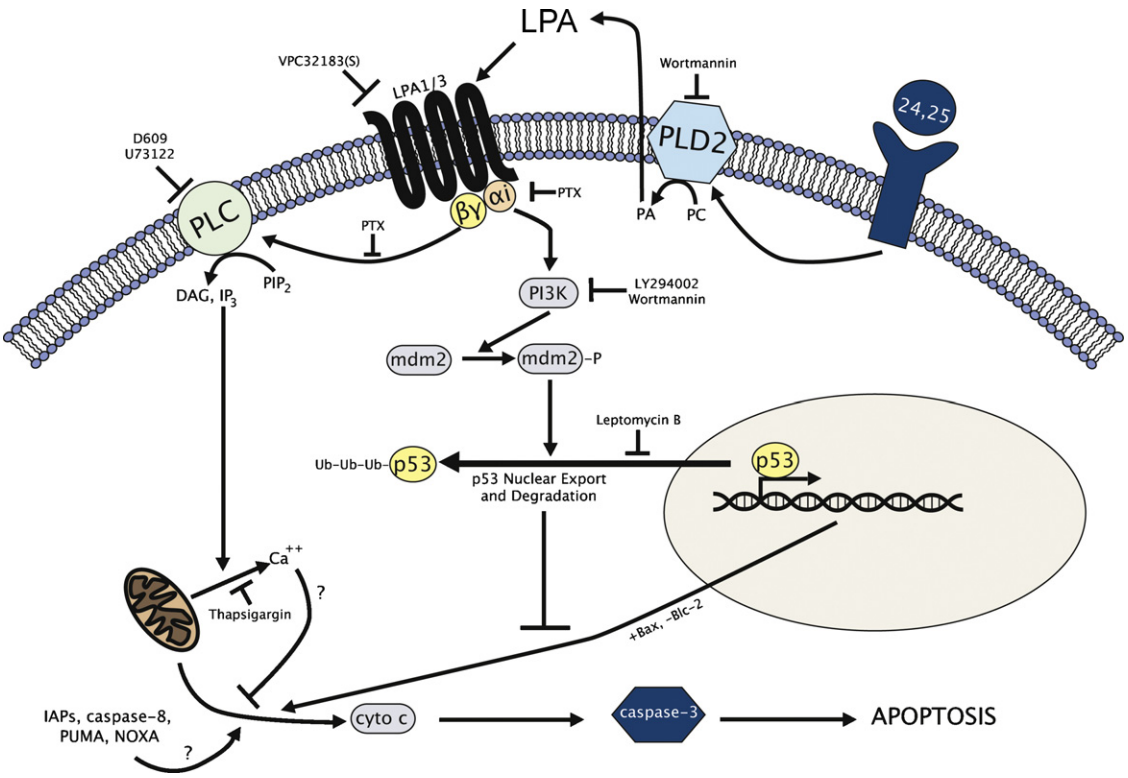

Fig. 8. Working model of mechanisms of 24R,25$(OH)_2D_3$ and LPA-mediated inhibition of apoptosis in resting zone chondrocytes. Resting zone chondrocytes respond to 24R,25$(OH)_2D_3$, resulting in increased phospholipase D (PLD) activity. Activation of PLD stimulates the production of lysophosphatidic acid (LPA). Stimulation of LPA1/3 receptor(s) by LPA leads to activation of $G_{\alpha i}$ and phoshoinositol 3-kinase (PI_3K) signaling. This results in increased phosphorylation of the E3-ubiquitin ligase murine double minute 2 (mdm2). Phospho-mdm2 translocates to the nucleus where it can mono- or poly-ubiquitinate p53 to promote its nuclear export and proteosomal degradation. A decrease in p53 protein abundance and nuclear localization yields reduced p53-mediated transcription and decreased caspase-3 activity, resulting in increased cell survival, maturation, and proliferation. 24R,25$(OH)_2D_3$ also stimulates PKC and PKA signaling and modulates intracellular calcium levels through $G_{\beta\gamma}$-mediated PLC activation, which may also pay a role in 24R,25$(OH)_2D_3$'s anti-apoptotic effects. The roles of other apoptotic regulatory proteins such as caspase-8, PUMA, NOXA, and IAPs in 24R,25$(OH)_2D_3$-mediated inhibition of apoptosis are not currently known.

lular calcium from the endoplasmic reticulum are necessary for the inhibition of Pi-induced caspase-3 activity by 24R,25$(OH)_2D_3$. Taken together, this suggests that 24R,25$(OH)_2D_3$ is initiating LPA-mediated stimulation of $G_{\beta\gamma}$-induced PLC activity, resulting in release of intracellular calcium. Whether the intracellular Ca^{2+} and diacylglycerol (DAG) resulting from the activation of PI-PLC by LPA1/3 contribute to the increase in PKC seen in cells treated with 24R,25$(OH)_2D_3$ is not known. Further studies are also needed to determine if other apoptosis regulators such as caspase-8, p53 up-regulated modulator of apoptosis (PUMA), NOXA, and inhibitors of apoptosis (IAPs) contribute to 24R,25$(OH)_2D_3$-mediated inhibition of Pi-induced cell death. Lastly, the effect of suppression of caspase-3 by 24R,25$(OH)_2D_3$ on cellular functions beyond apoptosis has yet to be determined.

Resting zone chondrocytes respond to 24R,25$(OH)_2D_3$ with increased proliferation, maturation, and matrix production [6,43]. In this study, it is shown 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. This implies that 24R,25$(OH)_2D_3$ modulates growth plate development by controlling the rate and extent of transition of chondrocytes from a resting zone to a growth zone phenotype. Additionally, our data suggest that 24R,25$(OH)_2D_3$ is a homeostatic regulator in the growth plate, acting to enhance cell survival in the presence of the apoptosis-permissive vitamin D metabolite 1,25-dihydroxyvitamin D3. Therefore, our findings may lead to the development of therapeutic agents that combat disruptions in endochondral formation and that enhance bone fracture healing. 24R,25$(OH)_2D_3$ may also prove to enhance cell survival in other cartilage types such as articular cartilage and may therefore be useful in the treatment of osteoarthritis.

In summary, these data identify a new signaling axis in the inhibition of apoptosis in the growth plate (Fig. 8). Chondrocytes respond to 24R,25$(OH)_2D_3$ which stimulates the signaling of its second messenger, LPA, resulting in inhibition of p53 signaling and enhanced cell survival. Information gleaned from this study provides new understanding into the maintenance of the pool of chondrocytes in the resting zone of the growth plate. Therefore, this study identifies this signaling axis as a potential therapeutic target for the treatment of bone fracture repair and as a regulatory agent of endochondral ossification during skeletal development and long bone growth.

Acknowledgements

This work was supported by Children's Healthcare of Atlanta, Atlanta, GA and the Price Gilbert, Jr. Foundation. We would like to thank Sharon Hyzy, Erin Plute, and Leang Chhun for their assistance with this project.

References

[1] K. Mansfield, B. Pucci, C.S. Adams, I.M. Shapiro, Induction of apoptosis in skeletal tissues: phosphate-mediated chick chondrocyte apoptosis is calcium dependent, Calcif. Tissue Int. 73 (2) (2003) 161–172.
[2] 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).

[3] 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) C833–839.
[4] Z. Schwartz, H. Ehland, V.L. Sylvia, D. Larsson, R.R. Hardin, V. Bingham, D. Lopez, D.D. Dean, B.D. Boyan, 1Alpha,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.
[5] D.D. Dean, B.D. Boyan, Z. Schwart, O.E. Muniz, M.R. Carreno, S. Maeda, D.S. Howell, Effect of 1alpha,25-dihydroxyvitamin D3 and 24R,25-dihydroxyvitamin D3 on metalloproteinase activity and cell maturation in growth plate cartilage in vivo, Endocrine 14 (3) (2001) 311–323.
[6] 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.
[7] B.D. Boyan, V.L. Sylvia, D.D. Dean, Z. Schwartz, 24,25-(OH) (2)D (3) regulates cartilage and bone via autocrine and endocrine mechanisms, Steroids 66 (3–5) (2001) 363–374.
[8] B.D. Boyan, V.L. Sylvia, N. McKinney, Z. Schwartz, Membrane actions of vitamin D metabolites 1alpha,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.
[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] 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 1793 (5) (2009) 836–846.
[11] J. Chun, Lysophospholipid receptors: implications for neural signaling, Crit. Rev. Neurobiol. 13 (2) (1999) 151–168.
[12] C.W. Lee, R. Rivera, S. Gardell, A.E. Dubin, J. Chun, GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, LPA5, J. Biol. Chem. 281 (33) (2006) 23589–23597.
[13] E.J. Goetzl, S. An, A subfamily of G protein-coupled cellular receptors for lysophospholipids and lysosphingolipids, Adv. Exp. Med. Biol. 469 (1999) 259–264.
[14] K. Noguchi, S. Ishii, T. Shimizu, Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family, J. Biol. Chem. 278 (28) (2003) 25600–25606.
[15] T.M. McIntyre, A.V. Pontsler, A.R. Silva, A. St. Hilaire, Y. Xu, J.C. Hinshaw, G.A. Zimmerman, K. Hama, J. Aoki, H. Arai, G.D. Prestwich, Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPARgamma agonist, Proc. Natl. Acad. Sci. U.S.A. 100 (1) (2003) 131–136.
[16] X. Fang, M. Schummer, M. Mao, S. Yu, F.H. Tabassam, R. Swaby, Y. Hasegawa, J.L. Tanyi, R. LaPushin, A. Eder, R. Jaffe, J. Erickson, G.B. Mills, Lysophosphatidic acid is a bioactive mediator in ovarian cancer, Biochim. Biophys. Acta 1582 (1–3) (2002) 257–264.
[17] E.J. van Corven, A. Groenink, K. Jalink, T. Eichholtz, W.H. Moolenaar, Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins, Cell 59 (1) (1989) 45–54.
[18] G.B. Mills, W.H. Moolenaar, The emerging role of lysophosphatidic acid in cancer, Nat. Rev. Cancer 3 (8) (2003) 582–591.
[19] W. Deng, L. Balazs, D.A. Wang, L. Van Middlesworth, G. Tigyi, L.R. Johnson, Lysophosphatidic acid protects and rescues intestinal epithelial cells from radiation- and chemotherapy-induced apoptosis, Gastroenterology 123 (1) (2002) 206–216.
[20] J. Ren, Y.J. Xiao, L.S. Singh, X. Zhao, Z. Zhao, L. Feng, T.M. Rose, G.D. Prestwich, Y. Xu, Lysophosphatidic acid is constitutively produced by human peritoneal mesothelial cells and enhances adhesion, migration, and invasion of ovarian cancer cells, Cancer Res. 66 (6) (2006) 3006–3014.
[21] K. Hama, J. Aoki, M. Fukaya, Y. Kishi, T. Sakai, R. Suzuki, H. Ohta, T. Yamori, M. Watanabe, J. Chun, H. Arai, Lysophosphatidic acid and autotaxin stimulate cell motility of neoplastic and non-neoplastic cells through LPA1, J. Biol. Chem. 279 (17) (2004) 17634–17639.
[22] Y. Xu, Z. Shen, D.W. Wiper, M. Wu, R.E. Morton, P. Elson, A.W. Kennedy, J. Belinson, M. Markman, G. Casey, Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers, JAMA 280 (8) (1998) 719–723.
[23] 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.
[24] N. Panupinthu, J.T. Rogers, L. Zhao, L.P. Solano-Flores, F. Possmayer, S.M. Sims, S.J. Dixon, P2X7 receptors on osteoblasts couple to production of lysophosphatidic acid: a signaling axis promoting osteogenesis, J. Cell. Biol. 181 (5) (2008) 859–871.
[25] N. Panupinthu, L. Zhao, F. Possmayer, H.Z. Ke, S.M. Sims, S.J. Dixon, P2X7 nucleotide receptors mediate blebbing in osteoblasts through a pathway involving lysophosphatidic acid, J. Biol. Chem. 282 (5) (2007) 3403–3412.
[26] A. Facchini, R.M. Borzi, F. Flamigni, Induction of ornithine decarboxylase in T/C-28a2 chondrocytes by lysophosphatidic acid: signaling pathway and inhibition of cell proliferation, FEBS Lett. 579 (13) (2005) 2919–2925.
[27] S.A. Karagiosis, N.J. Karin, Lysophosphatidic acid induces osteocyte dendrite outgrowth, Biochem. Biophys. Res. Commun. 357 (1) (2007) 194–199.
[28] 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.
[29] 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.
[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] Y.J. Chang, Y.L. Kim, Y.K. Lee, S.J. Sacket, K. Kim, H.L. Kim, M. Han, Y.S. Bae, F. Okajima, D.S. Im, Dioleoyl phosphatidic acid increases intracellular Ca^{2+} through endogenous LPA receptors in C6 glioma and L2071 fibroblasts, Prostaglandins Other Lipid Mediat. 83 (4) (2007) 268–276.
[32] 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 1alpha,25-(OH)(2)D(3) is mediated by phospholipase C, Steroids 66 (9) (2001) 683–694.
[33] M.M. Murph, L.A. Scaccia, L.A. Volpicelli, H. Radhakrishna, Agonist-induced endocytosis of lysophosphatidic acid-coupled LPA1/EDG-2 receptors via a dynamin2- and Rab5-dependent pathway, J. Cell Sci. 116 (Pt. 10) (2003) 1969–1980.
[34] H. Mototani, A. Iida, M. Nakajima, T. Furuichi, Y. Miyamoto, T. Tsunoda, A. Sudo, A. Kotani, A. Uchida, K. Ozaki, Y. Tanaka, Y. Nakamura, T. Tanaka, K. Notoya, S. Ikegawa, A functional SNP in EDG2 increases susceptibility to knee osteoarthritis in Japanese, Hum. Mol. Genet. 17 (12) (2008) 1790–1797.
[35] J.J. Contos, I. Ishii, N. Fukushima, M.A. Kingsbury, X. Ye, S. Kawamura, J.H. Brown, J. Chun, Characterization of lpa(2) (Edg4) and lpa(1)/lpa(2) (Edg2/Edg4) lysophosphatidic acid receptor knockout mice: signaling deficits without obvious phenotypic abnormality attributable to lpa(2), Mol. Cell. Biol. 22 (19) (2002) 6921–6929.
[36] J. Downward, PI 3-kinase, Akt and cell survival, Semin. Cell Dev. Biol. 15 (2) (2004) 177–182.
[37] M. Li, C.L. Brooks, F. Wu-Baer, D. Chen, R. Baer, W. Gu, Mono- versus polyubiquitination: differential control of p53 fate by Mdm2, Science 302 (5652) (2003) 1972–1975.
[38] D. Milne, P. Kampanis, S. Nicol, S. Dias, D.G. Campbell, F. Fuller-Pace, D. Meek, A novel site of AKT-mediated phosphorylation in the human MDM2 oncoprotein, FEBS Lett. 577 (1–2) (2004) 270–276.
[39] A. Arcaro, M.P. Wymann, Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses, Biochem. J. 296 (Pt. 2) (1993) 297–301.
[40] G.C. Blobe, L.M. Obeid, Y.A. Hannun, Regulation of protein kinase C and role in cancer biology, Cancer Metastasis Rev. 13 (3–4) (1994) 411–431.
[41] A.V. Smrcka, P.C. Sternweis, Regulation of purified subtypes of phosphatidylinositol-specific phospholipase C beta by G protein alpha and beta gamma subunits, J. Biol. Chem. 268 (13) (1993) 9667–9674.
[42] A.V. Smrcka, G protein betagamma subunits: central mediators of G protein-coupled receptor signaling, Cell. Mol. Life Sci. 65 (14) (2008) 2191–2214.
[43] 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.