Lysophosphatidic Acid Signals through Multiple Receptors in Osteoclasts to Elevate Cytosolic Calcium Concentration, Evoke Retraction, and Promote Cell Survival†§

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Lysophosphatidic acid (LPA) is a bioactive phospholipid whose functions are mediated by multiple G protein-coupled receptors. We have shown that osteoblasts produce LPA, raising the possibility that it mediates intercellular signaling among osteoblasts and osteoclasts. Here we investigated the expression, signaling and function of LPA receptors in osteoclasts. Focal application of LPA elicited transient increases in cytosolic calcium concentration ([Ca2+]i), with 50% of osteoclasts responding at ~400 nM LPA. LPA-induced elevation of [Ca2+]i was blocked by pertussis toxin or the LPA1/3 receptor antagonist VPC-32183. LPA caused sustained retraction of osteoclast lamellipodia and disrupted peripheral actin belts. Retraction was insensitive to VPC-32183 or pertussis toxin, indicating involvement of a distinct signaling pathway. In this regard, inhibition of Rho-associated kinase stimulated reexpanding after LPA-induced retraction. Real-time reverse transcription-PCR revealed transcripts encoding LPA1, and to a lesser extent LPA2, LPA4, and LPA5 receptor subtypes. LPA induced nuclear translocation of Nfatc1 and enhanced osteoclast survival, effects that were blocked by VPC-32183 or by a specific peptide inhibitor of NFAT activation. LPA slightly reduced the resorptive activity of osteoclasts in vitro. Thus, LPA binds to at least two receptor subtypes on osteoclasts: LPA1, which couples through G13 to elevate [Ca2+]i, activate NFATc1, and promote survival, and a second receptor that likely couples through G12/13 and Rho to evoke and maintain retraction through reorganization of the actin cytoskeleton. These findings reveal a signaling axis in bone through which LPA, produced by osteoblasts, acts on multiple receptor subtypes to induce pleiotropic effects on osteoclast activity and function.

The actions of osteoclasts (bone-resorbing cells) and osteoblasts (bone-forming cells) are vital for skeletal development and remodeling (1). Bone cells continuously receive signals from adjacent cells, soluble mediators, and the extracellular matrix to regulate their proliferation, activity, and survival. Balance between resorption and formation is critical for skeletal homeostasis, and imbalance leads to diseases such as osteoporosis (2).

Lysophosphatidic acid (LPA)§ is a potent bioactive phospholipid present at low levels in plasma (~100 nM) (3) and elevated levels at sites of tissue injury and inflammation (3, 4). Produced by several cell types including activated platelets, LPA signals through five well described G protein-coupled receptors, LPA1–LPA5, each of which can couple to multiple heterotrimeric G proteins (5). Downstream responses can include elevation of cytosolic free calcium concentration ([Ca2+]i), activation of Ras and extracellular signal-regulated kinases (ERK), and stimulation of phosphatidylinositol 3-kinase/Akt signaling (6). Moreover, LPA receptors couple to Rho and Rac to elicit changes in cytoskeletal organization, thereby regulating cell migration and chemotaxis (7). Lysophospholipids have the capacity to evoke and modulate immune responses by attracting and activating T cells, B cells and macrophages and influencing their interactions with other cell types (8). In neoplasia, LPA has been implicated in tumor initiation, progression, and metastasis (9). In this regard it has been shown that overexpression of LPA1 in breast cancer cells enhances the growth of bone metastases (10).

Relatively little is known about the effects of LPA on bone cells. Investigations in osteoblasts have demonstrated that LPA acts to increase DNA synthesis (11) and induce chemotaxis (12) through interactions with the LPA1 receptor. In addition, LPA, which is elevated in the synovial fluid of patients with rheumatoid arthritis, stimulates the migration of synovioocytes and the production of inflammatory cytokines, implicating this lysophospholipid in the progression of rheumatoid arthritis (13). Interestingly, the related lipid mediator sphingosine 1-phosphate has been shown to regulate the migration of osteoclast precursors (14). We have previously shown that signaling...
through the P2X7 nucleotide receptor stimulates LPA production in osteoblasts, resulting in increased differentiation and osteogenesis (15). The discovery that osteoblasts are a source of LPA in bone raises the possibility that LPA serves as an autocrine and paracrine signaling molecule, regulating the function of both osteoblasts and osteoclasts within the bone microenvironment. However, despite our understanding of the roles of LPA in osteoblasts, little is known as to the effects of LPA on osteoclasts.

**EXPERIMENTAL PROCEDURES**

**Materials**—Medium 199 (M199) buffered with 25 mM HEPES and 26 mM HCO₃⁻, HCO₃⁻-free M199 buffered with 25 mM HEPES, α-minimum essential medium buffered with HCO₃⁻ (26 mM), heat-inactivated fetal bovine serum (FBS), antibiotic solution (penicillin 10,000 units/ml; streptomycin 10,000 μg/ml; and amphotericin B 25 μg/ml), fura-2 acetoxy-methyl ester (fura-2 AM), and Alexa Fluor® 488 phalloidin were purchased from Invitrogen. Bovine albumin (crystallized) was purchased from ICN Biomedicals Inc. (Aurora, OH). 1-Oleoyl-sn-glycero-3-phosphate (LPA), 1-oleoyl-rac-glycerol (oleoyl glycerol), and Dulbecco’s modified Eagle’s medium were purchased from Sigma. The LPA₁/₃ antagonist VPC-32183 (16) was from Avanti Polar Lipids, Inc. (Alabaster, AL). The cell-permeable peptide inhibitor of NFAT 1R-VIVIT (RRRRRRRRRRRR-GGG-MAG-PHPVIVITGPHEE) (17) was synthesized and purified by Invitrogen. IL-1 receptor antagonist was from R&D Systems (Minneapolis, MN). Stock solutions of LPA, VPC-32183, and oleoyl glycerol were prepared in phosphate-buffered saline (PBS) containing bovine albumin (3% w/v). Y-27632 was from EMD Bioscience, Inc. (La Jolla, CA) and was dissolved in dimethyl sulfoxide. Pertussis toxin (holotoxin) was from List Biological Laboratories, Inc. (Campbell, CA) and was reconstituted in 0.5 M NaCl in 0.1 M sodium phosphate buffer at pH 7.0. Unless otherwise indicated, serum-free media were supplemented with bovine albumin (1 mg/ml) and antibiotic solution (1% v/v).

**Osteoclast Isolation**—Osteoclasts were isolated from the long bones of neonatal Wistar rats and New Zealand White rabbits as previously described (18). All procedures were approved by the Council on Animal Care of The University of Western Ontario and were in accordance with the guidelines of the Canadian Council on Animal Care. Briefly, long bones were dissected free of soft tissue and minced with a scalpel in HCO₃⁻-buffered, serum-free M199 medium while maintaining the tissue viability. Cells from the mince were dispersed by repeated passage through a glass pipette and plated on glass coverslips. Freshly isolated rat osteoclasts were incubated at 37 °C in 5% CO₂ for 1 h, washed gently with PBS to remove non-adherent cells, and incubated for 0.5–2 h before experiments in medium consisting of HCO₃⁻-buffered, serum-free M199 supplemented with FBS (15% v/v) and antibiotic solution (1%). The resulting cells were suspended by repeated passage through a glass pipette and plated on glass coverslips. Freshly isolated rat osteoclasts were incubated at 37 °C in 5% CO₂ for 1 h, washed gently with PBS to remove non-adherent cells, and incubated for 0.5–2 h before experiments in medium consisting of HCO₃⁻-buffered, serum-free M199 supplemented with FBS (15% v/v) and antibiotic solution (1%). The resulting cells were suspended by repeated passage through a glass pipette and plated on glass coverslips. Freshly isolated rat osteoclasts were incubated at 37 °C in 5% CO₂ for 1 h, washed gently with PBS to remove non-adherent cells, and incubated for 0.5–2 h before experiments in medium consisting of HCO₃⁻-buffered, serum-free M199 supplemented with FBS (15% v/v) and antibiotic solution (1%). The resulting cells were suspended by repeated passage through a glass pipette and plated on glass coverslips. Freshly isolated rat osteoclasts were incubated at 37 °C in 5% CO₂ for 1 h, washed gently with PBS to remove non-adherent cells, and incubated for 0.5–2 h before experiments in medium consisting of HCO₃⁻-buffered, serum-free M199 supplemented with FBS (15% v/v) and antibiotic solution (1%). The resulting cells were suspended by repeated passage through a glass pipette and plated on glass coverslips. Freshly isolated rat osteoclasts were incubated at 37 °C in 5% CO₂ for 1 h, washed gently with PBS to remove non-adherent cells, and incubated for 0.5–2 h before experiments in medium consisting of HCO₃⁻-buffered, serum-free M199 supplemented with FBS (15% v/v) and antibiotic solution (1%).

**Bone Marrow-derived Osteoclasts**—Bone marrow cells from the femurs and tibias of 6–10-week-old male C57Bl/6 mice were used to prepare osteoclasts as described previously (19). After isolation, cells were suspended in α-minimum essential medium supplemented with FBS (10%) and antibiotics (1%) and cultured in T75 tissue culture flasks (15 × 10⁶ cells per flask) with recombinant human macrophage colony-stimulating factor (PeproTech, 25 ng/ml). After 24 h, non-adherent cells were removed and resuspended in α-minimum essential medium containing FBS (10%), antibiotics (1%), macrophage colony-stimulating factor (50 ng/ml), and recombinant human RANKL (huRANKL-LZ, a gift from Amgen Inc., 100 ng/ml) and plated at 10 ± 10⁶ cells/cm² in suspension culture dishes (Corning). The resulting cells were cultured for an additional 3 days. Cells were then suspended by incubation for 10 min in Ca²⁺/Mg²⁺-free PBS at 4 °C, and osteoclasts were enriched by unit gravity sedimentation through FBS (2 times).

**RAW-264.7-derived Osteoclast-like Cells**—The murine leukemic monocyte macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium containing FBS (10%) and antibiotic solution (1%). RAW 264.7 cells were cultured at a density of 1.3 × 10⁵ cells/cm² and treated with huRANKL-LZ (100 ng/ml) for 4 days to give rise to multinucleated osteoclast-like cells.

**Fluorescence Measurement of [Ca²⁺]i**—Isolated rat and rabbit osteoclasts were plated on glass coverslips and loaded with fura-2 by incubation with 1.5 μM fura-2 AM for 30 min at room temperature in HEPES-buffered, HCO₃⁻-free M199. [Ca²⁺], was monitored using a Deltascan system (Photon Technology International, Birmingham, NJ) with the 510-nm emission detected using a photometer. Test substances were applied locally to cells by pressure ejection from a micropipette.

**Time-lapse Microscopy**—Osteoclast morphology was monitored using an inverted Nikon Eclipse TE300 phase contrast microscope, and images were captured using Image Master 5 Software (Photon Technology International). To perform time-lapse recordings, the culture medium was removed and replaced with HEPES-buffered, serum-free M199 medium (HCO₃⁻ free). Cells were plated on a heated stage and maintained at ~35 °C. For data analysis, the periphery of each osteoclast was traced at 5-min intervals to quantify the planar area using Image Master Software. The planar area is expressed as a percentage of the initial area at time 0. Initial planar area was 5530 ± 3600 μm² (mean ± S.D., n = 197 osteoclasts).

**Real-time RT-PCR Analyses**—Total RNA was isolated from purified bone marrow-derived osteoclasts using TRIZOL reagent and the RNeasy Mini kit (Qiagen). Primers and probes for murine LPA₁ (Edg2, Mm00439145_m1), LPA₂ (Edg4, Mm00469694_m1), LPA₃ (Edg7, Mm00469694_m1), LPA₄ (GPR23, Mm01228533_m1), LPA₅ (GPR92, Mm02621109_s1), calcitonin receptor (Calcr, Mm00432277_m1), glyceraldehyde-3-phosphate dehydrogenase (Gapdh, product no. 4308313),
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and 18 S ribosomal RNA (product no. 4308329) were from Applied Biosystems (Gene Expression Assay). Real-time RT-PCR was performed using TaqMan One-step RT-PCR Master Mix Reagents kit (Applied Biosystems) and the ABI Prism 7900HT Sequence Detector (Applied Biosystems) according to the manufacturer’s recommendations. Samples were amplified in triplicate. Dilutions of total RNA obtained from murine bone marrow-derived osteoclasts, small intestine, ovaries, and MC3T3-E1 cells were used to validate relative amplification efficiencies of primer/probe sets. The amounts of mRNA were normalized to levels of 18 S ribosomal RNA in the same samples.

**Immunoassay of Phospho-ERK1/2**

Using a two-site sandwich ELISA (catalogue no. 7A6, catalogue no. sc-7294, Santa Cruz Biotechnology) specific for phospho-ERK1/2 followed by biotinylated goat-antimouse IgG (Vector Laboratories) and fluorescein-conjugated streptavidin (Vector Laboratories). Distribution of fluorescent label was assessed in all osteoclasts on each coverslip (usually 40–70 per coverslip) using a LSM 510 confocal microscope (Zeiss) as described (21). In some samples, nuclei were counterstained with 4,6-diamidino-2-phenylindole (Vector Laboratories).

**Immunoassay of Phospho-ERK1/2**—To quantify ERK phosphorylation, we used a two-site sandwich ELISA (catalogue no. SUV1018, R&D Systems, Minneapolis MN) according to the manufacturer’s instructions.

**Osteoclast Survival and Apoptosis**—Rat osteoclasts were isolated and plated on 12-mm glass coverslips in HCO$_3$-buffered M199 supplemented with FBS (15%) and antibiotic solution (1%), and incubated at 37 °C in 5% CO$_2$ for 1 h. Coverslips were then washed gently with PBS to remove non-adherent cells and incubated for 0.5–1 h in HCO$_3$-buffered, serum-free M199. Osteoclasts were counted using phase-contrast microscopy, as described previously (22). Test substances were added to the same medium at $t = 0$, and cultures were then incubated for 15–18 h at 37 °C and 5% CO$_2$. The number of osteoclasts per coverslip was counted again, and survival was expressed as a percentage of the initial osteoclast number on the same coverslip. Number of osteoclasts per coverslip was 94 ± 40 (mean ± S.D., $n = 105$ coverslips).

To examine apoptosis, rat osteoclasts were plated on 12-mm coverslips in HCO$_3$-buffered M199 with serum (15%) and antibiotics (1%) and incubated at 37 °C in 5% CO$_2$ for 1 h. Coverslips were washed gently with PBS to remove non-adherent cells and incubated for 0.5–1 h in HCO$_3$-buffered, serum-free M199. Test substances were added to the same medium at $t = 0$. After 6 h incubation, cells were stained with Hoechst 33342 (5 μg/ml, for at least 10 min). Nuclear morphology was examined using a Zeiss Axiovert 40 CFL microscope. Osteoclasts exhibiting condensed and/or fragmented nuclei were scored as apoptotic, and the numbers of apoptotic cells were expressed as percentages of the total number of osteoclasts per dish (23).

**Pit Formation Assay**—Slices (~ 25 mm$^2$) were prepared from elephant ivory obtained as a gift from the Canadian Wildlife Service. Osteoclasts were isolated from neonatal rabbits and plated directly on ivory slices in HCO$_3$-buffered M199 with serum (15%) and antibiotics (1%). After 3 h of incubation, slices were washed to remove nonadherent cells. Cultures were incubated for an additional 24 h in the same medium, then fixed in 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase by using the leukocyte acid phosphatase kit from Sigma. The number of osteoclasts per slice was determined by counting multinucleated, tartrate-resistant acid phosphatase-positive cells. Slices were then sonicated in water for 20 min to remove cells and stained with toluidine blue (1% w/v). Resorption pits were counted, and the planar area of each pit was measured using a stereo optical light microscope (Zeiss). To determine pit depth, slices were sputter coated with platinum and imaged using a Leo/Zeiss 1540XB focused ion beam/scanning electron microscope. The samples were positioned at the coincidence point between the electron and ion beams. Each sample was tilted 54° relative to the electron beam and normal to the ion beam. A horizontal line was cut across the diameter of each pit using the ion beam. The scanning electron microscope image of the line was “tilt-corrected” to obtain maximum pit depth.

**Statistical Analyses**—Results are presented as the means ± S.E. unless otherwise indicated. Differences between two groups were evaluated by $t$ tests. Differences among three or more groups were evaluated by one-way or two-way analysis of variance followed by Tukey’s or Bonferroni post-hoc tests. In some cases, data were normalized using an arc sine transformation before statistical analysis. Differences were accepted as statistically significant at $p < 0.05$.

**RESULTS**

**LPA Elicits Elevations of Cytosolic-free Calcium Concentration**—To investigate whether LPA stimulates elevation of [Ca$^{2+}$], freshly isolated rat and rabbit osteoclasts were examined using microspectrofluorimetry. LPA (300 nm to 10 μM) caused an acute, transient rise in [Ca$^{2+}$], in rat (Fig. 1A) and rabbit (Fig. 1B) osteoclasts. In contrast, both vehicle and the inactive LPA metabolite oleoyl glycerol (3 μM) failed to elicit an appreciable rise in [Ca$^{2+}$], (Fig. 1C), although subsequent application of LPA confirmed cells to be responsive. Moreover, bath addition of the LPA$_{1/3}$ antagonist VPC-32183 (1 μM) inhibited the Ca$^{2+}$ rise in response to LPA (5 μM) (Fig. 1D) and (E), implicating signaling through LPA$_{1/3}$ receptors in this response.

When the concentration of LPA was varied, the percentage of responding cells (cells that exhibited a rise of [Ca$^{2+}$], exceeding 15 nM) increased with increasing concentrations of LPA. Whereas ~35% of cells responded to 300 nM LPA, more than 95% of osteoclasts stimulated with 10 μM LPA exhibited a rise of [Ca$^{2+}$], (Fig. 1F). Interestingly, the magnitude of [Ca$^{2+}$] elevation in responsive cells was also dependent on the concentration of LPA (Fig. 1G).

**LPA Induces Retraction of Osteoclast Lamellipodia**—To determine whether LPA elicits changes in osteoclast morphology, cultures were monitored using time-lapse phase-contrast microscopy. Freshly isolated rat osteoclasts were bathed in HEPES-buffered, serum-free M199 with or without VPC-32183 (1 μM) at 35 °C. Images were recorded for 20 min before vehicle (Fig. 2A), oleoyl glycerol (5 μM, Fig. 2B), or LPA (5 μM, Fig. 2C) were added to the medium. Before addition, osteoclast lamellipodia...
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FIGURE 2. LPA induces retraction of osteoclast lamellipodia. Rat osteoclasts were bathed in HEPES-buffered, serum-free medium at 35 °C and imaged using time-lapse phase-contrast microscopy. At time 0, osteoclast lamellipodia were well spread. A–C, at time 20 min, vehicle, oleoyl glycerol (5 μM), or LPA (5 μM) was added to the bath. Only LPA induced prompt retraction of lamellipodia, which was sustained for at least 40 min. D, VPC-32183 (1 μM) was added to the bath at time 0 followed by LPA (5 μM) at 20 min. Despite the presence of VPC-32183, the osteoclast exhibited sustained retraction, with retraction fibers prominent at 60 min. Images are representative of the responses of 9–18 osteoclasts from 3–5 independent preparations. See also supplemental Video 1 of the response illustrated in C.

LPA elicits an acute rise of [Ca²⁺], in rat and rabbit osteoclasts. Osteoclasts were loaded with the Ca²⁺-sensitive dye fura-2 and bathed in physiological buffer, and changes in [Ca²⁺], were measured using microspectrofluorimetric techniques. Test substances were applied by pressure ejection from a micropipette (except VPC-32183), indicated by the horizontal bars below the traces. A, illustrated is the response of a rat osteoclast to LPA (300 nM). B, calcium transient elicited in a rabbit osteoclast by LPA (5 μM) (representative of 5 cells) is shown. C, the inactive LPA metabolite oleoyl glycerol (3 μM) failed to alter [Ca²⁺], in a rat osteoclast (representative of 7 cells). D, the LPA₁/₃ receptor antagonist VPC-32183 (1 μM) in the bath greatly attenuated the Ca²⁺ transient in a rat osteoclast induced by LPA (5 μM). E, the amplitude of Ca²⁺ transients was quantified as the maximal rise of [Ca²⁺], above base line. The bar graph represents responses of rat osteoclasts to LPA (5 μM) or to LPA (5 μM) in the presence of VPC-32183 (1 μM). Data are the means ± S.E. (n = 4 independent preparations; a total of 16 cells for LPA and 12 cells for VPC-32183+LPA). # indicates significant effect of VPC-32183, p < 0.001. F, osteoclasts were considered responsive if they exhibited a 15 nm or greater rise of [Ca²⁺], above base line. Data points illustrate the percentage of rat osteoclasts responding to the indicated concentrations of LPA. Data are based on n = 4–8 independent preparations (a total of 16–40 cells in each treatment group). G, data points illustrate the amplitude of responses to the indicated concentrations of LPA for responsive cells only. Data are based on n = 4–8 independent preparations (a total of 14–32 cells in each treatment group). Sigmoid curves were fit by nonlinear regression.

Pseudopodia were still observed (60 min, see also supplemental Video 1), indicating that the cells were not quiescent, as occurs when retraction is induced by calcitonin (24). VPC-32183 failed to block LPA-induced retraction of lamellipodia (Fig. 2D); however, prominent retraction fibers were noted, a distinct feature of the LPA response when pretreated with VPC-32183.

To quantify the change in morphology, the planar area of osteoclasts was measured over time, with values expressed as a percentage of the initial area. Unlike vehicle (Fig. 3A) or oleoyl glycerol (Fig. 3B), LPA elicited a significant decrease in the planar area within 5 min of application (Fig. 3C). On its own, VPC-32183 had no significant effect on osteoclast planar area (Fig. 3D). VPC-32183 also had no effect on the ability of LPA to elicit a sustained decrease in the planar area of osteoclasts, suggesting the involvement of a receptor other than LPA₁ or LPA₃ (Fig. 3E).

We next investigated whether retraction of lamellipodia was associated with changes in the actin cytoskeleton. Rat osteoclasts, plated on glass coverslips, were fixed, permeabilized, and incubated with fluorescently tagged phalloidin that specifically labels filamentous actin. LPA (5 μM) caused rapid disruption of peripheral filamentous actin belts (supplemental Fig. S1).

Expression of LPA Receptors in Osteoclasts—Real-time RT-PCR revealed that the LPA₁ subtype was predominantly expressed at a level ~20% that of the calcitonin receptor, a well established osteoclast marker (Fig. 4). We also found evidence
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for lower levels of transcripts encoding LPA₁, LPA₄, and LPA₅. The presence of LPA₅ was not detectable in osteoclasts; however, LPA₅ was detectable in RNA samples from murine small intestine, confirming the effectiveness of the primer/probe set.

LPA-induced Rise of Cytosolic Free Calcium Is Sensitive to Pertussis Toxin—To investigate the signaling pathway involved in LPA-induced elevation of [Ca²⁺]ᵢ, we examined the effect of pertussis toxin (PTX), a specific inhibitor of Gᵢ₉ proteins. Rat osteoclasts were preincubated with PTX (1 μg/ml) or its vehicle (control) in HCO₃⁻-buffered, serum-free medium for at least 3 h and subsequently treated with LPA (5 μM). The rise in [Ca²⁺]ᵢ elicited by LPA was practically abolished in PTX-pretreated cells (Fig. 5, A and E) compared with the robust responses seen in control cells (Fig. 5, B and E).

Osteoclasts respond to extracellular nucleotides such as UTP, which activate P2Y receptors (25). P2Y receptors couple at least in part through pertussis toxin–insensitive G proteins to induce release of Ca²⁺ from intracellular stores (26). Therefore, we compared the response of PTX-pretreated and control osteoclasts to UTP. The response to UTP was only partially attenuated in PTX-pretreated osteoclasts (Fig. 5, C and D, and E). Mean values obtained from 4–5 independent preparations show that pretreatment with PTX significantly decreased the rise of [Ca²⁺]ᵢ induced by LPA but not by UTP (Fig. 5E). These data

![FIGURE 3. Quantification of LPA-induced retraction of osteoclasts. Data were obtained as described in the legend to Fig. 2. Image analysis software was used to calculate the planar area of osteoclasts at the times indicated. Data are the means ± S.E., expressed as a percentage of the initial area at time 0. A, vehicle had no significant effect on osteoclast area (n = 5 independent preparations, a total of 10 osteoclasts). B, the control compound oleoyl glycerol (5 μM) had no significant effect on osteoclast planar area (n = 3 independent preparations, a total of 14 cells). C, in contrast, LPA (5 μM) caused a significant, sustained decrease in osteoclast area (n = 4 independent preparations, a total of 9 cells). The asterisk indicates significant difference compared with vehicle at the corresponding times, p < 0.001. D, VPC-32183 (1 μM) alone caused no significant change in osteoclast area (n = 4 independent preparations, a total of 18 cells). E, in osteoclasts pretreated with VPC-32183, LPA (5 μM) still elicited a significant sustained decrease in area when compared with cells treated with VPC-32183 and vehicle (D); *, p < 0.001. Moreover, there was no significant difference in the response compared with cells treated with LPA alone (C). n = 4 independent preparations, a total of 14 cells.

![FIGURE 4. Real-time RT-PCR analysis of LPA receptor expression in osteoclasts. Real-time RT-PCR was performed on RNA isolated from murine bone marrow-derived osteoclasts. Data were normalized to levels of 18S ribosomal RNA, and primer sets were confirmed to have similar amplification efficiencies. Transcript levels of LPA₁–5 were compared with levels of mRNA encoding the calcitonin receptor (Calcr, a marker of mature osteoclasts) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression of LPA₁ was dominant, ~20% that of the calcitonin receptor. Expression of LPA₂, LPA₄, and LPA₅ was also detectable. Data are the means ± S.E., n = 5 independent preparations each performed in triplicate.

![FIGURE 5. LPA-induced elevation of [Ca²⁺]ᵢ in rat osteoclasts is blocked by pertussis toxin. Rat osteoclasts were pretreated with PTX (1 μg/ml) or its vehicle (control) in HCO₃⁻-buffered, serum-free medium for a minimum of 3 h before stimulation. Cells were then loaded with the Ca²⁺-sensitive dye fura-2 and bathed in physiological buffer, and changes in [Ca²⁺]ᵢ were measured using microspectrofluorimetric techniques. Test substances were applied by pressure ejection from a micropipette, where indicated by the horizontal bars below the traces. A, a PTX-pretreated osteoclast shows no response to LPA (5 μM), B, robust response to LPA (5 μM) was elicited in a control osteoclast. C, a PTX-pretreated osteoclast was responsive to UTP (a P2Y receptor agonist, 10 μM) but not LPA (5 μM). D, a control osteoclast was responsive to both UTP (10 μM) and LPA (5 μM), E, the amplitude of Ca²⁺ transients was quantified as the maximal rise of [Ca²⁺]ᵢ above base line. The bar graph represents responses to LPA (5 μM) or UTP (10 μM) in osteoclasts pretreated with vehicle (open bars, Con) or PTX (filled bars). Data are the means ± S.E. # indicates significant effect of PTX, p < 0.001 (n = 4–5 independent preparations; a total of 28 cells for Con/LPA, 22 cells for PTX/LPA, 7 cells for Con/UTP and 8 cells for PTX/UTP).]
Inhibition of Rho-associated Kinase Promotes Osteoclast Respreading—The pertussis toxin-insensitive G proteins $G_{12/13}$ are activated by LPA in several other cell types and regulate the actin cytoskeleton through the Rho family of GTPases. Moreover, Rho-associated kinase mediates morphological changes downstream of LPA in fibroblasts and osteoblasts (27, 28). To examine the participation of Rho-associated kinase in LPA-induced morphological changes of osteoclasts, cells were pretreated with the specific inhibitor Y-27632 (10 $\mu M$) (29) or its vehicle (control). Although Y-27632 did not inhibit the initial retraction of osteoclast lamellipodia elicited by LPA (5 $\mu M$), it did promote respreading (Fig. 6B). In control cells, LPA-induced retraction that was sustained for at least 60 min; whereas in Y-27632-treated cells, lamellipodia re-formed, and osteoclast planar area was significantly greater 25 min after LPA addition (see also supplemental Video 2). Treatment of osteoclasts with Y-27632 alone caused no significant changes in the osteoclast planar area (supplemental Fig. S2B) or motility. Thus, it appears that LPA acts through a pertussis toxin-insensitive G protein (likely $G_{12/13}$) to activate Rho/Rho-associated kinase, which sustains osteoclast retraction.

LPA Enhances Osteoclast Survival—Cell survival is a key factor regulating osteoclast number and, hence, bone turnover in vivo (2). Therefore, we examined the effects of LPA on this critical aspect of osteoclast biology. Survival was quantified by counting the number of rat osteoclasts before and after an 18-h incubation period and was expressed as the percentage of surviving cells. Osteoclasts were identified by phase contrast microscopy as multinucleated cells ($\geq 3$ nuclei) with broad lamellipodia. Only 18% of vehicle-treated osteoclasts survived 18 h of incubation in HCO$_3$-$^-$-buffered, serum-free M199 (Fig. 7A). However, the addition of LPA (2 and 5 $\mu M$) significantly enhanced survival, with 30 and 35% of osteoclasts surviving for 18 h, respectively. The LPA$_1/3$ receptor antagonist VPC-32183 (1 $\mu M$) alone had no significant effect on osteoclast survival; however, VPC-32183 blocked the effect of LPA on survival, indicating involvement of LPA$_1$.

We next examined whether survival involved LPA$_1$ signaling downstream of the LPA$_1$ receptor. In these experiments, freshly isolated osteoclasts were treated with PTX (500 ng/ml) or its vehicle (control) in HCO$_3$-$^-$-buffered serum-free medium for 3 h. The number of osteoclasts per dish was then counted followed by the addition of LPA (5 $\mu M$) or its vehicle (in the continued presence of PTX or its vehicle). After incubation for an additional 15 h, the number of osteoclasts per dish was recounted. PTX abolished the effect of LPA (Fig. 7B), indicating that LPA$_1$ couples through $G_{i/o}$ to promote survival. PTX treatment had no significant effect on osteoclast survival in the absence of LPA.

LPA has been shown to induce expression of IL-1$\beta$ mRNA in endothelial cells (30). Therefore, we assessed the possible involvement of IL-1 in mediating the effects of LPA on osteoclast survival using IL-1 receptor antagonist, an endogenous
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**FIGURE 7.** LPA enhances osteoclast survival. Rat osteoclasts were maintained in HCO\(_3^-\)-buffered, serum-free M199 containing either vehicle or LPA (1–5 \(\mu\)M). A, survival was assessed by counting the number of osteoclasts before and after 18 h of culture. The number of surviving osteoclasts on each coverslip at 18 h was expressed as a percentage of the initial number of osteoclasts on the same coverslip. LPA (2 and 5 \(\mu\)M) increased osteoclast survival. The asterisks indicate significant increase compared with vehicle, \(p < 0.01\). The LPA\(_{1/3}\) receptor antagonist VPC-32183 (1 \(\mu\)M) blocked the effect of LPA (5 \(\mu\)M) on survival (filled bars). B, at time 0, rat osteoclasts were treated with PTX (500 ng/ml, filled bars) or its vehicle (Con, open bars) in HCO\(_3^-\)-buffered, serum-free M199. At 3 h, the number of osteoclasts per coverslip was counted, and cells were treated with LPA (5 \(\mu\)M) or its vehicle in the continued presence or absence of pertussis toxin. At time 18 h, the number of surviving osteoclasts per coverslip was counted and expressed as a percentage of the number of cells at 3 h. PTX blocked the effect of LPA on survival. # indicates significant effect of PTX, \(p < 0.001\). For A and B, data are the means ± S.E., \(n = 3–4\) independent preparations, each performed in duplicate.

**FIGURE 8.** LPA inhibits apoptosis of osteoclasts. Rat osteoclasts were maintained in HCO\(_3^-\)-buffered, serum-free M199. At time 0, LPA (2 or 5 \(\mu\)M) or its vehicle was added to the medium. Where indicated, the LPA\(_{1/3}\) receptor antagonist VPC-32183 (1 \(\mu\)M) was also added to the medium. At time 6 h, cells were stained with Hoechst 33342 (5 \(\mu\)M, for 10–20 min) to reveal nuclear morphology. A and B, fluorescence (left) and phase contrast (right) images were obtained using a Zeiss Axiovert 40 CFL microscope. A, representative healthy osteoclast with characteristic lamellipodia and normal nuclear morphology is shown. B, representative apoptotic osteoclast with condensed and fragmented nuclei is shown. C, the bar graph illustrates the percentage of osteoclasts with apoptotic nuclear morphology at 6 h. LPA (2 or 5 \(\mu\)M) suppressed osteoclast apoptosis. The effect of LPA was blocked by VPC-32183 (filled bars). Data are the means ± S.E., \(n = 4\) independent preparations, each performed in duplicate. Bars labeled with different lowercase letters are significantly different from one another.

antagonist that binds specifically to IL-1 receptors (31). Interestingly, the stimulatory effects of LPA on osteoclasts survival were blocked in the presence of IL-1 receptor antagonist (0.1 \(\mu\)g/ml, supplemental Fig. S3). This observation raises the possibility that LPA stimulates the secretion of IL-1, which then acts through an autocrine loop to enhance osteoclast survival.

Because the ERK1/2 anti-apoptotic pathway has been reported to regulate osteoclast survival (32), we determined whether LPA induces ERK1/2 phosphorylation in osteoclast-like cells derived from RAW 264.7 cells. LPA (5 \(\mu\)M, 5 min) induced a small but significant increase in the levels of phospho-ERK1/2 (supplemental Fig. S4). In contrast, oleoyl glycerol (5 \(\mu\)M) had no effect. Both basal levels and the LPA-induced increase in ERK1/2 phosphorylation were suppressed by the MEK1/2 inhibitor U0126 (10 \(\mu\)M).

**LPA Suppresses Osteoclast Apoptosis**—To further investigate the mechanism by which LPA enhances survival, we evaluated its effects on osteoclast apoptosis. Apoptosis in osteoclasts is characterized by nuclear condensation and fragmentation, occurring in all nuclei simultaneously (33). We visualized nuclear morphology in live osteoclasts using the membrane-permeant dye Hoechst 33342. Apoptotic osteoclasts exhibited condensed and fragmented nuclei, clearly distinguishable from the nuclear morphology in non-apoptotic cells (Fig. 8, A and B). Approximately 47% of vehicle-treated rat osteoclasts were apoptotic after 6 h of incubation in HCO\(_3^-\)-buffered, serum-free M199 at 37 °C (Fig. 8C). LPA suppressed osteoclast apoptosis in a concentration-dependent manner. Moreover, these effects were abolished by VPC-32183, consistent with the involvement of LPA\(_{1}\). Treatment of osteoclasts with VPC-32183 alone had no significant effect on apoptosis. Together, these data demonstrate that LPA acts on LPA\(_{1}/G_{i/o}\) to suppress apoptosis and thereby promote survival.
Role of NFAT in Mediating the Effects of LPA on Osteoclast Survival—The transcription factor NFAT is activated by the Ca\(^{2+}\)/calmodulin-dependent protein phosphatase, calcineurin. NFATc1 is strongly induced during receptor activator of nuclear factor κB ligand (RANKL)-stimulated osteoclast differentiation (34, 35), and we have shown that NFAT mediates in part the stimulatory effects of RANKL on osteoclast survival (23). To investigate the possible involvement of NFAT in mediating the effects of LPA on survival, we used immunofluorescence to monitor the intracellular distribution of NFATc1, which accumulates in the nuclei upon activation (supplemental Fig. S5). LPA (5 μM, 3 h) induced nuclear accumulation of NFATc1 in rabbit osteoclasts (Fig. 9A). This effect was blocked by VPC-32183 and by 11R-VIVIT, a cell-permeable peptide that specifically inhibits NFAT activation (17). We also found that 11R-VIVIT blocked the ability of LPA to prolong the lifespan of osteoclasts (Fig. 9B), consistent with a role for NFAT in mediating the effects of LPA on survival.

**DISCUSSION**

Lyposphospholipids play important roles in development, wound healing and pathology (36). Moreover, LPA has recently been found to enhance osteogenesis (15); however, until the present study, nothing has been reported about the regulation of osteoclasts by LPA. It is possible that the effects of LPA on osteoclasts have not been recognized previously because LPA is abundant in the sera used routinely to supplement media for *in vitro* studies.

In the present study we characterized the expression of LPA receptors and the effects of LPA on osteoclasts. We found that LPA acts through multiple receptor subtypes to elevate [Ca\(^{2+}\)]\(_i\), induce cellular retraction, activate NFATc1, and prolong osteoclast survival (Fig. 10). Because LPA is produced by osteoblasts (15), our findings suggest that this lipid mediator could serve as both an autocrine and paracrine signaling molecule in bone, contributing to the coordination of osteoblast and osteoclast activity.

Several lines of evidence establish that the LPA-induced responses reported here are receptor-mediated. For example, application of vehicle or the inactive LPA metabolite oleoylglycerol failed to elicit elevation in [Ca\(^{2+}\)]\(_i\), or changes in osteoclast morphology. Moreover the LPA1/3 receptor antagonist VPC-32183 inhibited the rise of [Ca\(^{2+}\)]\(_i\) elicited by LPA. Many tissues express LPA receptors. Here we show that osteoclasts predominantly express LPA\(_1\), with some expression of LPA\(_2\), LPA\(_4\), and LPA\(_5\). Notably, we found no evidence for expression...
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Because preparations of osteoclasts contain a number of adherent cell types in addition to osteoclasts, it is possible that responses of osteoclasts to test substances are secondary to primary effects on other cell types. However, when LPA was applied locally to individual osteoclasts using a micropipette, acute elevations in [Ca\textsuperscript{2+}], were observed with short delay. Moreover, morphological retraction was observed within 1 min after the addition of LPA, establishing that effects are direct.

LPA appears to activate at least two distinct receptor subtypes on osteoclasts. VPC-32183 inhibited the rise of [Ca\textsuperscript{2+}], and translocation of NFATc1 elicited by LPA and blocked its effect on osteoclast survival. However, the same concentration of VPC-32183 did not block lamellipodia retraction, indicating that a receptor other than LPA\textsubscript{1} mediates this striking response. In addition, our data reveal that distinct LPA receptors are coupled to different G proteins in osteoclasts. The LPA-induced rise in [Ca\textsuperscript{2+}], and enhancement of survival were both sensitive to pertussis toxin, consistent with LPA\textsubscript{1} signaling through Gi/o. In other cell types, the \( \beta \gamma \) subunit of Gi/o can activate phospholipase C (46), which in turn produces inositol 1,4,5-trisphosphate and diacylglycerol, causing release of Ca\textsuperscript{2+} from intracellular stores and activation of protein kinase C, respectively. Moreover, the \( \beta \gamma \) subunit can either indirectly associate with the small GTPase Ras to activate the ERK pathway or directly activate phosphatidylinositol 3-kinase/Akt signaling to enhance cell survival (38). Indeed, we found that LPA induces a modest increase in the levels phospho-ERK. However, our data indicate that the LPA-induced rise of [Ca\textsuperscript{2+}], itself plays a role in promoting osteoclast survival. High levels of cytosolic calcium activate calmodulin, which in turn stimulates the phosphatase calcineurin and several members of the NFAT family of transcription factors (47). In previous studies, RANKL has been shown to induce activation of NFATc1, promoting osteoclast differentiation (34) and enhancing osteoclast survival (23). In the present study we used the specific peptide inhibitor 11R-VIVIT to show that the pro-survival effects of LPA in osteoclasts are dependent on NFAT signaling.

It will be of interest in future studies to examine cross-talk among the pathways downstream of LPA receptors and those activated by RANKL and macrophage colony-stimulating factor in osteoclasts. In this regard, David \textit{et al.} (48) recently reported that LPA increases osteoclast formation in response to macrophage colony-stimulating factor and RANKL. The action of LPA to promote osteoclast survival, reported in the present study, may have contributed to the increase in osteoclast numbers observed by David \textit{et al.} (48).

There is some evidence pointing to a role for LPA in skeletal development. Targeted deletion of the gene encoding LPA\textsubscript{1} in mice (\( lpa_{1} \)) results in distinct craniofacial defects, including shorter snouts and more widely spaced eyes (49). The authors suggest that these defects likely arise from the loss of LPA\textsubscript{1} that is normally expressed within developing facial bones. Interestingly, the double \( lpa_{1}/lpa_{2} \) knock-out mouse shows similar defects (50), suggesting that the effects of LPA\textsubscript{1} predominate in craniofacial development. Understanding the contribution of LPA receptors in osteoblasts and osteoclasts to this phenotype will be a fruitful area for future research.
LPA is a potent mitogen and motility factor that has been implicated in the metastasis of breast and ovarian tumors to bone (51). Moreover, breast cancer cells overexpressing LPA₃ promote the recruitment of osteoclasts to metastatic sites and stimulate bone resorption (10). Our previous findings demonstrate that osteoblasts can produce LPA (15). This LPA may attract and activate tumor cells, as well as regulate osteoclast motility and survival. Thus, LPA released from osteoblasts may be an important autocrine and paracrine mediator, physiologically regulating skeletal development and remodeling, and pathologically contributing to metastatic bone disease.

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