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Lysophosphatidic acid signaling promotes proliferation, differentiation, and cell survival in rat growth plate chondrocytes

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ABSTRACT

Growth plate cartilage is responsible for long bone growth in children and adolescents and is regulated by vitamin D metabolites in a cell zone-specific manner. Resting zone chondrocytes (RC cells) are regulated by 24,25-dihydroxyvitamin D3 via a phospholipase D-dependent pathway, suggesting downstream phospholipid metabolites are involved. In this study, we showed that 24R,25$(OH)_2D_3$ stimulates rat costochondral RC chondrocytes to release lysophosphatidic acid (LPA) and, therefore sought to determine the role of LPA signaling in these cells. RC cells expressed the G-protein coupled receptors LPA1–5 and peroxisome proliferator-activated receptor gamma (PPAR-γ). LPA and the LPA1/3 selective agonist OMPT increased proliferation and two maturation markers, alkaline phosphatase activity and [^{35}S]-sulfate incorporation. LPA and 24R,25$(OH)_2D_3$'s effects were inhibited by the LPA1/3 selective antagonist VPC32183(S). Furthermore, apoptosis induced by either inorganic phosphate or chelerythrine was attenuated by LPA, based on DNA fragmentation, TUNEL staining, caspase-3 activity, and Bcl-2:Bax protein ratio. LPA prevented apoptotic signaling by decreasing the abundance, nuclear localization, and transcriptional activity of the tumor-suppressor p53. LPA treatment also regulated the expression of the p53-target genes Bcl-2 and Bax to enhance cell survival. Collectively, these data suggest that LPA promotes differentiation and survival in RC chondrocytes, demonstrating a novel physiological function of LPA-signaling.

1. Introduction

Lysophosphatidic acid (LPA 18:1; 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate) is a bioactive lysophospholipid that consists of a single fatty acid chain and is produced by activated platelets and cancer cell types [1,2]. LPA is derived from a number precursor lipids including phosphatidic acid (PA) which is generated by the metabolism of phosphatidylcholine (PC) by phospholipase D (PLD) [3]. LPA exerts its effects on cells by activating the cell surface G-protein coupled receptors (GPCRs) LPA1/Edg2, LPA2/Edg4, LPA3/Edg7, LPA4/GPR23, and LPA5/GPR92 [4–7]. These receptors collectively stimulate the $G_{\alpha i}$, $G_{\alpha q}$, $G_{\alpha s}$, and $G_{\alpha 12/13}$ signaling pathways [4,5,8–10]. In addition to G-protein coupled receptors, LPA has been shown to activate the nuclear fatty acid receptor peroxisome proliferator-activated receptor gamma (PPAR-γ) [11]. LPA signaling has been implicated in a wide array of cellular processes including wound healing and smooth muscle contraction as well as cell proliferation, survival, and migration [12–17]. These latter functions support a role for LPA signaling in cancer progression, where LPA has been shown to promote tumorigenesis by enhancing adhesion, migration, and invasion [12,16,18].

LPA appears to be involved in regulation of bone and cartilage. LPA has been shown to regulate osteoblasts [19,20] and chondrocytes are also sensitive to the lipid mediator [21] These studies suggest that LPA may also be involved in endochondral ossification, a process involving the formation of bone upon a cartilage template and the mechanism by which long bones in children and adolescents lengthen [22]. This template is the result of growth, maturation, and calcification of growth plate cartilage [23], which is regulated in part by the vitamin D metabolites 1,25-dihydroxy vitamin D3 [1α,25$(OH)_2D_3$] and 24,25-dihydroxy vitamin D3 [24R,25$(OH)_2D_3$] [24]. LPA acts synergistically with 1α,25$(OH)_2D_3$ to promote osteoblast differentiation [25], providing evidence of a relationship between this metabolite and LPA signaling, but it is not known if there is a relationship between LPA and 24R,25$(OH)_2D_3$.

The resting zone of the growth plate provides a reservoir of chondrocytes that will eventually undergo terminal differentiation, hypertrophy, and apoptosis as the growth plate matures. The cells in the resting zone are surrounded by a proteoglycan-rich extracellular matrix and apoptosis is a relatively infrequent event [26,27]. These cells respond in particular to the 24R,25$(OH)_2D_3$, resulting in increased cell maturation, matrix synthesis, and cell survival [28–31]. 24R,25$(OH)_2D_3$ acts on resting zone chondrocytes via a PLD-dependent mechanism [32] and many 24R,25$(OH)_2D_3$-mediated

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effects in resting zone chondrocytes have been shown to be dependent upon PLD activation [31].

These observations implicate LPA as a second messenger during the promotion of cell maturation and survival in chondrocytes by 24R,25 $(OH)_2D_3$. However, the downstream targets by which LPA exerts its effect on growth plate chondrocytes are unknown. One possibility is that LPA acts by modulating the abundance of the tumor-suppressor p53. LPA has been shown to promote the degradation of p53 in several cancer cell types [33,34], resulting in increased cell survival. Reduction of p53 protein abundance is necessary in osteoblast maturation [35,36] suggesting that LPA-mediated decreases in p53 may be important in the maintenance of cartilage tissue as well.

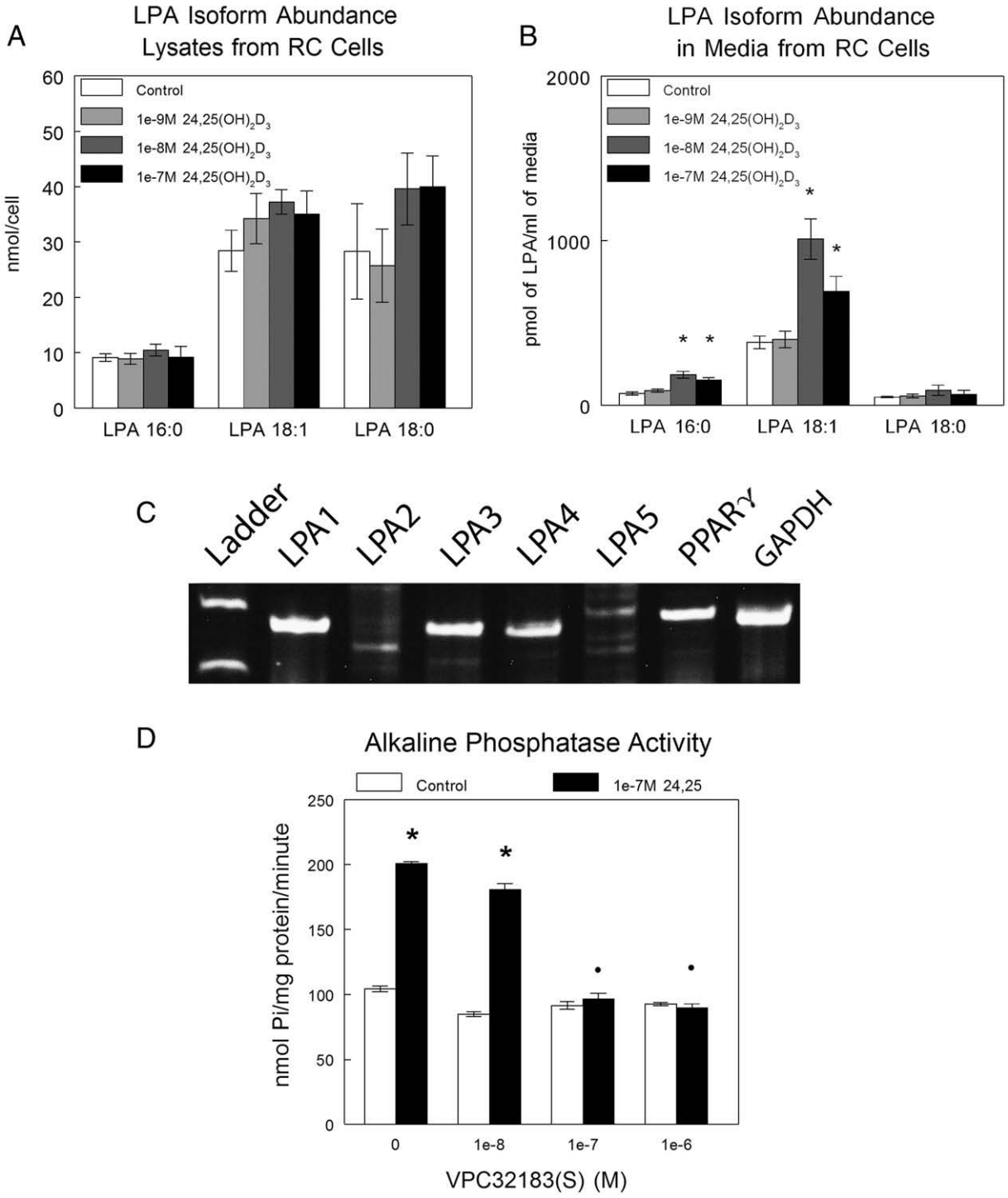


Fig. 1. Resting zone chondrocytes produce LPA and express LPA receptors. LPA isoform abundance was determined by LC ESI MS/MS from cell layer lysates (A) and media (B) collected from resting zone chondrocytes treated with media containing vehicle alone or 10^{-9} to 10^{-7} M 24R,25$(OH)_2D_3$. (C) Total RNA was isolated from confluent cultures of male rat resting zone chondrocytes using Trizol. LPA receptor cDNA was generated using specific reverse primers for LPA1–5, PPAR-γ, and GADPH and Omniscript Reverse Transcriptase (Qiagen). LPA receptors fragments were amplified via polymerase chain reaction using sequence specific primers. (D) Male rat resting zone chondrocytes were treated with complete media or varying concentrations of 24R,25$(OH)_2D_3$ in the presence or absence of the LPA1/3 selective antagonist VPC3218(S) for 24 h. Alkaline phosphatase specific activity was measured in cell layer lysates as a function of release of *para*-nitrophenol from *para*-nitrophenylphosphate at pH 10.2. (* = significant relative to untreated control, • = significant relative to 10^{-7} M 24R,25$(OH)_2D_3$).

The purpose of this study was to investigate the role of LPA signaling in the maintenance of the growth plate resting zone. Specifically, we assessed how LPA regulates proliferation, maturation, and apoptotic cell death in growth plate chondrocytes using resting zone cells isolated from adult rat costochondral growth plate cartilage as our model system. We found that LPA enhances two markers of chondrocyte maturation: alkaline phosphatase enzymatic activity and [^{35}S]-sulfate incorporation. In addition, LPA was found to be a potent stimulator of proliferation. Lastly, LPA protects resting zone chondrocytes from apoptotic cell death by decreasing the abundance of the tumor suppressor p53 to alter p53 target gene expression and protein abundance. Collectively, these data suggest that LPA signaling promotes cellular proliferation, maturation and survival in resting zone chondrocytes demonstrating a novel physiological function of LPA signaling and providing evidence that LPA produced by the cells in response to 24R,25$(OH)_2D_3$ stimulation may act to mediate its effects on resting zone chondrocytes.

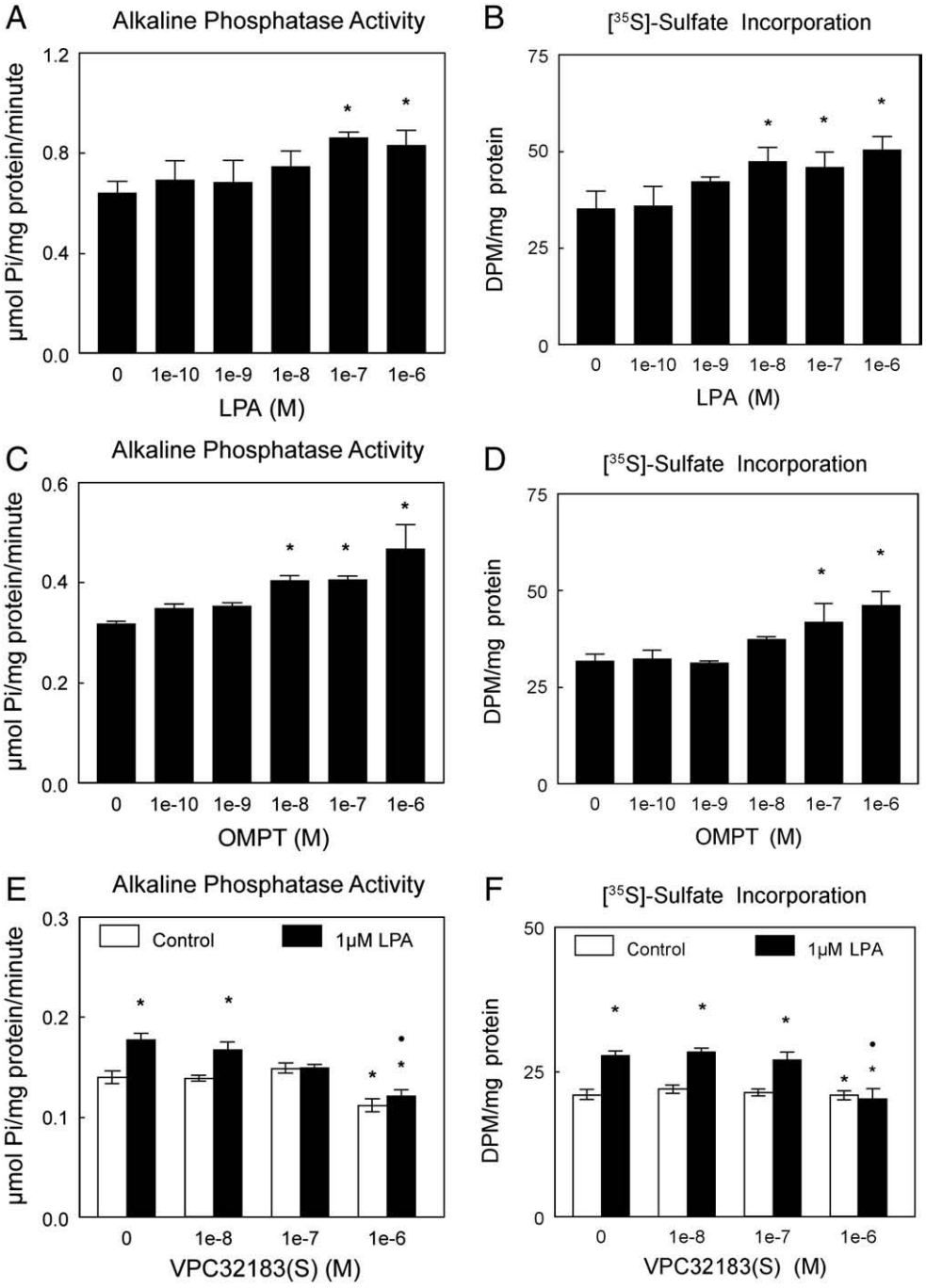


Fig. 2. LPA increases maturation in resting zone chondrocytes. Chondrocyte maturation was determined by measuring alkaline phosphatase specific activity (A, C, E) and [^{35}S]-incorporation (B, D, F). Male rat resting zone chondrocytes were treated with complete media or varying concentrations of LPA (A, B) or the LPA1/3 selective agonist OMPT (C, D) for 24 h prior to harvesting the cell monolayer. (E, F) Additionally, cells were treated with complete media or 1 µM LPA in the presence or absence of the LPA1/3 selective antagonist VPC3218(S) for 24 h. Alkaline phosphatase specific activity was measured in cell layer lysates as a function of release of *para*-nitrophenol from *para*-nitrophenylphosphate at pH 10.2. In order to measure sulfate incorporation, cells were labeled with [^{35}S]-sulfate 4 h prior to harvest. At harvest, the conditioned media were removed, the cell layers (cells and matrix) were collected, and the amount of [^{35}S]-sulfate incorporated determined as a function of protein in the cell layer. (* = significant relative to untreated control, • = significant relative to 1 µM LPA).

2. Materials and methods

2.1. Reagents

18:1 LPA (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate), OMPT ((2S)-1-oleoyl-2-O-methyl-glycero-3-phosphothionate), and VPC32183(S) ((S)-phosphoric acid mono-{2-octadec-9-enoylamino-3-[4-(pyridine-2-ylmethoxy)-phenyl]-propyl} ester) were purchased from Avanti Polar Lipids (Alabaster, AL). All lipid species were 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 [37]. 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.

2.3. LPA isoforms

The abundance of LPA isoforms in lysates and media collected from the resting zone chondrocyte cultures was determined by liquid chromatography electrospray ionization tandem mass spectrometry (LC ESI MS/MS). Resting zone chondrocytes were cultured in T75 flasks and grown until confluence. Cells were then treated for 30 min with starvation media (1% FBS) containing 1% bovine serum albumin (BSA) and 24R,25$(OH)_2D_3$ (10^{-9}, 10^{-8}, or 10^{-7}M) (BioMol, Plymouth Meeting, PA) or vehicle alone. After the treatment period, 1 ml of conditioned media was collected and cell monolayers were harvested using 0.05 M sodium hydroxide (NaOH). Media and lysate samples were spiked with 1 pmol of 17:0 LPA (internal standard) prior to lipid extraction via the Bligh-Dyer method [38]. LC ESI MS/MS analysis was conducted at the Georgia Institute of Technology Bioanalytical Mass Spectrometry Facility using a Shimadzu HPLC pump and a Q-TRAP 4000 (Applied Biosystems, Foster City, CA). Reverse phase chromatography was performed using a Supelco C18 column with a flow rate of 500 μl/min and an injection volume of 10 μl. The isocratic buffers were 75:25 methanol:water (v/v) and methanol each with 5 mM TEAA. Eluted samples were then analyzed on the QTRAP 4000 and LPA isoforms were compared to LPA standards supplied by Avanti Polar Lipids. Peak areas of LPA isoforms were normalized to 17:0 peaks to account for differences in lipid extraction efficiency.

2.4. LPA receptor, Bax, Bcl-2, and p53 expression

mRNA was harvested from resting zone chondrocytes using Trizol (Invitrogen, Carlsburg, CA) and reverse transcriptase polymerase chain reaction (RT-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′, LPA 2 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′. In order to determine the effect of LPA on p53, Bax, and Bcl-2 mRNA expression, cells were treated with 0, 0.01, 0.1, and 1 μM LPA for 6 h prior to harvesting the mRNA with Trizol. The following primers were used to amplify p53, Bax, and Bcl-2: p53 sense: 5′-CCGTCCCAGAAGGTTGCC-3′, p53 antisense: 5′-CGC TGC TCC GAA GGT GAT-3′, Bax sense: 5′-TTTGTTACAGGGTTTCATCC-3′, Bax antisense: 5′-CCAGTTCATCTCCAATTCG-3′, Bcl-2 sense: 5′-CTCGTGGCTGTCTCTGAAG-3′, Bcl-2 antisense: 5′-TCTGCTGACCTCACTTGTG-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′.

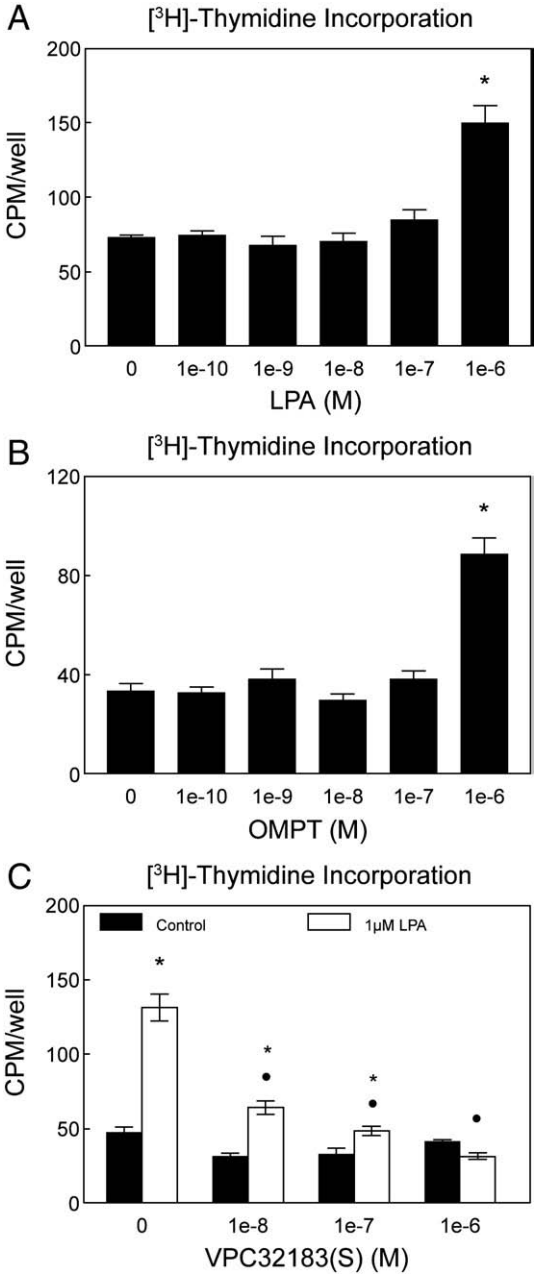
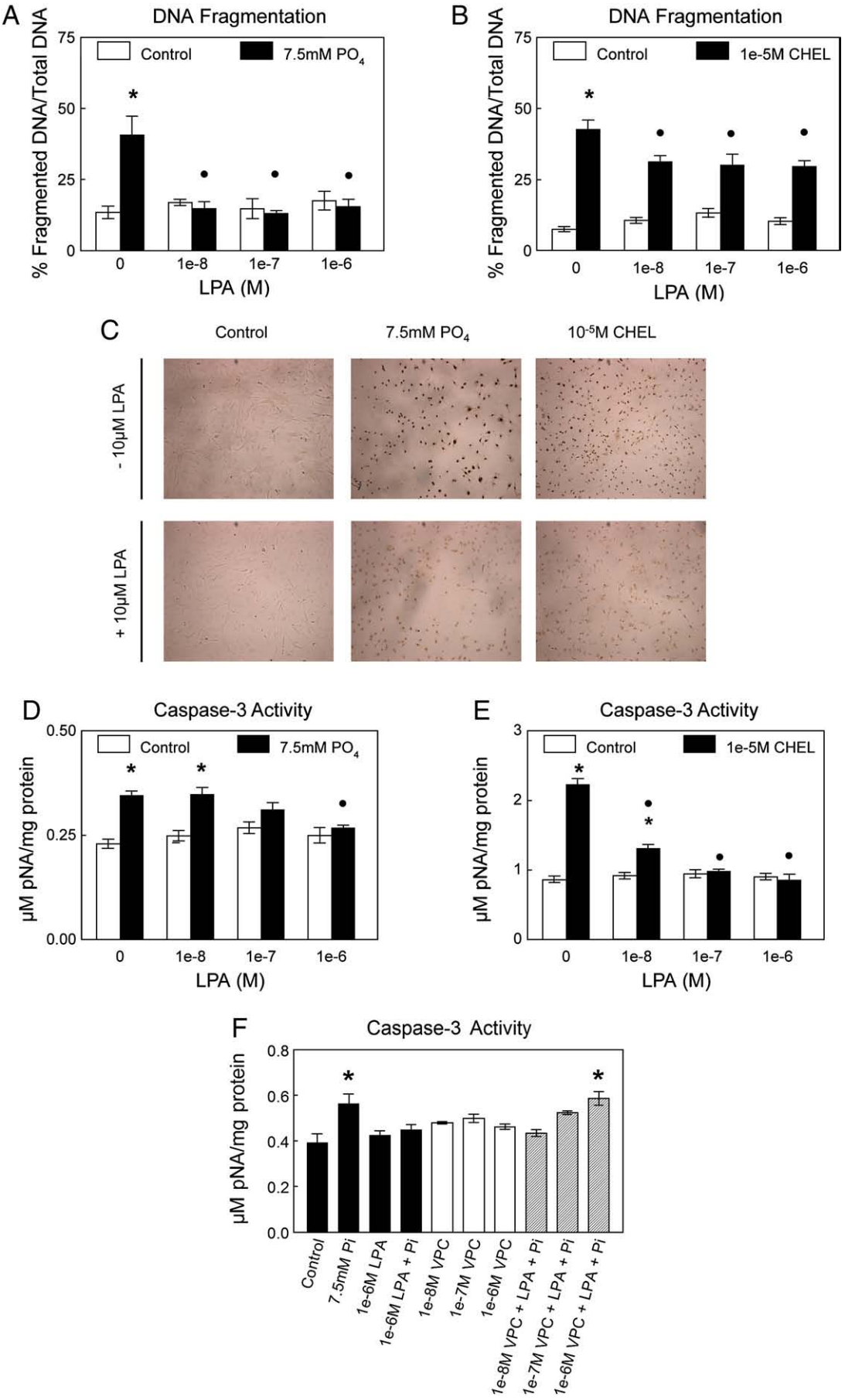


Fig. 3. LPA enhances proliferation. DNA synthesis was determined by measuring the incorporation of radio-labeled thymidine. Cells were grown to subconfluence and treated with DMEM containing 1% FBS for 48 h to induce quiescence. Male rat resting zone chondrocytes were treated with complete media or varying concentrations of LPA (A) or the LPA1/3 selective agonist OMPT (B) for 24 h prior to harvesting the cell monolayer. Cells were alternatively treated with complete media or 1 μM LPA in the presence or absence of the LPA1/3 selective antagonist VPC3218(S) for 24 h (C). Prior to harvest, cell were labeled for 3 h with [^{3}H]-thymidine. The monolayers were washed three times with PBS to remove unincorporated [^{3}H]. Cells were then fixed with cold 5% trichloroacetic acid followed by lysis with 1% sodium dodecyl sulfate. The amount of [^{3}H] activity was determined in each sample to determine the total amount of incorporated labeled thymidine. (* = significant relative to untreated control, • = significant relative to 1 μM LPA).


A
DNA Fragmentation
% Fragmented DNA/Total DNA
Control
7.5mM PO4
LPA (M)
B
DNA Fragmentation
1e-5M CHEL
C
Control
7.5mM PO4
10-5M CHEL
- 10μM LPA
+ 10μM LPA
D
Caspase-3 Activity
μM pNA/mg protein
E
Caspase-3 Activity
F
Caspase-3 Activity
Control
7.5mM Pi
1e-6M LPA
1e-6M LPA + Pi
1e-8M VPC
1e-7M VPC
1e-6M VPC
1e-8M VPC + LPA + Pi
1e-7M VPC + LPA + Pi
1e-6M VPC + LPA + Pi

2.5. Chondrocyte maturation assays

Confluent cultures were treated with LPA (0.01 nM to 1 μM) for the times indicated below. To determine if the LPA1/3 receptor was involved, cultures were also treated with LPA in the presence or absence of the LPA1/3-selective agonist (2S)-1-oleoyl-2-O-methyl-glycero-3-phosphothionate (OMPT) (Avanti Lipids, Alabaster, AL) (0.1 nM to 1 7μM) [39] or the LPA1/3-selective antagonist (S)-phosphoric acid mono-(2-octadec-9-enoylamino-3-[4-(pyridine-2-ylmethoxy)-phenyl]-propyl) ester (VPC32183(S)) (Avanti Lipids) (0.01 μM to 1 μM) [40]. Chondrocyte maturation was assessed by examining alkaline phosphatase specific activity and [^{35}S]-sulfate incorporation.

To determine that 24R,25$(OH)_2D_3$ elicited its effects via an LPA-dependent mechanism, confluent cultures of resting zone cells were cultured for 24 h in the presence and absence of the LPA1/3 selective antagonist VPC32183(S). Alkaline phosphatase specific activity was measured as described below.

2.5.1. Alkaline phosphatase specific activity

Initial experiments determined the optimal time course by treating confluent cultures with 1 μM LPA for 3, 6, 12, 18, and 24 h. Subsequent experiments were performed after treating the cells for 24 h. Following treatment, cell monolayers were lysed using 0.1% Triton X followed by sonication of each sample for 30 s. 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).

2.5.2. [^{35}S]-sulfate incorporation

Mature chondrocytes produce a proteoglycan-rich extracellular matrix that is characterized by sulfated glycosaminoglycans. To assess the effects of LPA on chondrocyte maturation, confluent cells were labeled with [^{35}S]-sulfate 3 h prior to harvest. At harvest, the conditioned media were removed, the cell layers (cells and matrix) were collected, and the amount of [^{35}S]-sulfate incorporated was determined as a function of protein in the cell layer [41].

2.6. DNA synthesis

To determine if LPA regulated chondrocyte proliferation, DNA synthesis was assessed by measuring the incorporation of radio-labeled thymidine. Cells were grown to subconfluence and treated with DMEM containing 1% FBS for 48 h to induce quiescence. Cells were then treated with LPA (0.1 nM to 1 μM) in the presence or absence of OMPT (0.1 nM to 1 μM) or VPC32183(S) (0.01 μM to 1 μM) for 24 h. Prior to harvest, cell were labeled for 3 h with [^{3}H]-thymidine. The monolayers were washed three times with phosphate buffer solution (PBS) to remove unincorporated [^{3}H]. Cells were then fixed with cold 5% trichloroacetic acid followed by lysis with 1% sodium dodecyl sulfate. The amount of [^{3}H] activity was determined in each sample to determine the total amount of incorporated radio-labeled thymidine.

2.7. Apoptosis assays

The role of LPA in chondrocyte survival was assessed by examining its ability to reduce apoptosis induced by two apoptogens, inorganic phosphate and chelerythrine [42,43]. Apoptotic cell death was determined by measuring by caspase-3 activity, TUNEL staining, and DNA fragmentation. Confluent cultures of resting zone chondrocytes were treated with either 10^{-5} M chelerythrine or 7.5 mM monobasic sodium phosphate to induce apoptosis. LPA (0.01 μM, 0.1 μM, or 1 μM), VPC32183(S) (0.01 μM, 0.1 μM, or 1 μM), or the vehicle was added to the cultures.

2.7.1. Caspase-3 activity

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 protein content as determined by the Pierce Macro BCA Protein Assay Kit.

2.7.2. 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.

2.7.3. TUNEL staining

DNA nicking was measured using the In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). After treatment, cells were fixed using 4% paraformaldehyde in PBS for 1hour. To detect nicks, cells were incubated with horse radish peroxidase-conjugated dUTP for 1 h at 37 °C. Nicks were visualized with DAB substrate (3,3-diaminobenzidinetetrahydrochloride) also purchased from Roche.

2.8. Regulation of p53

Confluent cultures in T75 flasks were treated for 6 h with 0, 0.01, 0.1, and 1 μM LPA. After treatment, the cell monolayer was washed twice with PBS and harvested with RIPA buffer. mRNA for p53 was determined as described above. The abundance of p53 protein in both the whole cell lysate and 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. Nuclear and cytoplasmic fractions were isolated by centrifuging whole cell lysates for 10 min at 13,000 rpm. The resulting supernatants (cytoplasmic fraction) were collected

Fig. 4. LPA protects cells from phosphate and chelerythrine-induced apoptosis. *DNA fragmentation:* Resting zone chondrocytes were labeled with [^{3}H]-thymidine for 4 h prior to treatment with the PKC inhibitor chelerythrine (A) or 7.5 mM monobasic sodium phosphate (B) in the presence and absence of LPA for 24 h. At the end of the treatment period, cell monolayers were washed with DMEM three times to remove unincorporated [^{3}H] and cell 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. *TUNEL:* (C) Resting zone chondrocytes were treated with complete media (control), chelerythrine or 7.5 mM monobasic sodium phosphate in the presence and absence of LPA for 24 h. Cells were fixed using 4% paraformaldehyde in PBS for 1 h. DNA nicking was assessed using the In situ Cell Death Detection Kit (Roche) followed by analysis with light microscopy. All images were obtained using an exposure time of 1.5 s. *Caspase-3 activity:* Caspase-3 activity was measured using the Colorimetric CaspACE™ Assay System (Promega) (D–F). Resting zone chondrocytes were harvested after treatment with complete media (control), chelerythrine or 7.5 mM monobasic sodium phosphate in the presence and absence of LPA and/or VPC32183(S) for 24 h. Cell lysates were incubated with the caspase-3 substrate DEVD-pNA. Cleavage of DEVD-pNA into pNA was measured at 405 nm and normalized to total protein. (* = significant relative to untreated control, • = significant relative to 7.5 mM PO_4 alone or 10^{-5} M CHEL alone).

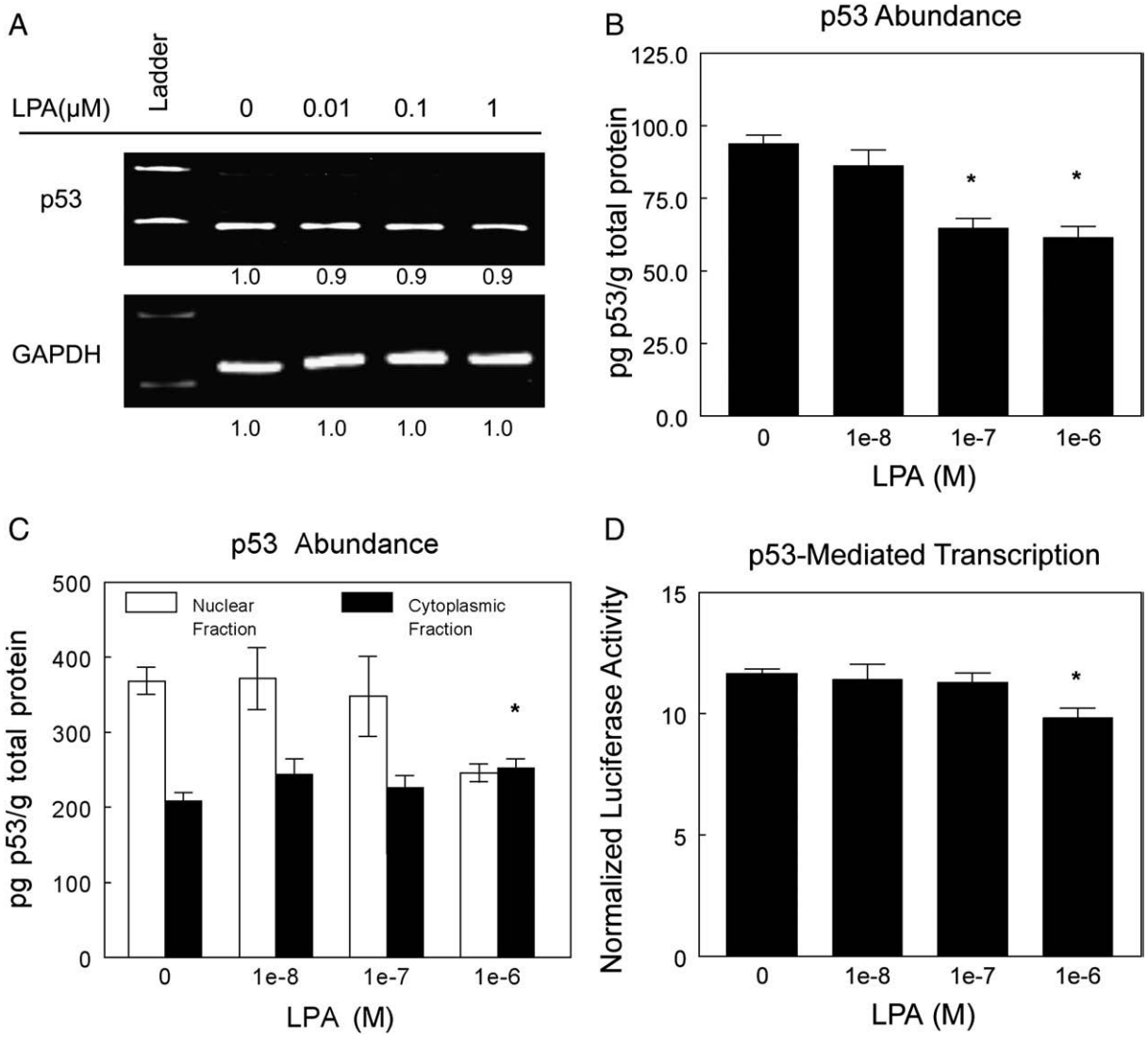


Fig. 5. LPA reduces p53 at the translational level but not at the transcriptional level. RNA from resting zone chondrocyte cultures treated with 0, 0.01, 0.1, or 1 μM LPA for 6 h was collected using Trizol (A). p53 and GAPDH cDNA were generated using specific reverse primers and Omniscript Reverse Transcriptase (Qiagen). Gene fragments were then amplified via polymerase chain reaction using sequence specific primers. Resting zone chondrocyte cultures were treated with complete media or with LPA for 6 h prior harvest. The abundance of p53 was measured in both the whole cell lysate (B) and the nuclear and cytoplasmic fractions (C) by p53 pan ELISA (Roche). p53-mediated transcription was assessed as a function of normalized luciferase activity (D). Cells were transfected with p53-controlled firefly luciferase and constitutively active *Renilla* luciferase. 24 h post-transfection, cells were treated with LPA for 16 h prior to measuring luciferase activity.

and the pellets (nuclear fraction) were resuspended in 500 μl RIPA buffer.

To assess changes in p53-mediated transcription, luciferase reporter gene assays were conducted as previously described [44]. 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, 0.01, 0.1, and 1 μM LPA for 16 h and luciferase activity was measured using the Dual Luciferase Reporter Assay kit (Promega, Madison, WI).

2.9. Abundance of Bax, Bcl-2, and p21 protein

Western blots were performed to determine the effect of LPA on the protein abundance of Bax, Bcl-2, and p21. 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 Bax (Δ 21, Santa Cruz Biotechnology, Inc.), Bcl-2 (DC 21, Santa Cruz Biotechnology, Inc.), p21 (BD Pharmingen, San Jose, CA), or GAPDH (MAB374 Chemicon, Billerica, MA). Immunoreactive bands were detected using 1:5000 dilutions of horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA), and visualized using enhanced chemiluminescence (Super-Signal WestPico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). mRNA for Bax and Bcl-2 were performed as described above.

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. Resting zone chondrocytes produce LPA and express LPA receptors

LC ESI MS/MS showed that three LPA isoforms, 16:0 LPA, 18:1 LPA, and 18:0 LPA, were present in both the cell monolayer (Fig. 1A) and conditioned media (Fig. 1B), indicating the existence of both intracellular and extracellular LPA in cultures of RC cells. 24R,25 $(OH)_2D_3$ increased the abundance of 16:0 and 18:1 LPA in the media, but not in the cell monolayer. The effect of 24R,25$(OH)_2D_3$ on both LPA forms was dose-dependent and was greatest in cultures treated with 10^{-8} M. RT-PCR using sequence-specific primers detected the presence of the five cell surface G-protein coupled receptors, LPA1–5, and the nuclear receptor PPAR-gamma (Fig. 1C). Distinct bands for LPA1, LPA3, and LPA4 were observed. Bands for LPA2 and LPA5 were present but to a lesser extent.

24R,25$(OH)_2D_3$ exerted its effects on chondrocyte maturation via an LPA1/3 dependent mechanism. The LPA1/3-selective antagonist VPC32183(S) attenuated 24R,25(OH)2D3-mediated increases in alkaline phosphatase activity (Fig. 1D).

3.2. Exogenous LPA enhances chondrocyte maturation

Initial time course experiments showed that LPA increased alkaline phosphatase specific activity at 24 h, but no LPA effect was observed prior to this time point (data not shown). For this reason, all future maturation experiments were conducted following a 24 hour exposure to the lipid mediator. Both LPA and the LPA1/3-selective agonist OMPT increased alkaline phosphatase activity in the resting zone chondrocytes in a dose dependent manner at concentrations ranging from 0.01 μM to 1 μM (Fig. 2A and C). The same concentrations of LPA and OMPT also increased [^{35}S]-sulfate incorporation (Fig. 2B and D). Furthermore, VPC32183(S) attenuated LPA-mediated increases in both alkaline phosphatase activity and [^{35}S]-sulfate incorporation in a dose dependent manner (Fig. 2E and F), indicating that the effects of LPA stimulation are dependent upon activation of LPA1 and/or LPA3.

3.3. LPA increases DNA synthesis in a dose-dependent manner

Treatment of pre-confluent cells culture with 1 μM LPA or OMPT enhanced DNA synthesis 100% over control cultures (Fig. 3A and B). Inhibition of LPA1 and LPA3 with VPC321283(S) inhibited LPA-mediated increases in proliferation (Fig. 3C). These data demonstrate LPA promotes proliferation in resting zone chondrocytes through activation of LPA1 and/or LPA3.

3.4. LPA reduces the stimulatory effects of phosphate and chelerythrine apoptosis

Both phosphate and chelerythrine increased DNA fragmentation relative to the control in a dose-dependent manner (Fig. 4A and B). LPA doses ranging from 0.01 μM to 1 μM completely and partially rescued phosphate and chelerythrine-induced DNA fragmentation, respectively. Similarly, LPA reduced DNA nicking induced by both apoptogens, evidenced by reduced TUNEL staining (Fig. 4C). Inorganic phosphate and chelerythrine also increased caspase-3 activity and LPA reduced this marker of apoptosis as well (Fig. 4D, E). The rescue of Pi-induced caspase-3 activity by LPA was attenuated by VPC32183(S) (4F).

3.5. LPA promotes cell survival via p53 signaling

Control cultures of resting zone chondrocytes expressed p53 mRNA (Fig. 5A) and protein (Fig. 5B). Treatment with LPA had no effect on p53 mRNA at 6 h, but there was a decrease in p53 protein at this time point. Nuclear p53 protein was decreased by LPA, whereas, cytoplasmic p53 did not change in response to the treatment (Fig. 5C). Both p53-mediated transcription (Fig. 5D) and the abundance of the p53-target gene p21 (data not shown) were decreased by LPA. Moreover, LPA decreased both the mRNA expression (Fig. 6A) and protein abundance (Fig. 6B) of Bax. Conversely, both Bcl-2 mRNA and protein abundance were increased by LPA (Fig. 6A, B).

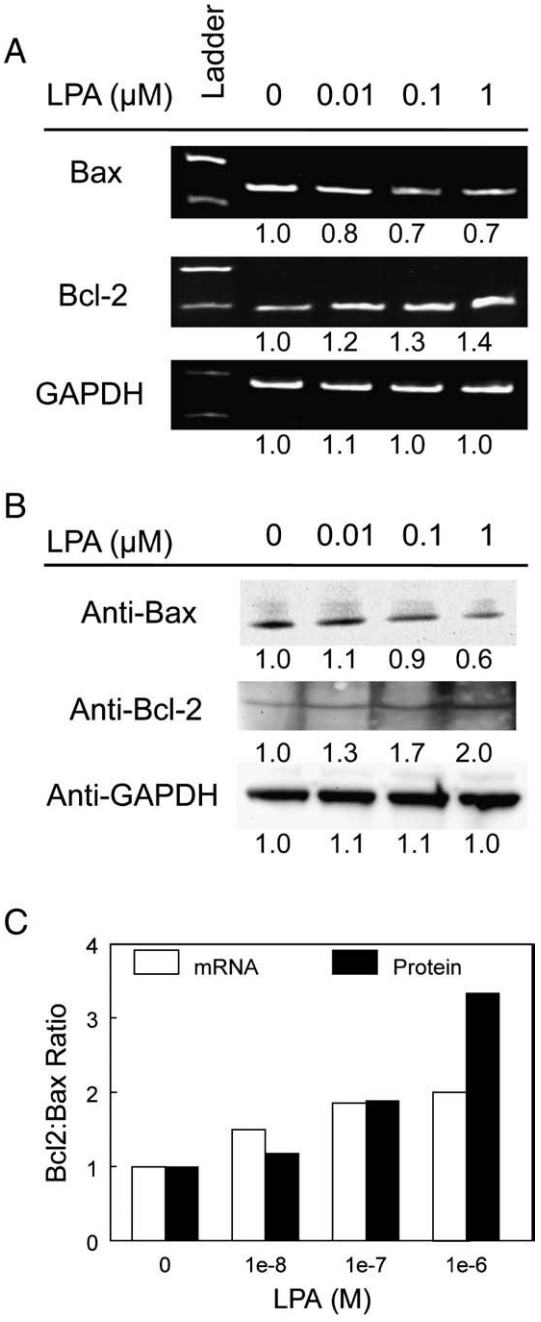


Fig. 6. Bax and Bcl-2 mRNA and protein abundance are regulated by LPA. (A) Total RNA was isolated from male rat resting zone chondrocyte cultures using Trizol. Bax, Bcl-2, and GAPDH cDNA was generated using specific reverse primers and Omniscript Reverse Transcriptase (Qiagen). Gene fragments were amplified via polymerase chain reaction using sequence specific primers. (B) Resting zone chondrocyte cultures were treated with complete media or with LPA for 6 h prior to immunoblotting of whole-cell extracts with mouse anti-Bcl-2, mouse anti-Bax, or mouse anti-GAPDH antibodies. (C) Calculated Bcl-2:Bax mRNA and protein ratios in response to LPA treatment from panels A and B.

4. Discussion

Our results indicate that LPA is an autocrine regulator in the growth plate resting zone. Resting zone chondrocytes contain intracellular LPA and secrete extracellular LPA. The LPA isoforms identified, 16:0, 18:1, and 18:0, are the most biologically relevant of the LPA isoforms [45–47]. In addition, LPA receptors are present in RC cells, demonstrating that they have the potential to respond to this phospholipid metabolite. Moreover, 24R,25$(OH)_2D_3$ increased the extracellular abundance of LPA 16:0 and LPA 18:1 and the LPA1/3-selective antagonist VPC32183(S) attenuated 24R,25$(OH)_2D_3$-mediated maturation, suggesting that LPA may act as downstream mediators of vitamin D metabolite effects on resting zone cells.

This study demonstrates that LPA signaling plays a role in the maintenance of the resting zone cartilage by promoting the survival of the chondrocyte pool (Fig. 7). At least two mechanisms are involved in the inhibition of apoptosis. LPA acts via reduced p53 and its downstream mediator p21, reduced Bax and increased Bcl-2. This is particularly important because resting zone cells serve as the pool for the growth zone and premature cell death in the resting zone could result in premature closure of the growth plate and limb shortening. In addition, LPA stimulated DNA synthesis, suggesting that LPA is involved in maintaining the pool of resting zone chondrocytes via proliferation. LPA has been shown to act as a mitogen in other systems [13,21,48–50], as well as in primary rat articular chondrocytes [21]. However, it decreased proliferation of T/C-28a2 cells, a human articular chondrocyte-like cell line [51], raising the possibility that its effects are cell specific.

In addition to its stimulatory effects on DNA synthesis, LPA increased [^{35}S]-sulfate incorporation, suggesting an increase in the synthesis of a sulfated proteoglycan extracellular matrix around the newly generated chondrocytes. Alkaline phosphatase specific activity also increased, supporting the hypothesis that the chondrocytes were producing a mature matrix containing alkaline phosphatase-rich extracellular matrix vesicles [52].

Our results strongly support a physiological role for LPA in promoting chondrocyte survival in the resting zone. We demonstrated by four different methods (DNA fragmentation, TUNEL staining, caspase-3 activity, and Bcl-2/Bax ratio) that LPA signaling reduced the induction of apoptosis by two agents shown previously to stimulate the apoptotic pathway in resting zone chondrocytes in vitro [42,43]. Moreover, the ability of LPA to rescue the apoptotic effect of Pi was attenuated by the LPA1/3 receptor antagonist, indicating that LPA1/3 signaling was responsible. The timing of programmed cell death is crucial in the maintenance of the growth plate. Inhibition of apoptotic signaling in hypertrophic chondrocytes prevents their terminal differentiation [53], resulting in lengthening of the growth plate as is typically seen in the vitamin D-deficient rickets. Phosphate plays an important role in this process [54], but it isn't known if the phosphate content is also a regulator of apoptosis in the resting zone.

Cell proliferation is associated with an increase in protein kinase C (PKC) in many cell types [55,56], and LPA reduced the effects of PKC inhibition by chelerythrine. Previously we have showed that 24R,25$(OH)_2D_3$ stimulates PKC and cell proliferation in resting zone chondrocytes via a PLD-dependent mechanism [57]. Our results suggest that LPA may mediate this response.

The LPA-induced decrease in the cellular abundance of the tumor suppressor p53 may be involved as well. The reduction of p53 correlates enhanced cell survival [58], indicating that the inhibition of p53 is the mechanism of LPA-mediated protection against cell death. The inhibition of p53 has also been implicated in the maturation of osteoblasts [35,36], suggesting that LPA-mediated decreases in p53 may be significant in chondrocyte maturation in addition to enhancing survival in these cells. LPA altered p53-mediated transcription and the expression of the p53-target genes p21, Bax and Bcl-2 at the transcriptional and translational level. The

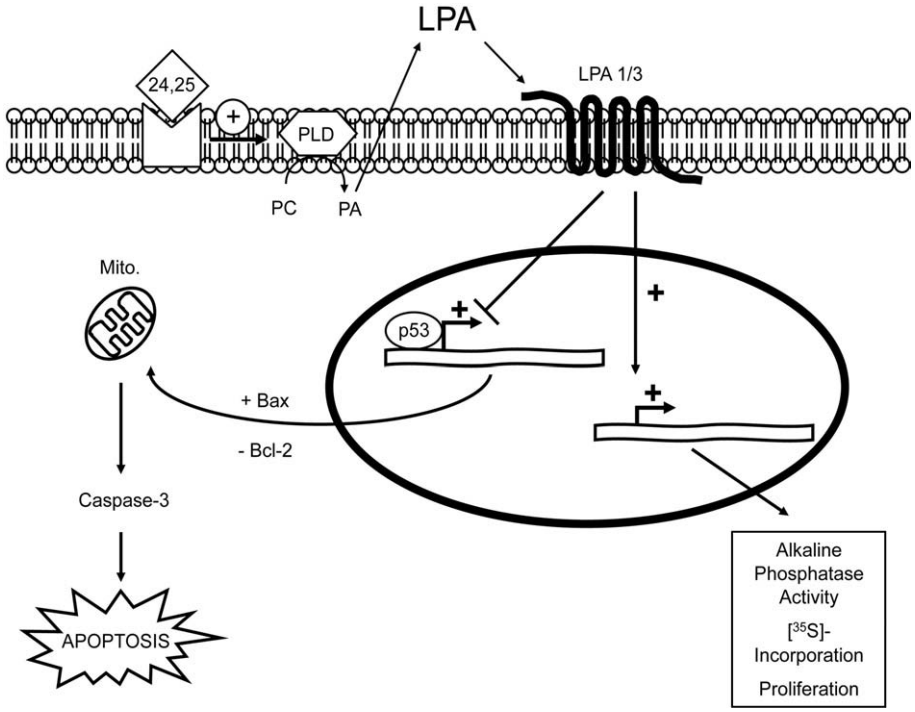


Fig. 7. Proposed mechanism of LPA signaling in the resting zone. 24R,25$(OH)_2D_3$-mediated stimulation of phospholipase D (PLD) promotes the conversion of phosphatidylcholine (PC) to phosphatidic acid (PA) leading to LPA production. LPA stimulates LPA1 and/or LPA3, resulting in increases alkaline phosphatase activity, [^{35}S]-incorporation, and proliferation. LPA also decreases the abundance and transcriptional activity of the tumor suppressor p53 causing an increase in the Bcl-2:Bax ratio and a decrease in caspase-3 activity.

change in the cellular Bax to Bcl-2 ratio would result in the inhibition of the release of cytochrome *c* from the mitochondria [59], halting the initiation of the apoptotic proteolytic caspase cascade. This is supported by our finding that LPA inhibits chelerythrine and phosphate-induced caspase-3 activity via an LPA1/3 mediated mechanism. Collectively, our results define a pathway for LPA-mediated enhancement of cell survival and chondrocyte maturation by which LPA decreases the abundance of p53 to alter p53-target gene expression resulting in the inhibition of caspase activity.

In summary, LPA was found to be a stimulator of resting zone chondrocyte proliferation and maturation and an inhibitor of chondrocyte apoptosis. This confirms a physiological role for LPA as a regulator of growth plate cartilage, and suggests that LPA produced via 24R,25$(OH)_2D_3$-stimulated PLD activity may mediate the actions of the secosteroid in growth plate resting zone chondrocytes. Additionally, this establishes LPA as a potential therapeutic regulatory agent in controlling the processes of endochondral bone formation during long bone growth and development and during fracture repair.

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