

DNA microarray analysis reveals a role for lysophosphatidic acid in the regulation of anti-inflammatory genes in MC3T3-E1 cells

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Abstract

Lysophosphatidic acid (LPA) is a bioactive lipid with functional properties that overlap those of growth factors and cytokines. LPA production *in vivo* is linked to platelet degranulation and the biological activities of this lipid are associated with wound healing. Osteoblasts and their progenitor cells are exposed to high levels of this lipid factor in regions adjacent to bone fractures and we postulate a role for LPA in skeletal healing. The regeneration of bone injuries requires a complex array of changes in gene expression, but the effects of LPA on mRNA levels in bone cells have not been investigated. We performed a genome-wide expression analysis in LPA-treated MC3T3-E1 pre-osteoblastic cells using Affymetrix GeneChip arrays. Cells exposed to LPA for 6 h exhibited 513 regulated genes, whereas changes in the levels of 54 transcripts were detected after a 24-h LPA treatment. Gene ontology analysis linked LPA-regulated gene products to biological processes that are known to govern bone healing, including cell proliferation, response to stress, organ development, chemotaxis/motility, and response to stimuli. Among the gene products most highly up-regulated by LPA were transcripts encoding the anti-inflammatory proteins sST2, ST2L, and heat-shock protein 25 (HSP25). RT-PCR analysis confirmed that these mRNAs were increased significantly in MC3T3-E1 cells and primary osteoblasts exposed to LPA. The response of cells to LPA is mediated by G-protein-coupled receptors, and the stimulation of anti-inflammatory gene expression in MC3T3-E1 cells was blocked by Ki16425, an inhibitor of LPA₁ and LPA₃ receptor forms. Pertussis toxin impaired only the LPA-induced expression of sST2. LPA-stimulated levels of sST2, ST2L and HSP25 mRNAs persisted if the cytosolic Ca²⁺ elevations elicited by this lipid were blocked with BAPTA. In contrast to the stimulatory effect of LPA, exposure of MC3T3-E1 cells to fluid shear reduced the transcript levels of all three anti-inflammatory genes. The induction of sST2, ST2L and HSP25 expression by LPA suggests a role for this lipid factor in the regulation of osteoblastic cell function during periods of inflammation.

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Introduction

Lysophosphatidic acid (LPA) is a bioactive lipid that acts as an autocrine/paracrine agent to regulate cell proliferation, survival, adhesion, migration, gene expression, cancer cell invasion, and membrane structural dynamics [1]. LPA has been detected in

a number of body fluids but it is particularly abundant in serum (1–5 μM) where it is generated by degranulating platelets during blood clot formation [2,3]. Although a variety of cell types are known to respond to LPA, many studies have focused on the ability of this lipid to stimulate the migration of endothelial cells during angiogenesis and soft tissue wound healing [4]. Osteoblasts and their progenitor cells are exposed to LPA that diffuses out of the hematoma that is formed as a result of bone fractures, but little is known about the potential role in skeletal healing of LPA and other lipid growth factors that originate from platelets. The proliferation of pre-osteoblastic cells and their

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directed migration (chemotaxis) to the fracture gap are essential for proper bone repair, and both of these processes are known to be regulated by platelet-derived proteins [5]. Similarly, LPA promoted osteoblastic cell mitogenesis and survival [6–9], and our data demonstrated that LPA is a potent inducer of pre-osteoblast chemotaxis [10]. We also recently reported that LPA-treated MLO-Y4 osteocytes exhibited profound increases in the outgrowth of dendritic membrane processes [11], a phenomenon that may be involved in the restoration of the osteocyte network in newly formed bone. Together, these observations suggest that platelet-derived LPA contributes to the regulation of bone formation during the repair of skeletal injuries.

Bone healing requires the coordinated activity of many genes by a variety of cell types [12], and we postulated that the mechanisms by which LPA could modulate fracture repair include changes in bone cell gene expression. In support of this hypothesis, MC3T3-E1 pre-osteoblast migration velocity increased only after a 6- to 8-h lag period following the addition of LPA to the medium, suggesting a requirement for new gene transcription [10]. Furthermore, LPA and related bioactive lipids are surface receptor-mediated agonists of MAP kinase activation and Ca^{2+} release from intracellular stores in osteoblastic cells, both of which are key signaling events in many regulatory processes including gene expression [8,13–15]. To determine whether LPA treatment leads to changes in bone cell transcript levels, we employed DNA microarrays to analyze global gene expression in mouse MC3T3-E1 pre-osteoblastic cells. We report here that the exposure of these cells to LPA was associated with the modulation of a large number of gene products that control a diverse set of cell functions, many of which relate to bone regeneration.

Fracture healing occurs most often via endochondral bone formation, a process that involves a highly regulated series of phases consisting of inflammation, chondrogenesis/angiogenesis, mineralized matrix deposition, and remodeling [12]. During the inflammatory response, macrophages and other immune cells attack potentially infectious microbes and remove necrotic tissue, and the effectiveness of this process is determined by the balance of regulation by pro-inflammatory and anti-inflammatory cytokines. The production of LPA by platelets at sites of bone injury coincides with the initiation of inflammation, but the role of LPA in the control of this response is not clear: LPA has been linked to both pro- and anti-inflammatory actions in a variety of tissues [16]. Among the data presented here are results showing a strong up-regulation in LPA-treated MC3T3-E1 cells of gene products linked to anti-inflammatory actions. These results suggest an ability of this lysophospholipid to modulate inflammation in bone, and are consistent with a growing body of evidence supporting a role for osteoblasts in the regulation of the immune response [17].

Materials and methods

Cell culture

MC3T3-E1 osteoblastic cells were grown in α MEM (Cellgro, Herndon, VA, USA) containing 10% fetal bovine serum (Valley Biomedical, Winchester, VA, USA) in a humidified 5% CO_2 /95% air atmosphere at 37 °C. The experiments

involving oscillating fluid flow employed MC3T3-E1 subclone 14 cells grown exactly as described above. Primary osteoblasts were isolated from the calvaria of 2- to 4-day-old Swiss Webster (CFW) mice (Charles River Laboratories, Wilmington, MA, USA) according to the method of Ryu et al. [18] and cultured in DMEM (Cellgro, Herndon, VA, USA) containing 10% fetal bovine serum, 2 mM L-glutamine and penicillin–streptomycin. Where indicated, cells were serum-starved by incubation in α MEM containing 0.1% fatty acid-free BSA (α MEM/BSA) prior to the addition of LPA (1-oleoyl-2-hydroxy-*sn*-3-glycerol-3-phosphate; Biomol, Plymouth Meeting, PA, USA) from 1.0 mM or 5.0 mM aqueous stock solutions.

DNA microarray analysis

Triplicate dishes of MC3T3-E1 cells were serum-starved for 16 h in α MEM/BSA and then incubated an additional 6 or 24 h in the absence or presence of 2.5 μM LPA. Total RNA was extracted separately from each dish using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA, USA). RNA quality was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Complementary DNA was synthesized from total RNA in the presence of an oligo-dT primer containing a T7 RNA polymerase promoter, and an *in vitro* transcription reaction was performed in the presence of mixture of biotin-labeled ribonucleotides to produce biotinylated cRNA from the cDNA template, according to manufacturer's protocols. Biotin-labeled cRNA (15 μg) was fragmented to a size range between 50 and 200 bases and used for hybridization to Mouse Genome 430 2.0 chips (Affymetrix, Santa Clara, CA, USA). After hybridization, the arrays were washed and stained with streptavidin–phycoerythrin. Arrays were scanned at a resolution of 2.5 μm using an Affymetrix GeneChip Scanner 3000. Quality control parameters were assessed throughout the experimental process to measure the efficiency of transcription, integrity of hybridization, and consistency of qualitative calls. The synthesis of the cDNA and cRNA, and the fragmentation of cRNA were assessed using the Agilent 2100 Bioanalyzer. Spike-in control transcripts also were monitored to verify hybridization integrity. Relative log expression and normalized unscaled standard error plots demonstrated that data from a single array, 24 h+LPA replicate #3, were outside the acceptable limits and were therefore excluded from further analyses. The remaining raw data files were normalized using the Robust Multi-Array Analysis (RMA) [19], and significantly regulated genes were identified with multiple testing and false discovery rate statistics [20] at $p < 0.01$ using the appropriate packages in Bioconductor [21]. Biological process enrichment was calculated using the MetaCore software suite (St. Joseph, MI, USA) to identify the most significant cellular processes affected by the 6-h LPA treatment. The statistical scores in MetaCore are calculated using a hypergeometric distribution where the p value essentially represents the probability of particular mapping arising by chance, given the number of genes in the set of all genes on processes, genes on a particular process and genes in your experiment.

RT-PCR analyses

Qualitative detection of transcripts encoding mouse LPA receptors was accomplished using RT-PCR as previously published [10]. Total RNA was extracted from mouse calvarial osteoblasts as described above and cDNA was synthesized from total RNA via reverse transcription with random hexamer priming (Promega, Madison, WI). Pairs of oligodeoxynucleotide PCR primers (Table 1) were designed using Primer Designer 5.11 software (Sci Ed Central, Cary, NC) based on the cDNA encoding murine LPA receptors. All primer pairs spanned introns to eliminate the potential amplification of contaminating genomic DNA.

Quantitative assessment of mRNA expression was performed by real-time RT-PCR. Complementary DNA was synthesized from total RNA via reverse transcription as described above but with oligo-dT priming. As above, all primer pairs (Table 1) spanned introns. PCR reactions were carried out using FastStart DNA Master^{PLUS} SYBR Green I reagents (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions in a Roche Lightcycler II. Cycle parameters were: denaturation at 95 °C for 10 s, annealing at 55 °C for 5 s, and elongation at 72 °C for 10 s for 45 cycles. Melting curve analyses were performed from 60 °C to 95 °C in 0.5 °C increments. Quantitative RT-PCR data were normalized to the level of cyclophilin A transcript levels and relative expression of each mRNA was determined using REST software [22]. Statistical

Table 1
Primers for qualitative and quantitative (in **bold italics**) RT-PCR

Target	Forward primer (5'→3')	Reverse primer (5'→3')	Product size	GenBank no.
LPA ₁	CGTGGTGGTGGTATTGTAG	GCCAGAATGGTGTGGTTGAG	573 bp	U70622
LPA ₂	GCAGTGTGATGGCGGTACAG	ACCAGTGAGTTGGCCTCAGC	478 bp	NM_020028
LPA ₃	GTCCAACCTCCTGGCCTTCT	CGCCTCTCGGTATTGCTGTC	381 bp	NM_022983
LPA ₄	CTACAGGCATGAGCACATTC	GAGGCACTGCTGGTTATTAG	409 bp	NM_175271
sST2	CCTCACGGCTCTGAGCTTAT	GGGTCCAGAAGAGAACTACTG	70 bp	NM_010743
ST2L	AGACCTGTTACTGGGCAAG	CACCTGTCTTCTGTAATCTGG	70 bp	NM_001025602
HSP25	AGCTCACAGTGAAGACCAAGG	CATGTTCTGCTGCCTTTCT	72 bp	NM_013560
Cyclophilin A	GAGCTGTTGCAGACAAAGTTC	CCCTGGCACATGAATCTCTGG	125 bp	NM_008907

analysis was performed using Student's *t* test, and results were considered to be significant if *p* < 0.05.

Intracellular Ca²⁺ measurements

LPA-induced Ca²⁺ signaling in MC3T3-E1 cells was measured essentially as described previously [10,15]. Trypsinized cells were suspended in HBSS (5.6 mM D-glucose, 1.26 mM CaCl₂, 5.4 mM KCl, 0.44 mM KH₂PO₄, 0.5 mM MgCl₂, 0.5 mM MgSO₄, 137 mM NaCl, 4.2 mM NaHCO₃, 0.34 mM Na₂PO₄, pH 7.4; GIBCO-Invitrogen, Carlsbad, CA, USA) supplemented with 0.1% fatty acid-free BSA (HBSS/BSA). Cells were suspended in HBSS/BSA at a density of 1 × 10⁷ cells/ml and loaded with 1.0 μM fluo-4 acetoxymethyl ester (Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C. The cells subsequently were washed in HBSS/BSA to remove extracellular dye and incubated for 30 min at room temperature to maximize dye de-esterification. Dye-loaded cells were placed in a cuvette in the chamber of a dual-excitation FluoroMax-2 spectrofluorometer (Jobin Yvon, Edison, NJ, USA) at room temperature with constant stirring. The cell suspension was excited 488 nm and the fluorescence emission determined at 520 nm. Where indicated, cells were loaded with the intracellular Ca²⁺ chelator 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetra (BAPTA) prior to the analysis of LPA-induced Ca²⁺ signaling and gene expression. Cells were incubated at 37 °C in αMEM/BSA containing 5 μM BAPTA-acetoxymethyl ester (Molecular Probes, Eugene, OR, USA). After 30 min, the medium was replaced with fresh αMEM/BSA and culture continued for 6 h in the presence or absence of LPA.

Mechanical loading of osteoblasts

Osteoblasts were exposed to fluid shear essentially as described previously [23]. MC3T3-E1 subclone 14 cells were seeded onto 75 × 38 mm glass microscope slides at a density of 2000 cells/cm². Forty-eight hours after seeding, the medium was replaced with flow medium (αMEM with 2% FBS) and cultured an additional 24 h before exposure to fluid shear stress. On the day of experimentation, slides were placed into a custom-milled parallel plate flow chamber in series with rigid wall tubing and a Hamilton glass syringe. The syringe was displaced by an electromagnetic loading device (ELF 3200, Bose Electroforce, Eden Prairie, MN, USA). The flow rate was selected to induce a peak shear stress of 20 dynes/cm² at a frequency of 1 Hz and was monitored in real time with an ultrasonic flow meter (Transonic Systems, Ithaca, NY, USA) during all experiments. Cells were exposed to oscillating fluid flow for 2 h, removed from their chambers, and then cultured for an additional 4 h in fresh flow medium prior to the isolation of total RNA. Sham-treated cells were similarly loaded into parallel plate flow chambers but were not exposed to fluid shear.

Results

Effects of LPA on pre-osteoblast gene expression

The mechanisms by which LPA modulates osteoblast function are not clear, but we predicted that the effects of this lipid on osteoblastic cells would involve changes in gene expression.

To address this potentiality, we employed DNA oligodeoxynucleotide microarrays corresponding to the entire mouse genome to analyze global gene expression in MC3T3-E1 pre-osteoblastic cells grown for 6 or 24 h in the presence or absence of LPA. These time points were selected to identify genes that are regulated relatively early in the response to LPA, such as during the lag period that precedes LPA-induced increases in osteoblastic cell migration velocity [10], and to reveal genes that are modulated after more chronic exposure to this growth factor. Cells treated with LPA for 6 h exhibited statistically significant changes in the expression of 634 transcripts representing the products of 513 genes (Supplementary Table S1). In contrast, little effect on gene expression was seen in pre-osteoblasts treated with LPA for 24 h: only 54 gene products exhibited significant up- or down-regulation, and none exhibited changes exceeding 2-fold compared to the expression in control cells (data not shown). Only one gene product, encoding the pleiotropic receptor neogenin, was common to both data sets where it exhibited expression decreases of 2.1- and 1.5-fold after 6 and 24 h of LPA treatment, respectively. Based on the kinetics of gene regulation by LPA, we focused on the transcripts identified after the 6-h treatment.

Gene ontology (GO) analysis revealed that LPA altered the expression of genes linked to distinct functional categories (Fig. 1; individual gene products in each category are listed in Supplementary Table S2). Consistent with the mitogenic effects of this lipid factor on osteoblastic cells [6–8], we observed LPA-induced changes in the expression of 66 genes associated with the GO *cell proliferation* category. Most have not been studied in the context of bone physiology, but several have been linked previously to the regulation of osteoblast function, such as Fos-related antigen 1 (Fra-1), which stimulated bone matrix deposition [24], and c-Fos, which is regulated in osteoblasts by mechanical stimulation [25]. Also in this category was the LPA-stimulated gene product Flt-1, which is a receptor for vascular endothelial growth factor (VEGF), an angiogenic stimulus known to promote osteoblast differentiation and bone regeneration [26]. Fracture healing recapitulates many aspects of embryonic bone formation [12], and 84 genes were classified as related to *organ development* (Fig. 1; Table S2). Notable among the development-associated genes modulated by LPA were six transcripts related to the *Wnt* signaling pathway which plays a key role in osteoblast differentiation [27]: Axin2, Ccnd2, Tle4, Fzd5, sFRP-2, and Daam2. The response of cells to LPA is

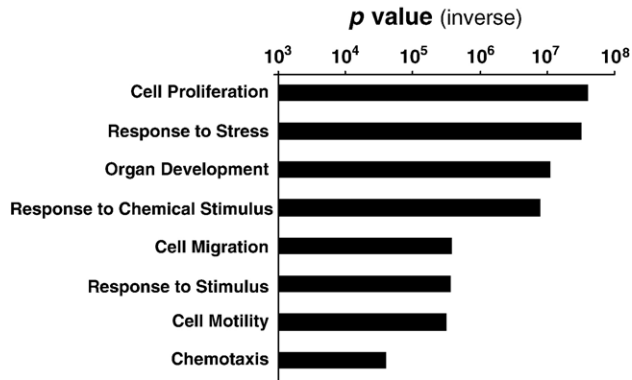


Fig. 1. Gene ontology analysis of MC3T3-E1 cellular processes modulated by 6-h LPA treatment. Biological process enrichment was calculated using the MetaCore software suite as described in Materials and methods. This algorithm calculates a p value based on the probability that a process appears in the data set relative to that expected by random chance. Multifunctional gene products may be assigned to multiple biological processes. The data are shown plotted versus the inverse of the p values to more clearly show relative significance.

mediated by G-protein-coupled receptors, and a large number of lysophospholipid-modulated transcripts were classified in the GO categories *response to chemical stimulus* (43 genes) and *response to stimulus* (96 genes) (Fig. 1; Table S2). Consistent with the ability of LPA to stimulate pre-osteoblastic cell locomotion [10], MC3T3-E1 cells treated for 6 h with LPA also displayed changes in the expression genes linked to the GO categories *cell migration* (28 genes), *cell motility* (38 genes), and *chemotaxis* (19 genes) (Fig. 1; Table S2). Among the LPA-regulated genes in these groupings were the integrins $\alpha 3$, $\alpha 6$, $\beta 3$ and $\beta 5$, as well as Flt-1. Other motility-associated gene products elevated by LPA treatment were the disintegrin ADAM12, tissue inhibitor of metalloproteinases 1 (TIMP-1), and the cell adhesion molecule VCAM1. Included in the LPA-regulated gene products were transcripts encoding proteins linked to the regulation of bone mass but for which no previous functional connection to this lipid growth factor existed, such as receptor activity modifying protein 3 (RAMP3), thrombospondin 1, and thrombospondin 2 [28–30].

Recent evidence suggests that osteoblastic cells play an active role in the control of the inflammatory response [17], and GO analysis categorized a number of LPA-regulated genes in MC3T3-E1 cells as being associated with *response to stress* (Fig. 1; Table S2). LPA treatment stimulated the expression of genes encoding pro-inflammatory cytokines/chemokines or their receptors, such as CXCR6, CXCL7, CXCL12, CCL2, CCL7, and four members of the tumor necrosis factor superfamily, TNFRsf9, TNFRsf12a, TNFRsf22, and TNFRsf23. The expression of IL-1 receptor antagonist (IL1RN), which inhibits IL-1-induced inflammatory bone resorption [31], was increased 6-fold. Among the LPA-regulated gene products that exhibited the largest increases were the sST2 and ST2L mRNAs (elevated 26-fold and 10-fold, respectively), which are two alternatively spliced products of the *IL-1 receptor-like 1* gene (*IL1RL1*; also called *ST2*), and the *HSPB1* gene product (elevated 13-fold), which encodes heat shock protein 25 (HSP25). Inflammation is governed by bioactive molecules that establish a balance

between the stimulation and dampening of the immune response, but relatively little is known about the role of anti-inflammatory factors during bone healing. ST2L, sST2 and HSP25 proteins recently were identified as anti-inflammatory agents (see Discussion) and we chose to target these LPA-regulated transcripts for further analysis.

Kinetics of LPA-induced sST2, ST2L and HSP25 expression in pre-osteoblasts

The microarray data revealed elevations in the expression of transcripts encoding sST2, ST2L and HSP25 in MC3T3-E1 cells treated with LPA for 6 h. We employed quantitative RT-PCR to measure the relative levels of mRNA encoding these proteins over an LPA treatment time course of 0 to 24 h. Maximum expression of sST2 and ST2L mRNA was observed after 6 h of LPA exposure, and HSP25 transcript abundance was maximal after 3 h of treatment (Fig. 2). The relative increase in expression compared to untreated control cells for sST2 (17.3-fold), ST2L (7.6-fold), and HSP25 (12.0-fold) revealed by RT-PCR agreed well with the changes determined by the microarray analysis (Table S1). Also consistent with the results of the microarray screen, the levels of all three transcripts returned to near baseline after 24 h of LPA treatment (Fig. 2).

The regulatory actions of LPA are attributed to its ability to bind one or more of a group of at least five G-protein-coupled receptors consisting of LPA₁ (previously named *Edg2*), LPA₂ (*Edg4*), LPA₃ (*Edg7*), LPA₄ (*GPR23*) and LPA₅ (*GPR92*) [32,33]. MC3T3-E1 cells express primarily the LPA₁, LPA₂ and LPA₄ receptor forms [10]. The LPA responsiveness of murine osteoblasts *in vivo* has not been reported, and we isolated primary osteoblasts from neonatal mouse calvaria and found that they express four LPA receptors (Fig. 3A); LPA₅ mRNA

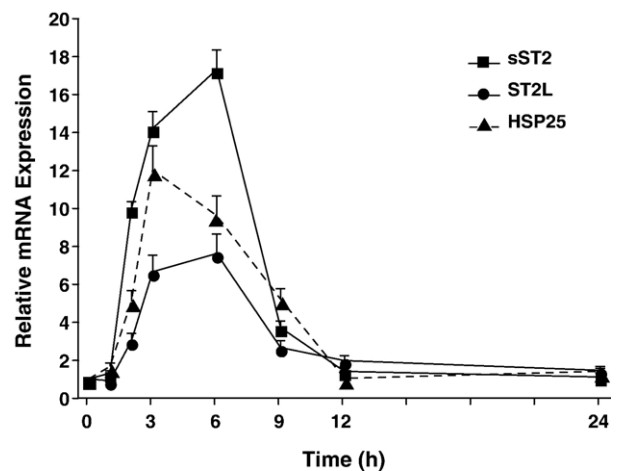


Fig. 2. LPA stimulates the time-dependent expression of sST2, ST2L and HSP25 transcripts in MC3T3-E1 cells. Cells were treated with 2.5 μ M LPA in α MEM/BSA for 0 to 24 h, after which RNA was isolated and subjected to quantitative RT-PCR analysis using primers specific for sST2, ST2L, and HSP25. The expression of each gene was normalized to the level of cyclophilin A mRNA in parallel samples. The data are presented relative to expression in untreated (0 h) cells and represent duplicate determinations \pm S.E. The expression of all three gene products was unchanged in cells incubated for 1 to 24 h in α MEM/BSA in the absence of LPA (data not shown).

expression was not detected in any of the mouse bone cell types (N.J. Karin, unpublished data). The relative levels of mRNA encoding each receptor in primary osteoblasts were similar to the expression stoichiometry we measured in MC3T3-E1 cells although, in contrast to the calvarial cells, MC3T3-E1 cells expressed only trace amounts of LPA₃ [10]. Calvarial osteoblasts also exhibited LPA-induced elevations sST2, ST2L and HSP25 expression (Fig. 3B). However, although the time course of induction was similar to MC3T3-E1 cells, the magnitude of stimulation in primary osteoblasts was smaller for all three gene products.

Receptor-linked signaling components involved in LPA-stimulated gene expression

We previously employed Ki16425, an inhibitor of LPA₁ and LPA₃ receptors [34], to demonstrate a functionally dominant role for LPA₁ in LPA-induced osteoblastic cell chemotaxis [10]. Ki16425 significantly attenuated LPA-induced expression of sST2, ST2L and HSP25 compared to cells treated with the lipid growth factor alone (Fig. 4). This demonstrated a major contribution of LPA₁-coupled pathways to the induction by LPA of

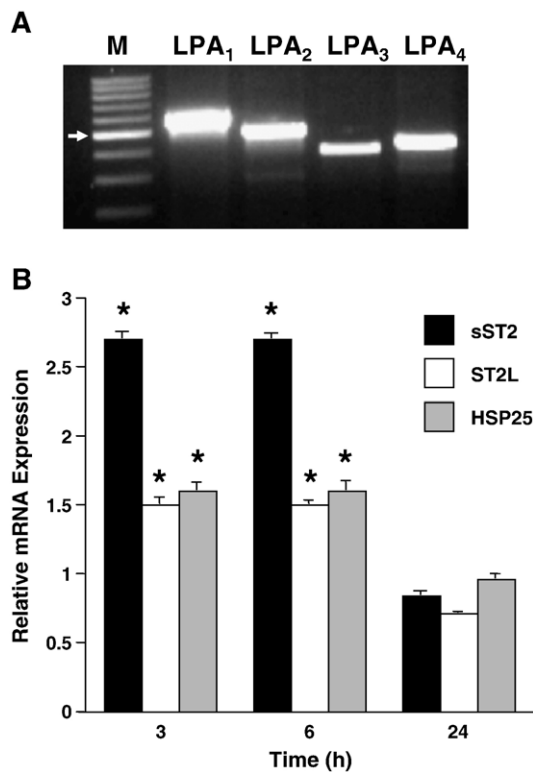


Fig. 3. Primary mouse osteoblasts express LPA receptors and respond to LPA with elevated anti-inflammatory gene expression. (A) Samples from RT-PCR reactions were analyzed by electrophoresis in Tris–borate–EDTA buffer through a 1% agarose gel. Expected amplicon sizes were: LPA₁, 573 base pairs (bp); LPA₂, 478 bp; LPA₃, 381 bp; and LPA₄, 409 bp. M=markers (100 bp ladder); the 500-bp marker is indicated with an arrow. (B) Calvarial osteoblasts were serum-starved in α MEM/BSA for 8 to 33 h prior to the addition of 2.5 μ M LPA for the times indicated; the total incubation time in serum-free medium was 36 h for all cells. Gene expression was measured by quantitative RT-PCR and presented as in Fig. 2. * p <0.015.

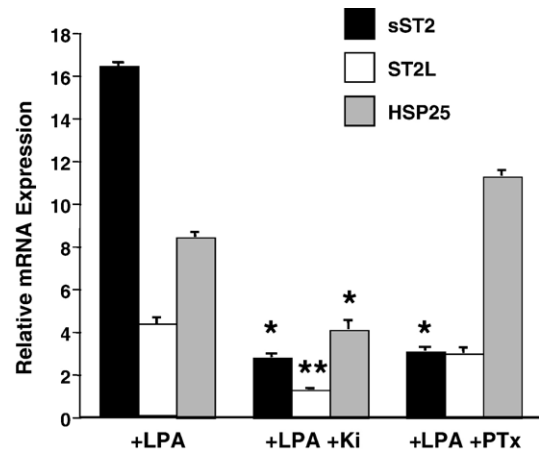


Fig. 4. The effects of receptor antagonists on LPA-induced sST2, ST2L and HSP25 mRNA expression in MC3T3-E1 cells. To determine the effects of a specific inhibitor of LPA receptors on gene expression, cells were serum-starved in α MEM/BSA for 16 h followed by incubation in the presence or absence of 2.5 μ M LPA and, where indicated, 5.0 μ M Ki16425 (Ki). Another set of osteoblasts was maintained in α MEM/BSA for 24 h in the presence or absence of 100 ng/ml pertussis toxin (PTx). PTx-treated cells were incubated during the final 6 h with or without 2.5 μ M LPA. Gene expression was measured by quantitative RT-PCR and presented as in Fig. 2. * p <0.002 vs. LPA alone; ** p <0.05 vs. LPA alone.

anti-inflammatory gene expression in MC3T3-E1 cells. LPA receptors are G-protein-coupled, and ligand binding led to robust increases in cytosolic Ca²⁺ in MC3T3-E1 cells that could be inhibited by Ki16425 [10,15]. Therefore, we sought to determine whether cytosolic Ca²⁺ elevations are necessary for the induction of sST2, ST2L and HSP25 expression by LPA. To this end, we analyzed mRNA levels in MC3T3-E1 cells loaded with the Ca²⁺ chelator BAPTA. The chelating agent abrogated LPA-induced Ca²⁺ elevations (Fig. 5, inset) but did not block the ability of this lipid growth factor to induce sST2 and HSP25 expression (Fig. 5). BAPTA-loaded cells displayed a small but statistically significant decrease in LPA-stimulated ST2L mRNA levels (Fig. 5). LPA-induced Ca²⁺ signaling in MC3T3-E1 cells also can be eliminated by U73122, an inhibitor of phospholipase C activity that prevents the generation of inositol-1,4,5-trisphosphate (data not shown). Pre-treatment of MC3T3-E1 cells with U73122 had no effect on the ability of LPA to up-regulate the levels of mRNA encoding sST2 and ST2L; the inhibitor alone induced HSP25 expression (data not shown).

Because cytosolic Ca²⁺ signaling, which is regulated primarily by the coupling of the G_q protein to lipid receptors [32], appeared to have little or no role in the LPA-induced expression of the anti-inflammatory genes, we measured the ability of LPA to regulate gene expression in cells pre-treated with pertussis toxin, an inhibitor of G_{i/o}-coupled receptors. Pertussis toxin effectively blocked LPA-induced expression of sST2 but had no significant inhibitory effects on ST2L and HSP25 transcript levels (Fig. 4).

Fluid shear suppresses the expression of sST2, ST2L and HSP25 in osteoblastic cells

Physical stimuli are important physiological regulators of bone mass, and LPA was reported to enhance the response of endothelial cells and smooth muscle cells to fluid shear [35].

ST2 gene expression was induced in cardiomyocytes by mechanical stimulation [36]. Therefore, we sought to determine whether the anti-inflammatory genes that were modulated in osteoblastic cells by LPA are also targets for regulation by mechanical stimuli. MC3T3-E1 cells were exposed for 2 h to oscillating fluid shear or sham-flow conditions then cultured for an additional 4 h prior to the measurement of sST2, ST2L, and HSP25 mRNA levels. While quantitative variation in *ST2* gene expression was observed among replicate experiments, the exposure of cells to fluid shear led to strong reductions in the expression of sST2, ST2L and HSP25 transcripts (Fig. 6).

Discussion

Osteoblastic cells are target cells for LPA but the effects of this lysophospholipid growth factor on bone cell gene expression had not been investigated. Our analysis of global gene expression in MC3T3-E1 pre-osteoblasts revealed that 566 genes were regulated in response to LPA treatment. The modulation of gene activity was much more extensive after 6 h (513 regulated genes) than after 24 h (54 regulated genes) of LPA exposure, which probably reflects receptor desensitization in

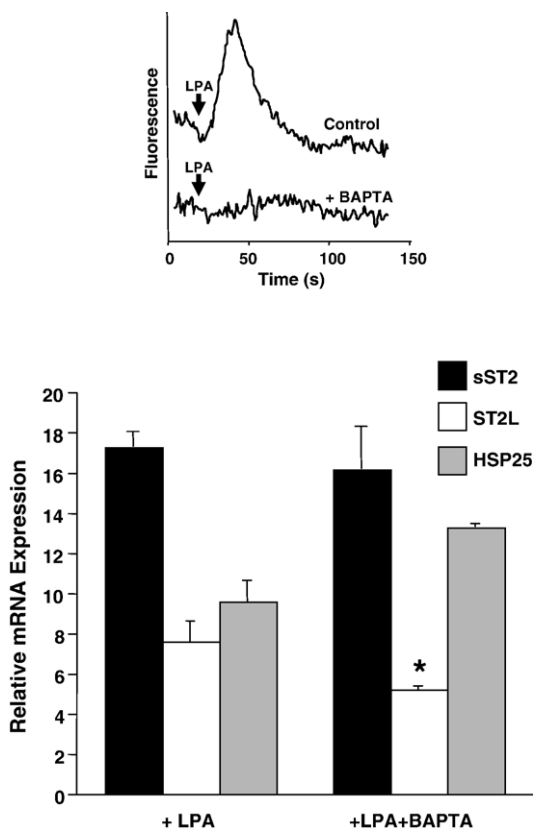


Fig. 5. The effect of cytoplasmic BAPTA loading on LPA-induced gene expression in MC3T3-E1 cells. Cells were serum-starved in α MEM/BSA for 16 h then incubated 30 min longer in the same medium in the presence or absence of 5.0- μ M BAPTA-acetoxymethyl ester for 30 min. The medium was replaced with fresh α MEM/BSA with or without 2.5 μ M LPA and the cells were incubated for 6 h. Gene expression was measured by quantitative RT-PCR and presented as in Fig. 2. * $p < 0.025$ vs. LPA alone. Inset: The effect of LPA on relative cytoplasmic Ca^{2+} levels (fluo-4 fluorescence) in control and BAPTA-loaded MC3T3-E1 cells.

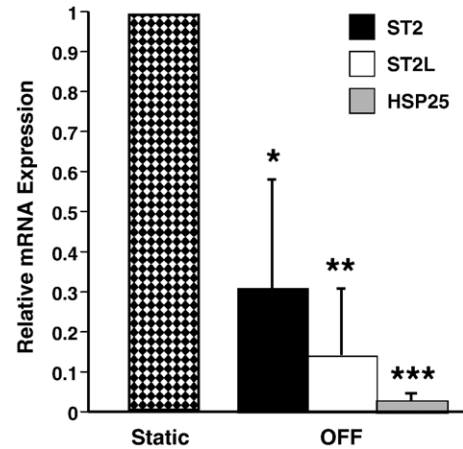


Fig. 6. Oscillating fluid flow depresses the expression of sST2, ST2L and HSP25 mRNA in MC3T3-E1 cells. Cells were grown on glass slides and incubated in the absence (Static) or presence (OFF) of oscillating fluid flow as described in Materials and methods. Gene expression was measured by quantitative RT-PCR as in Fig. 2 and normalized to mRNA levels in the absence of fluid shear (stippled bar). The data represent the mean (\pm S.E.) of duplicate measurements from each of three experiments. * $p < 0.02$ vs. static cells; ** $p < 0.005$ vs. static cells; *** $p < 0.0005$ vs. static cells.

the chronic presence of ligand. The number of LPA-regulated genes and the diversity of the functions with which they are associated imply that this lipid factor is involved in the regulation of a complex array of osteoblast functions, but many genes modulated by this lysophospholipid have particular relevance to bone formation and regeneration. LPA production *in vivo* is associated with platelet degranulation, and this growth factor is postulated to be among the paracrine agents involved in wound healing [4]. Based on previous data showing that LPA triggers the proliferation and migration of osteoblastic cells [6–8,10], we postulate that LPA plays a role in the regulation of bone cell function during fracture healing, and a number of results from the genomic screen support this hypothesis.

Gene ontology analysis showed that the products of the LPA-regulated genes are involved in diverse aspects of cell physiology including cell proliferation, response to stress, organ development, cell migration, cell motility and chemotaxis. That LPA-modulated genes were linked to cell proliferation and movement is consistent with reports by us and others that this lysophospholipid was both mitogenic and chemotactic for osteoblasts [6–8,10]. One of the motility-related gene products up-regulated by LPA encodes integrin $\alpha 6$, a receptor for the basement membrane protein laminin. Laminin is expressed early in fracture healing in association with neovascularization [37], and the LPA-induced expression of the laminin receptor may facilitate the adhesion of osteoblasts and the deposition of mineralized matrix around new blood vessels. Many aspects of fracture healing resemble embryonic and early postnatal bone formation [12], and LPA treatment led to the regulation of 84 genes related to the GO group, *organ development*. Exposure of MC3T3-E1 cells to LPA also led to the modulation of 62 genes in the GO category *response to stress*, which suggests a potential role for this lipid in the early inflammatory phase of bone healing (see below).

A number of LPA-regulated gene products have been linked previously to the control of bone metabolism. RAMP3 mRNA expression was elevated 16-fold in LPA-treated MC3T3-E1 cells. The RAMP3 protein heterodimerizes with calcitonin-like receptor to form a receptor for adrenomedullin, an anabolic regulator of bone mass [28]. TNFRsf9 (also called 4-1BB), which displayed a 10-fold increase in mRNA expression, also promoted osteogenesis by inhibiting receptor activator of NF- κ B ligand (RANKL)-mediated osteoclast differentiation [38]. Although these and other LPA-regulated osteoblast genes were associated with pathways that mediate bone anabolic effects, we also observed a stimulation of the expression of thrombospondin 1 and thrombospondin 2 transcripts which encode proteins linked to the inhibition of osteoblast-mediated mineralization *in vitro* and the suppression of bone formation *in vivo* [30,39].

LPA-induced changes in MC3T3-E1 cell gene expression included transcripts encoding cytokines/chemokines and their receptors. Most of the up-regulated cytokine/receptor genes are pro-inflammatory, such as the CXCR6 receptor which was proposed to foster the pathogenesis of rheumatoid arthritis [40]. However, LPA treatment also led to significant increases in the levels of mRNAs encoding three products of two genes, *ST2* and *HSBP1*, which encode proteins with anti-inflammatory actions. The *ST2* gene is the template for two alternatively spliced transcripts encoding ST2L, a membrane-bound glycoprotein, and sST2, a soluble protein corresponding to the extracellular domain of ST2L with nine additional amino acids at the C-terminus [41]. The two *ST2* gene transcripts and the HSP25 mRNA product of *HSBP1* also were elevated in LPA-treated calvarial osteoblasts but the relative stimulation was less than that observed in MC3T3-E1 cells. It is likely that this difference reflects the fact that the primary cells comprised a heterogeneous population of osteoblasts at various stages of differentiation while MC3T3-E1 cells are clonal and pre-osteoblastic [42]. *ST2* gene products originally were identified on the basis of their induction in fibroblasts by serum, glucocorticoids, phorbol ester, or oncogene expression [43–45]. The temporal pattern of sST2 and ST2L expression we observed in LPA-treated MC3T3-E1 cells, with peak expression at 6 h that returns to near-baseline levels at 24 h, is the same as that observed in mouse fibroblasts exposed to serum [43,45]. LPA is abundant in serum [2] and it seems likely that at least part of the induction of *ST2* expression observed in serum-treated fibroblasts was due to this lipid.

ST2L and sST2 are members of the Toll-Interleukin-1 receptor superfamily, and the *ST2* gene is designated as *interleukin-1 receptor-like-1* in the Human Gene Nomenclature Database due to high similarity to the gene encoding the IL-1 receptor. Neither sST2 nor ST2L exhibited high-affinity binding of IL-1, but a recent report provided evidence that ST2L is a receptor for a newly discovered cytokine, IL-33 [46]. The functions of sST2 and ST2L proteins in bone physiology are as yet unknown but osteoblasts and their progenitor cells are exposed to LPA during the early inflammatory phase of fracture healing, and the induction of *ST2* gene expression by a platelet-derived lipid growth factor suggests a possible role for these gene products in the control of fracture-associated inflammation. Both ST2L and sST2 exhibited anti-inflammatory acti-

vities *in vivo*, particularly in the context of arthritis [47,48]. sST2 also suppressed the inflammatory response in mice treated with lipopolysaccharide via a mechanism that directly involved macrophages [49]. Thus, elevated sST2 production by osteoblasts exposed to LPA may help to prevent uncontrolled inflammation during early bone repair. Putative sST2 binding proteins also have been identified on bone marrow macrophages, and the binding activity was elevated in response to inflammatory stimuli [49]. Macrophages and osteoclasts are functionally and ontologically related, and an intriguing possibility is that osteoblast-derived sST2 may regulate osteoclast-mediated bone resorption.

HSP25 mRNA expression in MC3T3-E1 cells was induced within 3 h of LPA treatment. Sphingosine-1-phosphate, a platelet-derived lipid growth factor with structural and functional similarities to LPA, stimulated HSP25 expression in MC3T3-E1 cells with kinetics similar to those reported here [50]. The role of HSP25 in bone cell physiology has not been elucidated, but in other cell types, it is an anti-apoptotic agent and a chaperone that prevents the aberrant protein aggregation that can result from the exposure of cells to stressful stimuli [51,52]. LPA promotes osteoblast survival from apoptotic cell death *in vitro* during serum starvation [9], and elevated HSP25 expression may be a component of the survival pathways associated with this lipid factor. HSP25 also was reported to stabilize actin microfilaments in cultured Chinese hamster cells, and the expression of the heat shock protein in endothelial cells was linked to cell migration and the formation of slender lamellipodia [53,54]. These results suggest that increased HSP25 expression may be an important step in the stimulation by LPA of osteoblast chemotaxis and osteocyte membrane outgrowth [10,11]. The expression of HSP25 has been associated with anti-inflammatory activity: this protein protected mice against inflammatory liver injury and endotoxic shock, and treatment of human monocytes with exogenous HSP27 (the human homolog of murine HSP25) led to the production of the anti-inflammatory cytokine, IL-10 [55–57].

LPA is an agonist of intracellular Ca²⁺ signaling in MC3T3-E1 cells, and Ca²⁺ elevations are impaired if the cells are also treated with the LPA receptor antagonist Ki16425 [10,15]. This agent blocks the function of LPA₁ and LPA₃ receptor forms, but because MC3T3-E1 cells express only trace amounts of LPA₃, we attributed the effects of Ki16425 to the inhibition of LPA₁ [10]. The induction of sST2, ST2L and HSP25 expression by LPA was suppressed if the cells were treated with Ki16425. However, the stimulation of sST2, ST2L and HSP25 expression by LPA was not prevented when cytosolic Ca²⁺ elevations were blocked. Thus, although Ca²⁺ is known to regulate the activity of a variety of genes [58], the stimulation of sST2, ST2L and HSP25 expression in osteoblasts by LPA appears to be independent of Ca²⁺ signals. The LPA-induced expression of sST2, but not ST2L, was impaired in cells pre-treated with pertussis toxin, an inhibitor of receptors coupled to G_{i/o} proteins. The transcription of the two alternatively spliced *ST2* gene products in fibroblasts and mast cells is initiated at separate promoters [59], and our data indicate that the two promoters controlling *ST2* gene expression in osteoblasts are regulated by different

G proteins. The LPA₁, LPA₂ and LPA₃ members of the LPA receptor family exhibit a complex relationship with heterotrimeric G proteins in that each receptor form is coupled to G_{i/o}, G_q and G_{12/13}; the G protein coupling status of LPA₄ is not known [32]. The inhibition of LPA-induced sST2 and ST2L expression by pertussis toxin revealed a link to a G_{i/o}-coupled pathway that most likely involves changes in cyclic AMP levels. In contrast, the stimulation of HSP25 expression by LPA was not impaired by pertussis toxin. Therefore, although our data revealed LPA₁ receptor-coupled, Ca²⁺-independent regulation of HSP25 transcript levels in MC3T3-E1 cells, the identity of the G protein(s) involved in transducing LPA binding events and the intracellular second messengers that control HSP25 expression remains unknown.

There is reason to postulate that LPA and physical stimuli would have synergistic effects on osteoblast functions. LPA-treated MC3T3-E1 cells exhibited alterations in the actin cytoskeleton and robust elevations in Ca²⁺ [10,15], effects that resemble the phenotype of osteoblastic cells exposed to fluid shear [60]. LPA and mechanical stimulation had additive effects on Ca²⁺ signaling in endothelial cells and smooth muscle cells [35]. ST2L and sST2 mRNA levels were induced in heart tissue by mechanical overload and in cardiomyocytes exposed to mechanical deformation *in vitro* [36]. Bone cells have complex responses to mechanical stimulation [25,61], and while LPA-induced anti-inflammatory gene expression in MC3T3-E1 cells was independent of Ca²⁺ signaling, we predicted that the exposure of osteoblastic cells to fluid shear would result in an elevated expression of ST2 gene products. In contrast, we detected significant decreases in the levels of both sST2 and ST2L transcripts when cells were exposed to fluid shear. Similarly, oscillating fluid flow led to a profound reduction in the level of HSP25 mRNA in MC3T3-E1 cells. We are not aware of any previous studies that link mechanical stimuli to the regulation of heat shock protein expression in osteoblasts. Little is known about the potential link between inflammation and the mechanical stimulation of bone cells, but fluid shear is known to increase the production of pro-inflammatory molecules, such as nitric oxide and prostaglandin E₂ [61]. This appears to be consistent with the down-regulation of anti-inflammatory gene expression we measured in MC3T3-E1 cells exposed to oscillating flow. Regardless of the physiological significance of the effects of fluid shear on sST2, ST2L and HSP25 expression in osteoblasts, it is clear that there are major differences between heart and bone cells with respect to the mechanisms that couple mechanical stimuli to the regulation of ST2 gene expression.

In conclusion, the treatment of osteoblasts with LPA led to changes in the expression of pre-osteoblast genes associated with a variety of cellular functions, many of which have potential relevance to our postulate that LPA stimulates bone formation during the repair of skeletal injuries. Our data also revealed LPA receptor-mediated increases in the expression of genes encoding inflammatory mediators, including the anti-inflammatory proteins sST2, ST2L and HSP25. These results provide new insights into the mechanisms by which bioactive lipids regulate osteoblast function, and add to an emergent body of literature that links bone cells to the control of inflammation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bone.2007.06.029](https://doi.org/10.1016/j.bone.2007.06.029).

References

- Goetzl EJ. Pleiotypic mechanisms of cellular responses to biologically active lysophospholipids. *Prostaglandins* 2001;64:11–20.
- Eichholtz T, Jalink K, Fahrenfort I, Moolenaar WH. The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem J* 1993;291(Pt 3):677–80.
- Sano T, Baker D, Virag T, Wada A, Yatomi Y, Kobayashi T, et al. Multiple mechanisms linked to platelet activation result in lysophosphatidic acid and sphingosine 1-phosphate generation in blood. *J Biol Chem* 2002;277:21197–206.
- Lee H, Goetzl EJ, An S. Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing. *Am J Physiol Cell Physiol* 2000;278:C612–8.
- Themistocleous GS, Kontou SE, Lembessis P, Katopodis HA, Kaseta MA, Themistocleous MS, et al. Skeletal growth factor involvement in the regulation of fracture healing process. *In Vivo* 2003;17:489–503.
- Caverzasio J, Palmer G, Suzuki A, Bonjour JP. Evidence for the involvement of two pathways in activation of extracellular signal-regulated kinase (Erk) and cell proliferation by Gi and Gq protein-coupled receptors in osteoblast-like cells. *J Bone Miner Res* 2000;15:1697–706.
- Dziak R, Yang BM, Leung BW, Li S, Marzec N, Margarone J, et al. Effects of sphingosine-1-phosphate and lysophosphatidic acid on human osteoblastic cells. *Prostaglandins Leukot Essent Fat Acids* 2003;68:239–49.
- Grey A, Banovic T, Naot D, Hill B, Callon K, Reid I, et al. Lysophosphatidic acid is an osteoblast mitogen whose proliferative actions involve Gi proteins and protein kinase C, but not P42/44 mitogen-activated protein kinases. *Endocrinology* 2001;142:1098–106.
- Grey A, Chen Q, Callon K, Xu X, Reid IR, Cornish J. The phospholipids sphingosine-1-phosphate and lysophosphatidic acid prevent apoptosis in osteoblastic cells via a signaling pathway involving Gi proteins and phosphatidylinositol-3 kinase. *Endocrinology* 2002;143:4755–63.
- Masiello LM, Fotos JS, Galileo DS, Karin NJ. Lysophosphatidic acid induces chemotaxis in MC3T3-E1 osteoblastic cells. *Bone* 2006;39:72–82.
- Karagiosis SA, Karin NJ. Lysophosphatidic acid induces osteocyte dendrite outgrowth. *Biochem Biophys Res Commun* 2007;57:194–9.
- Einhorn TA. The cell and molecular biology of fracture healing. *Clin Orthop Relat Res* 1998;S7–S21.
- Liu R, Farach-Carson MC, Karin NJ. Effects of sphingosine derivatives on MC3T3-E1 pre-osteoblasts: psychosine elicits release of calcium from intracellular stores. *Biochem Biophys Res Commun* 1995;214:676–84.
- Liu R, Xu Y, Farach-Carson MC, Vogel JJ, Karin NJ. 1,25 dihydroxyvitamin D₃ activates sphingomyelin turnover in ROS17/2.8 osteosarcoma cells without sphingolipid-induced changes in cytosolic Ca²⁺. *Biochem Biophys Res Commun* 2000;273:95–100.
- Lyons JM, Karin NJ. A role for G protein-coupled lysophospholipid receptors in sphingolipid-induced Ca²⁺ signaling in MC3T3-E1 osteoblastic cells. *J Bone Miner Res* 2001;16:2035–42.
- Gräler MH, Goetzl EJ. Lysophospholipids and their G protein-coupled receptors in inflammation and immunity. *Biochim Biophys Acta* 2002;1582:168–74.

- [17] Marriott I. Osteoblast responses to bacterial pathogens: a previously unappreciated role for bone-forming cells in host defense and disease progression. *Immunol Res* 2004;30:291–308.
- [18] Ryu J, Kim HJ, Chang EJ, Huang H, Banno Y, Kim HH. Sphingosine 1-phosphate as a regulator of osteoclast differentiation and osteoclast–osteoblast coupling. *EMBO J* 2006;25:5840–51.
- [19] Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 2003;4:249–64.
- [20] Dudoit S, van der Laan MJ, Pollard KS. Multiple testing: Part I. Single-step procedures for control of general type I error rates. *Stat Appl Genet Mol Biol* 2004;3 [Article 13].
- [21] Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 2004;5:R80.
- [22] Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 2002;30:e36.
- [23] Haut Donahue TL, Genetos DC, Jacobs CR, Donahue HJ, Yellowley CE. Annexin V disruption impairs mechanically induced calcium signaling in osteoblastic cells. *Bone* 2004;35:656–63.
- [24] Eferl R, Hoebertz A, Schilling AF, Rath M, Karreth F, Kenner L, et al. The Fos-related antigen Fra-1 is an activator of bone matrix formation. *EMBO J* 2004;23:2789–99.
- [25] Iqbal J, Zaidi M. Molecular regulation of mechanotransduction. *Biochem Biophys Res Commun* 2005;328:751–5.
- [26] Street J, Bao M, de Guzman L, Bunting S, Peale Jr FV, Ferrara N, et al. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc Natl Acad Sci U S A* 2002;99: 9656–61.
- [27] Krishnan V, Bryant HU, Macdougald OA. Regulation of bone mass by Wnt signaling. *J Clin Invest* 2006;116:1202–9.
- [28] Cornish J, Naot D, Reid IR. Adrenomedullin—a regulator of bone formation. *Regul Pept* 2003;112:79–86.
- [29] Hankenson KD, Ausk BJ, Bain SD, Bornstein P, Gross TS, Srinivasan S. Mice lacking thrombospondin 2 show an atypical pattern of endocortical and periosteal bone formation in response to mechanical loading. *Bone* 2006;38:310–6.
- [30] Ueno A, Miwa Y, Miyoshi K, Horiguchi T, Inoue H, Ruspita I, et al. Constitutive expression of thrombospondin 1 in MC3T3-E1 osteoblastic cells inhibits mineralization. *J Cell Physiol* 2006;209:322–32.
- [31] Strand V, Kavanaugh AF. The role of interleukin-1 in bone resorption in rheumatoid arthritis. *Rheumatology (Oxford)* 2004;43(Suppl 3):iii10–6.
- [32] Ishii I, Fukushima N, Ye X, Chun J. Lysophospholipid receptors: signaling and biology. *Annu Rev Biochem* 2004;73:321–54.
- [33] Lee CW, Rivera R, Gardell S, Dubin AE, Chun J. GPR92 as a new G_{12/13}- and G_q-coupled lysophosphatidic acid receptor that increases cAMP, LPA5. *J Biol Chem* 2006;281:23589–97.
- [34] Ohta H, Sato K, Murata N, Damirin A, Malchinkhuu E, Kon J, et al. Ki16425, a subtype-selective antagonist for EDG-family lysophosphatidic acid receptors. *Mol Pharmacol* 2003;64:994–1005.
- [35] Ohata H, Tanaka KI, Maeyama N, Ikeuchi T, Kamada A, Yamamoto M, et al. Physiological and pharmacological role of lysophosphatidic acid as modulator in mechanotransduction. *Jpn J Pharmacol* 2001;87:171–6.
- [36] Weinberg EO, Shimpo M, De Keulenaer GW, MacGillivray C, Tominaga S, Solomon SD, et al. Expression and regulation of ST2, an interleukin-1 receptor family member, in cardiomyocytes and myocardial infarction. *Circulation* 2002;106:2961–6.
- [37] Mark H, Penington A, Nanmark U, Morrison W, Messina A. Microvascular invasion during endochondral ossification in experimental fractures in rats. *Bone* 2004;35:535–42.
- [38] Shin HH, Lee EA, Kim SJ, Kwon BS, Choi HS. A signal through 4-1BB ligand inhibits receptor for activation of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis by increasing interferon (IFN)-β production. *FEBS Lett* 2006;580:1601–6.
- [39] Hankenson KD, Bain SD, Kyriakides TR, Smith EA, Goldstein SA, Bornstein P. Increased marrow-derived osteoprogenitor cells and endosteal bone formation in mice lacking thrombospondin 2. *J Bone Miner Res* 2000;15:851–62.
- [40] Nanki T, Shimaoka T, Hayashida K, Taniguchi K, Yonehara S, Miyasaka N. Pathogenic role of the CXCL16–CXCR6 pathway in rheumatoid arthritis. *Arthritis Rheum* 2005;52:3004–14.
- [41] Trajkovic V, Sweet MJ, Xu D. T1/ST2—an IL-1 receptor-like modulator of immune responses. *Cytokine Growth Factor Rev* 2004;15: 87–95.
- [42] Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J Cell Biol* 1983;96:191–8.
- [43] Klemenz R, Hoffmann S, Werenskiold AK. Serum- and oncoprotein-mediated induction of a gene with sequence similarity to the gene encoding carcinoembryonic antigen. *Proc Natl Acad Sci U S A* 1989;86: 5708–12.
- [44] Tominaga S. A putative protein of a growth specific cDNA from BALB/c-3T3 cells is highly similar to the extracellular portion of mouse interleukin 1 receptor. *FEBS Lett* 1989;258:301–4.
- [45] Werenskiold AK, Hoffmann S, Klemenz R. Induction of a mitogen-responsive gene after expression of the Ha-ras oncogene in NIH 3T3 fibroblasts. *Mol Cell Biol* 1989;9:5207–14.
- [46] Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005;23:479–90.
- [47] Leung BP, Xu D, Culshaw S, McInnes IB, Liew FY. A novel therapy of murine collagen-induced arthritis with soluble T1/ST2. *J Immunol* 2004;173: 145–50.
- [48] Xu D, Chan WL, Leung BP, Huang F, Wheeler R, Piedrafita D, et al. Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J Exp Med* 1998;187:787–94.
- [49] Sweet MJ, Leung BP, Kang D, Sogaard M, Schulz K, Trajkovic V, et al. A novel pathway regulating lipopolysaccharide-induced shock by ST2/T1 via inhibition of Toll-like receptor 4 expression. *J Immunol* 2001;166: 6633–9.
- [50] Kozawa O, Niwa M, Matsuno H, Tokuda H, Miwa M, Ito H, et al. Sphingosine 1-phosphate induces heat shock protein 27 via p38 mitogen-activated protein kinase activation in osteoblasts. *J Bone Miner Res* 1999;14: 1761–7.
- [51] Ehrmsperger M, Graber S, Gaestel M, Buchner J. Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. *EMBO J* 1997;16:221–9.
- [52] Garrido C, Gurbuxani S, Ravagnan L, Kroemer G. Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochem Biophys Res Commun* 2001;286:433–42.
- [53] Lavoie JN, Lambert H, Hickey E, Weber LA, Landry J. Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. *Mol Cell Biol* 1995;15:505–16.
- [54] Piotrowicz RS, Hickey E, Levin EG. Heat shock protein 27 kDa expression and phosphorylation regulates endothelial cell migration. *FASEB J* 1998;12:1481–90.
- [55] De AK, Kodys KM, Yeh BS, Miller-Graziano C. Exaggerated human monocyte IL-10 concomitant to minimal TNF-α induction by heat-shock protein 27 (Hsp27) suggests Hsp27 is primarily an antiinflammatory stimulus. *J Immunol* 2000;165:3951–8.
- [56] Sumioka I, Matsura T, Kai M, Yamada K. Potential roles of hepatic heat shock protein 25 and 70i in protection of mice against acetaminophen-induced liver injury. *Life Sci* 2004;74:2551–61.
- [57] Wischmeyer PE, Kahana M, Wolfson R, Ren H, Musch MM, Chang EB. Glutamine induces heat shock protein and protects against endotoxin shock in the rat. *J Appl Physiol* 2001;90:2403–10.
- [58] Mellstrom B, Naranjo JR. Mechanisms of Ca²⁺-dependent transcription. *Curr Opin Neurobiol* 2001;11:312–9.
- [59] Gächter T, Werenskiold AK, Klemenz R. Transcription of the interleukin-1 receptor-related T1 gene is initiated at different promoters in mast cells and fibroblasts. *J Biol Chem* 1996;271:124–9.
- [60] Chen NX, Ryder KD, Pavalko FM, Turner CH, Burr DB, Qiu J, et al. Ca²⁺ regulates fluid shear-induced cytoskeletal reorganization and gene expression in osteoblasts. *Am J Physiol Cell Physiol* 2000;278:C989–C9897.
- [61] Liedert A, Kaspar D, Blakytyn R, Claes L, Ignatius A. Signal transduction pathways involved in mechanotransduction in bone cells. *Biochem Biophys Res Commun* 2006;349:1–5.