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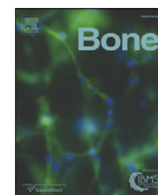

Original Full Length Article

The role of the sphingosine-1-phosphate signaling pathway in osteocyte mechanotransduction

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ABSTRACT

Osteocytes are proposed to be the mechanosensory cells that translate mechanical loading into biochemical signals during the process of bone adaptation. The lipid mediator sphingosine-1-phosphate (S1P) has been reported to play a role in the mechanotransduction process of blood vessels and also in the dynamic control of bone mineral homeostasis. Nevertheless, the potential role of S1P in bone mechanotransduction has yet to be elucidated. In this study, we hypothesized that a S1P cascade is involved in the activation of osteocytes in response to loading-induced oscillatory fluid flow (OFF) in bone. MLO-Y4 osteocyte-like cells express the necessary components of a functional S1P cascade. To examine the involvement of S1P signaling in osteocyte mechanotransduction, we applied OFF (1 Pa, 1 Hz) to osteocyte-like MLO-Y4 cells under conditions where the S1P signaling pathway was modulated. We found that decreased endogenous S1P levels significantly suppressed the OFF-induced intracellular calcium response. Addition of extracellular S1P to MLO-Y4 cells enhanced the synthesis and release of prostaglandin E2 (PGE_2) under static cells and amplified OFF-induced PGE_2 release. The stimulatory effect of OFF on the gene expression levels of osteoprotegerin (OPG) and receptor activator for nuclear factor κB ligand (RANKL) was S1P dependent. Furthermore, the $S1P_2$ receptor subtype was shown to be involved in OFF-induced PGE_2 synthesis and release, as well as down-regulation of RANKL/OPG gene expression ratio. In summary, our data suggest that S1P cascade is involved in OFF-induced mechanotransduction in MLO-Y4 cells and that extracellular S1P exerts its effect partly through $S1P_2$ receptors.

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Introduction

Bones can dynamically adapt their structure and mass to accommodate changes in mechanical load [1]. Although the concept of loading induced bone remodeling is well accepted, much less is known about the underlying cellular signaling processes. There is growing evidence that osteocytes are the primary mechanosensitive cells in the bone, which not only directly modify bone structure but also regulate remodeling activities of other cell types in the bone (i.e., osteoblasts, osteoclasts [2–9]). Osteocytes are the most abundant cell type in bone (90–95%). They are embedded in the lacunae of mineralized bone matrix and form extensive communication network with their cellular processes encased in narrow channels known as canaliculi [2,4,10,11]. Previous studies have indicated that the dynamic fluid flow in the osteocytic network of lacunae-canaliculi is one of the mechanical stimuli that osteocytes respond to under physiological mechanical loads in vivo [12–14]. The flow-induced cellular signals elicited in one osteocyte have been shown to propagate to other cells directly through cell–cell contacts at the osteocytic processes and/or through paracrine mediators transported in the lacunar–canalicular system [3,11].

Several intracellular signaling events have been shown to be involved in osteocyte mechanotransduction, among them the release of Ca^{2+} from intracellular stores, PKC-, RhoA/Rho kinase- and MAP kinase-linked pathways [15–17]. However, the upstream mediators of these various signaling cascades in osteocytes remain to be characterized.

In this regard, the bioactive lipid mediator sphingosine-1-phosphate (S1P) is a primary candidate. This molecule has recently been shown to be potentially involved in bone mechanotransduction [18,19] and links to all signaling pathways that have been assigned a role in osteocyte mechanotransduction yet [16–18]. S1P is the phosphorylation product of the membrane lipid component sphingosine.

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The phosphorylation step is catalyzed by two sphingosine kinase (Sphk) isoforms, Sphk1 (localized at the membrane when activated) and Sphk2 (cytosolic). Sphk1 has been shown to be functionally antagonized by the S1P phosphohydrolase 1 (SPP1) [20]. The resulting rheostat plays a major role in resistance artery smooth muscle cell mechanotransduction [21,22].

The fraction of newly synthesized S1P, which is released to the intracellular compartment, acts as a second messenger and has been primarily linked to the IP3-independent release of Ca^{2+} from intracellular stores. The extracellular fraction signals through five distinct G-protein-coupled receptors ($S1P_{1–5}$) [23] that bind to a large variety of intracellular signaling pathways [24], some of which are involved in the intracellular release of Ca^{2+} (primarily phospholipase C) [15]. S1P's ability to either directly (intracellular S1P) or indirectly (extracellular S1P through GPCRs) modify intracellular Ca^{2+} led us to hypothesize about a possible role in osteocyte mechanotransduction where the mobilization of calcium from intracellular stores is believed to be a primary early signal in response to changes in mechanical load [25]. As an example, changes in the expression of specific genes [3] and the activity of p38 MAPK [16] by oscillatory fluid flow (OFF) at physiological shear stress levels (1 Pa) are entirely dependent on increases in osteocyte Ca^{2+} levels. However, the underlying mechanism of OFF-induced intracellular calcium response in osteocytes is not completely understood.

In further support of our hypothesis regarding the involvement of S1P in bone mechanotransduction, extracellular S1P activates signaling pathways (i.e., RhoaA, Rho kinase, Rac1 and PKC) in resistance artery smooth muscle cells exposed to pressure increases that are similar to those involved in loading-induced bone remodeling [17–19,25].

For the present study, we employed the murine long bone osteocyte Y4 (MLO-Y4) cell line, an immortalized cell line with a morphology and cell marker expression pattern similar to osteocytes [26]. To characterize the contribution of S1P signaling to established mechanosensitive responses in osteocytes, MLO-Y4 were either subjected to oscillatory fluid flow (OFF) at 1 Pa or no flow. Under both conditions, either exogenous S1P was added or components of the signaling pathway (i.e., Sphk1 or the $S1P_2$ receptor) were inhibited. The cellular responses determined comprised intracellular Ca^{2+} (immediate response) [25], prostaglandin E2 (PGE_2) release [27,28] and mRNA (COX-2 gene) expression (intermediate response) [28–30] and receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) and osteoprotegerin (OPG) mRNA expression (late-stage response) [9,31–33].

The present study shows that all these parameters induced by OFF were markedly affected by changes in cellular S1P levels, suggesting an important modulatory role for S1P in bone mechanotransduction.

Materials and methods

Cell culture

Murine long bone osteocyte Y4 (MLO-Y4) cells, kindly provided by Dr. Lynda Bonewald (University of Missouri—Kansas City, Kansas City, MO), were cultured on type I rat tail collagen (BD Laboratory)-coated 100-mm tissue culture dishes in α-MEM (GIBCO™) supplemented with 2.5% (v/v) fetal bovine serum (FBS) (Hyclone), 2.5% calf serum (CS) (Hyclone), as well as 1% penicillin and streptomycin (PS) (GIBCO™). Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator (Thermo Scientific). Cell subculture was performed when the cells reached 70% confluence. For calcium imaging experiments, cells were seeded on UV transparent quartz slides (76 mm × 26 mm × 1.6 mm) at 100,000 cells/slide 48 h prior to fluid flow exposure to ensure the 70–80% confluence at the time of experiment. For flow experiments for protein and mRNA quantification, MLO-Y4 cells were cultured on type I rat tail collagen coated glass slides (75 mm × 38 mm × 1 mm) at 150,000 cells/slide 48 h prior to fluid flow exposure to ensure the 70–80% confluence at the time of experiment.

Table 1
PCR primers.

Gene (species)	Primer sequence	Amplicon length (bp)
Sk1	F: 5′-GAACCATAACTCTGTGCCTTTGTCT-3′ R: 5′-AGCAATGGGGAGTGTCTTCTATATG-3′	244
Sk2	F: 5′-GCCCCGAGATGGTCTAGTCT-3′ R: 5′-GTGGGTAGGTGTAGATGCAGA-3′	107
SPP1	F: 5′-GGGTGCTGGTCATGTACCTG-3′ R: 5′-CCCGTAGATAAGAGGATACTGCC-3′	203
S1PR1	F: 5′-CTGGAAACGTCAATTCTTCTTCCTA-3′ R: 5′-TGAGAGATCACAACACTTCCTCTTG-3′	395
S1PR2	F: 5′-GCAGTGACAAAAGCTGCCGAATGCTGATG-3′ R: 5′-AGATGGTGACCACGCAGAGCACGTAGTG-3′	170
S1PR3	F: 5′-TCAGTATCTTCACCGCCATT-3′ R: 5′-AATCACTACGGTCCGCAGAA-3′	137
18S (RT-PCR)	F: 5′-AGGAATTGACGGAAGGGCAC-3′ R: 5′-GGACATCTAAGGGCATCACA-3′	317
COX-2	F: 5′-AGAAGGAAATGGCTGCAGAA-3′ R: 5′-GCTCGGCTTCCAGTATTGAG-3′	194
OPG	F: 5′-TGTCACCCTGTGTGAAGAGG-3′ R: 5′-CTCTCGGCATTCACTTTGGT-3′	114
RANKL	F: 5′-CTGGTCGGGCAATTCT-3′ R: 5′-CCCAAAGTACGTCGCAT-3′	139
18S (qPCR)	F: 5′-GAGAAACGGCTACCACATCC-3′ R: 5′-CCTCCAATGGATCCTCGTTA3′	158

Expression of S1P signaling components in MLO-Y4 cells

After the MLO-Y4 cells reached 70–80% confluence in the culture dish, total RNA was extracted and purified using TRIzol (GIBCO™) reagent for cDNA synthesis. Specific primer pairs for sphingosine kinase 1 and 2 (Sk1/Sk2), S1P phosphohydrolase 1 (SPP1), and S1P receptors 1, 2 and 3 ($S1P_1$/$S1P_2$/$S1P_3$) were designed using published gene sequences (PubMed, NCBI Entrez Nucleotide Database) (Table 1). SuperScript™ III RT (Invitrogen, USA) was used for reverse transcription steps. Traditional PCR was run using the primers mentioned, and gel electrophoresis was run to observe if band of desired molecular weight was found.

Oscillatory fluid flow

A previously described fluid flow device was used to deliver laminar oscillatory fluid flow to MLO-Y4 cells [14]. In brief, the laminar oscillatory fluid flow was driven by an electro-mechanical loading device (Mechanical & Industrial Engineering, University of Toronto) mounted with a Hamilton glass syringe in series with rigid walled tubing and a parallel plate flow chamber as previously described [14]. During the calcium imaging experiments, MLO-Y4 cells on UV transparent quartz slides were mounted on the microscope parallel plate flow chamber (chamber size: 38 mm × 10 mm × 0.254 mm) and exposed to a total of 3 min of oscillating fluid flow (OFF) with peak sinusoidal wall shear stress of 1.0 Pa at 1 Hz. For PGE_2 and bone marker gene experiments, MLO-Y4 cells on collagen-coated glass slides were mounted in the large parallel plate flow chambers (chamber size: 75 mm × 34 mm × 0.28 mm) and subjected to the same level of oscillatory fluid flow for 2 h at 37 °C and 5% CO_2. Control slides were incubated in the parallel flow chamber, and were not subjected to oscillatory fluid flow (static cells). Fresh culture media (flow media) and culture media supplemented with 100 nM S1P (S1P media) were used in both PGE_2 and mRNA experiment. This S1P concentration is decided according to physiological level of S1P in the serum in mammals (up to 400 nM) and previous studies [34].

Modification of S1P components

In order to suppress the sphingosine kinase activity, MLO-Y4 cells were treated with 3 μM sphingosine kinase inhibitor N, N-

dimethylsphingosine (DMS) (Cayman Chemical, USA) 1 h prior the flow experiment [35]. JTE-013 (Cayman Chemical, USA), a selective $S1P_2$ receptor antagonist that binds to human and rat receptors (10 μM, 10 min prior to OFF), was used to block $S1P_2$ receptor in MLO-Y4 cells [36]. (The concentrations of the inhibitors were chosen according to previous dose-dependent studies [35,36] and manufacturer's instruction.)

Calcium imaging

Real-time intracellular calcium levels were quantified as described previously [37]. Prior to exposure to OFF, MLO-Y4 cells on quartz slides were incubated with 10 μM Fura-2 AM (Molecular Probes, Eugene OR) for 30 min at 31 °C (to reduce dye compartmentalization), then washed with fresh working media (α-MEM without phenol-red (GIBCO™) supplemented with 1% FBS and 1% CS). Following Fura-2 AM loading, each quartz slide was mounted in a microscope parallel plate flow chamber and fixed to a pre-heated stage (37 °C) of a fluorescent microscope (Eclipse Ti-S, Nikon, Japan). For 30 min, the cells were left undisturbed. Fresh working media were used as flow media during the experiment. Calcium images were taken continuously employing a calcium imaging system (PTI, USA), and Fura-2 340 nm/380 nm ratio values were converted to $[Ca^{2+}]_i$ values using image analysis software (EasyRatioPro, PTI). Oscillatory fluid flow (1 Pa, 1Hz) was applied for 3 min, 3 min after the imaging start point (baseline period). Temporal profiles were determined for approximately 40 cells per field. A cell response was defined as a transient increase in the $[Ca^{2+}]_i$ of at least 4 times the maximum oscillation recorded during the 3 min baseline period.

PGE_2 release

MLO-Y4 cells seeded on type I rat tail collagen coated glass slides were serum-starved with α-MEM supplemented with 0.05% fetal bovine serum and 0.05% calf serum, 12 h prior to flow experiment. During the flow experiment, glass slides with serum-starved MLO-Y4 cells were mounted in large parallel flow chambers and subjected to oscillatory fluid flow with peak sinusoidal wall shear stress of 1.0 Pa at 1 Hz for 2 h. MLO-Y4 cells in parallel flow chambers without OFF treatment were defined as no flow control. Fresh culture media were used as flow media during the experiment. Immediately following OFF exposure, all the conditioned media from the flow system were collected and centrifuged at 12,000 *g*. Supernatant PGE_2 levels were measured using Prostaglandin E_2 EIA Kit (sensitivity: 50% B/B_0: 50 pg/ml; 80% B/B_0: 15 pg/ml) (Cayman Chemical, USA). PGE_2 levels of each experimental group

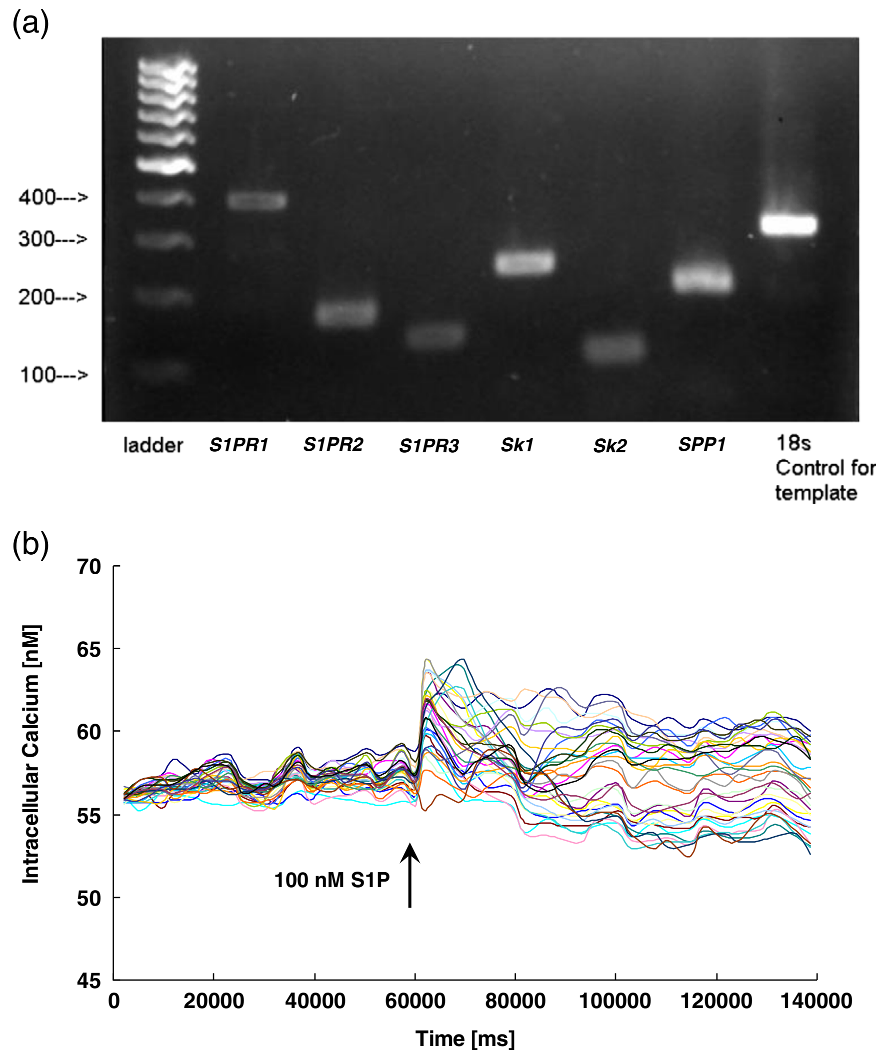

Fig. 1. Sphingosine-1-phosphate signaling components in MLO-Y4 cells. (a) Expression of S1P signaling components in MLO-Y4 cells. All components were expressed, and bands corresponded to expected molecular weights. (b) 100 nM S1P induced intracellular calcium mobilization in MLO-Y4 cells (as arrow indicated).

were normalized to total cell number on each slide and then to the average level in no flow control group.

mRNA quantification

Immediately following the flow experiment, glass slides with MLO-Y4 cells were transferred from flow chambers to 100-mm tissue culture dishes. Cells were trypsinized, and total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, USA). Extracted RNA was treated with DNase I (Fermentas), and was used for cDNA synthesis by SuperScript™ III RT (Invitrogen, USA). The cDNA samples were subjected to quantitative PCR (qPCR) using gene-specific primers and SYBR Green I in LightCycler 480 (Roche, USA). Specific primer pairs for COX-2, OPG, RANKL and 18S were designed using published gene sequences (PubMed, NCBI Entrez Nucleotide Database) (Table 1). mRNA levels of each gene of interest for experimental groups were normalized to 18S (housekeeping gene) rRNA levels and to a no flow control group.

Statistical analysis

Student's *t*-test was used for two sample comparisons. ANOVA were used to compare observations from more than two groups, followed by Tukey post-hoc test. Confidence level of 95% ($p < 0.05$) was considered statistically significant for all statistical analyses. Data were reported as mean value ± standard deviation (SD).

Results

Expression of S1P signaling components in MLO-Y4 cells

RT-PCR experiments detected the expression of sphingosine kinases (*Sk1* and *Sk2*), S1P phosphohydrolase 1 (*SPP1*), and S1P receptors $S1P_1$, $S1P_2$, and $S1P_3$ (*S1PR1*, *S1PR2* and *S1PR3*, respectively) in MLO-Y4 osteocyte-like cells (Fig. 1(a)), demonstrating that MLO-Y4 cells express all the components of the integrated S1P signaling pathway. In addition, a transient increase of intracellular calcium concentration ($[Ca^{2+}]_i$) was induced in MLO-Y4 cells treated with 100 nM S1P (Cayman Chemical) (Fig. 1(b)), implicating a functional S1P cascade MLO-Y4 cells.

Intracellular calcium mobilization in MLO-Y4 cells exposed to OFF

MLO-Y4 cells exposed to oscillatory fluid flow typically exhibit one rapid initial response, and some of the cells showed such initial response followed by multiple oscillations (the initial response was the largest in magnitude) (Fig. 2(a)). Specifically, 48% of MLO-Y4 cells showed immediate calcium response to the flow, and 28% of MLO-Y4 cells showed multiple responses after the initial response during the 3 min flow period (Fig. 3). MLO-Y4 cells treated with 3 μM sphingosine kinase inhibitor N, N-dimethylsphingosine (DMS) were also exposed to the same level of oscillatory fluid flow. The DMS treated cells exhibited similar intracellular calcium response compared with normal MLO-Y4 cells (Fig. 2(b)). However, only 28% of cells showed rapid calcium increase in response to flow, and 15% of cells showed the initial calcium response followed by multiple oscillations ($p < 0.01$ OFF vs. DMS + OFF) (Fig. 3).

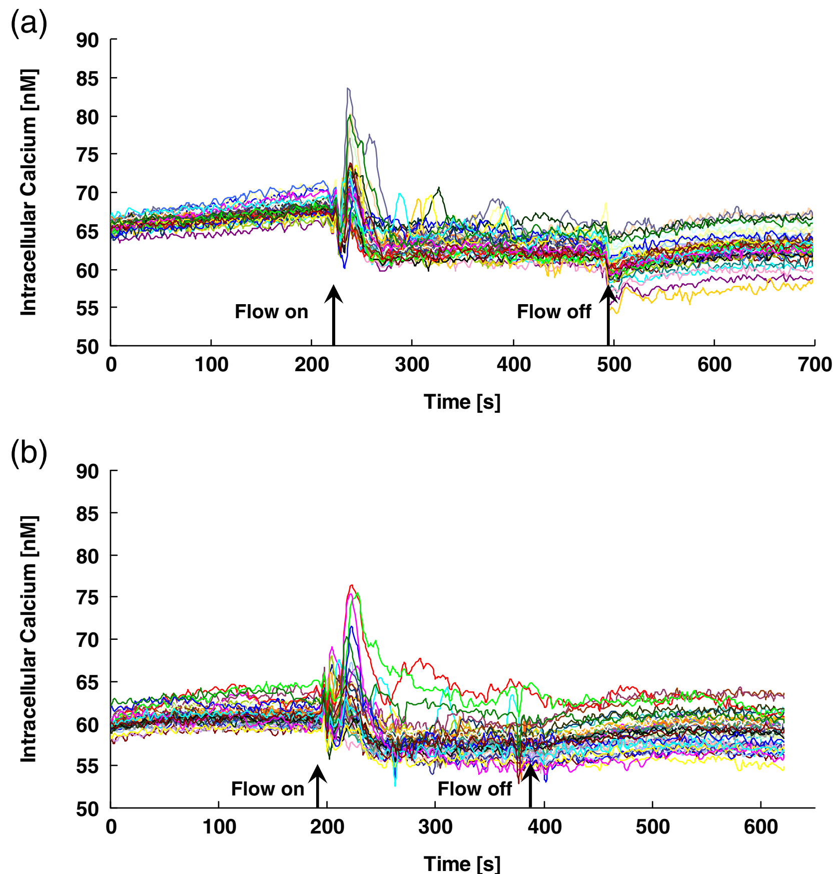

Fig. 2. Representative temporal profile of intracellular calcium concentration in (a) MLO-Y4 cells and (b) DMS treated MLO-Y4 cells exposed to OFF at 1 Pa, 1 Hz (3 min pre-flow period, 3 min flow period). Arrows indicate when flow was initiated. MLO-Y4 cells (a) typically exhibited one rapid initial response followed by multiple oscillations to OFF, and the initial response was the largest in magnitude. MLO-Y4 cells treated with DMS (3 μM, 1 h prior to OFF) (b) showed the initial response to OFF with similar magnitude compared with un-treated cells.

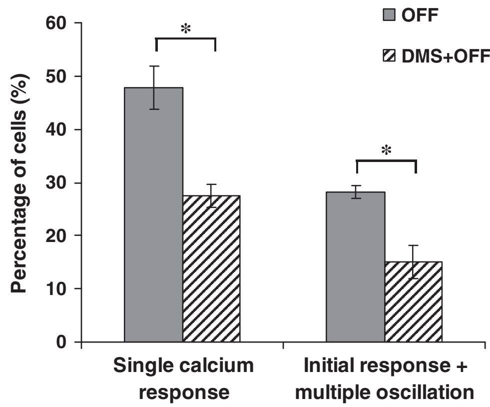

Fig. 3. Percentage of cells showing calcium response and percentage of cells showing multiple responses to OFF (1 Pa, 1 Hz); in MLO-Y4 cells and DMS treated MLO-Y4 cells, respectively. DMS treatment significantly decreased intracellular calcium mobilization in MLO-Y4 cells in response to OFF. At least 40 individual cells were analyzed in each sample, n = 4 for all groups. $^{*}p < 0.05$.

S1P in OFF regulated PGE_2 release and synthesis in MLO-Y4 cells

Both exogenous S1P treatment and OFF showed a stimulatory effect on PGE_2 release levels in MLO-Y4 cells (Fig. 4(a)). Static group of MLO-Y4 cells that were placed in parallel flow chambers, which were filled with 100 nM S1P supplemented flow media (S1P media), showed a 1.7-fold PGE_2 release compared with no flow controls ($p < 0.05$). 2 h OFF (1 Pa, 1 Hz) resulted in a 4.2-fold PGE_2 release in MLO-Y4 cells compared to no flow controls ($p < 0.05$). The OFF-induced increase in PGE_2 release was significantly reduced in DMS (3 μM, 1 h prior to OFF) treated MLO-Y4 cells (2.7-fold increase compared with no flow control, $p < 0.05$ OFF vs. DMS + OFF) (Fig. 4(b)) In addition, MLO-Y4 exposed to OFF using S1P supplemented media showed significant increases in PGE_2 release compared with OFF-loaded cells with normal flow media (5-fold increase compared with no flow control, $p < 0.05$ OFF vs. OFF + S1P) (Fig. 4(c)). The total effect of exogenous S1P and OFF on PGE_2 release was significantly decreased by JTE-013 (Cayman Chemical, USA), a selective $S1P_2$ receptor antagonist that binds to human and rat receptors (10 μM, 10 min prior to OFF) ($p < 0.01$ OFF + S1P vs. JTE + OFF + S1P) (Fig. 4(d)). However, JTE-treated MLO-Y4 still showed increased PGE_2 release level when exposed to OFF (1.6-fold increase compared with no flow control, $p < 0.05$).

To investigate whether S1P also plays a role in PGE_2 synthesis, we examined COX-2 expression in MLO-Y4 cells following similar manipulations in PGE_2 release analysis. Exogenous S1P (100 nM) induced significant increase in COX-2 mRNA level in static cells ($p < 0.05$). 2 h OFF (1 Pa, 1 Hz) resulted in an approximately 4-fold increase in COX-2 mRNA level in MLO-Y4 cells compared to no flow controls ($p < 0.05$) (Fig. 4(a)). OFF-induced increase in COX-2 mRNA level was slightly decreased by suppressing the sphingosine kinase activity (DMS treatment) ($p < 0.05$ DMS + OFF vs. no flow control) (Fig. 4(b)); on the other hand, 100 nM S1P in the flow media slightly enhanced the OFF-increased COX-2 mRNA level ($p < 0.05$ OFF + S1P vs. no flow control) (Fig. 4(c)). However, these changes due to altered exogenous S1P level are not statistically significant compared with OFF-induced changes in COX-2 mRNA expression. In comparison, the increased COX-2 expression in MLO-Y4 cells by OFF and exogenous S1P was significantly suppressed via blockage of S1P receptor $S1P_2$ ($p < 0.05$ JTE + S1P + OFF vs. OFF + S1P) (Fig. 4(d)).

S1P in OFF-regulated RANKL/OPG ratio at mRNA level in MLO-Y4 cells

The RANKL/OPG ratio at the mRNA level is an important indicator to evaluate the anti-osteoclastogenic response to mechanical loading [31]. The data in this study showed that OFF decreased the RANKL/OPG ratio

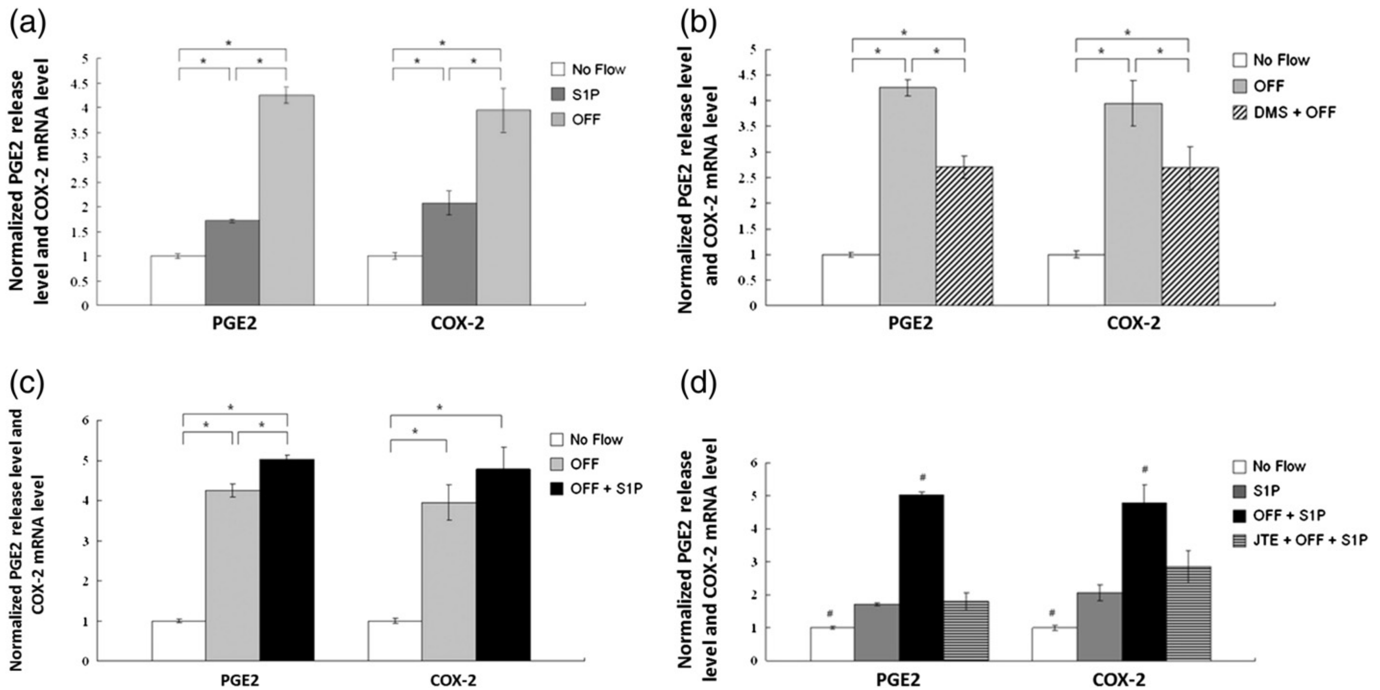

Fig. 4. Normalized PGE_2 release level and relative mRNA level of COX-2 in MLO-Y4 cells subjected to OFF (1 Pa, 1 Hz, 2 h). (a) MLO-Y4 cells in parallel flow chambers without OFF treatment were defined as no flow control. MLO-Y4 cells sitting in parallel flow chambers with 100 nM S1P supplemented flow media (S1P media), which were not exposed to OFF, were defined as S1P group. $^{*}p < 0.05$. (b) DMS (3 μM, 1 h prior to OFF) treated MLO-Y4 cells, which were subjected to flow media were defined as DMS + OFF group. $^{*}p < 0.05$. (c) OFF treated MLO-Y4 cells, which were exposed to S1P media were defined as OFF + S1P group. $^{*}p < 0.05$. (d) JTE-013 (10 μM, 10 min prior to OFF) treated MLO-Y4 cells, which were exposed to S1P media were defined as JTE + OFF + S1P group. $^{*}p < 0.05$, $\#p < 0.05$ vs. all groups. PGE_2 release levels were normalized to total cell amount per slide and no flow control; mRNA levels of COX-2 were normalized to 18s rRNA and no flow control. n = 4 for all groups.

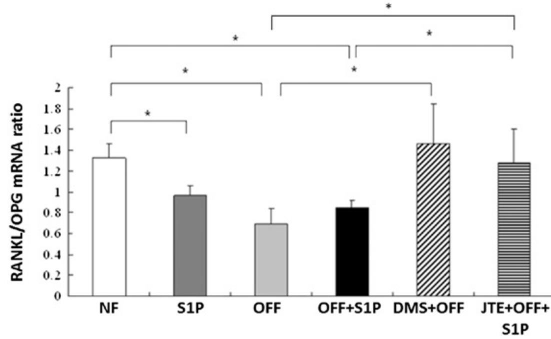

Fig. 5. Relative RANKL/OPG ratio at mRNA level in MLO-Y4 cells subjected to OFF (1 Pa, 1 Hz, 2 h). OFF treated MLO-Y4 cells were defined as OFF group, OFF treated MLO-Y4 cells, which were exposed to S1P media, were defined as OFF + S1P group, JTE-013 (10 μM, 10 min prior to OFF) treated MLO-Y4 cells, which were exposed to S1P media, were defined as JTE + OFF + S1P group. *$p < 0.05$ vs. No Flow, #$p < 0.05$ vs. JTE + OFF + S1P. mRNA levels were normalized to 18s rRNA and no flow control, n = 4 for all groups.

at the mRNA level in MLO-Y4 cells by about 50% ($p < 0.05$, OFF vs. No Flow). As well, exogenous S1P decreased the RANKL/OPG ratio (17% decrease, $p < 0.05$ OFF + S1P vs. No Flow) (Fig. 5). Although exogenous S1P alone did not affect the OFF-induced decrease in the RANKL/OPG ratio, DMS eliminated the decreasing effect of OFF. The combined effect of OFF and S1P was abolished by inhibition of $S1P_2$.

Discussion

The present study characterizes the phospholipid mediator sphingosine-1-phosphate (S1P) as an important mediator of mechanosensitive responses in osteocytes. Different signaling pathways serve to link S1P to the temporally separate levels of the cellular response to oscillatory fluid flow (OFF), which regulates the mechanical loading-induced bone remodeling process [9,10,28,38,39] to ultimately reduce bone resorption [1,40,41].

Specifically, S1P modulates the OFF-induced Ca^{2+} increase in MLO-Y4 cells (Fig. 3), promotes PGE_2 production and release (Fig. 4) and reduces the RANKL/OPG mRNA ratio (Fig. 5). The effects of exogenously added S1P on PGE_2 and the RANKL/OPG mRNA ratio appeared to be mediated by the $S1P_2$ receptor (Figs. 4(d) and 5). Our observations nicely tie into growing evidence of S1P being an important mediator of several mechanosensitive processes [20–22], bone remodeling [17,25,42] and, now, osteocyte mechanotransduction. We have chosen to focus on $S1P_2$, since it activates Rho-Akt [43] and, subsequently, canonical Wnt which has been shown to be one of major mechanotransduction pathways in osteocytes [3].

Although S1P signaling components appear to be ubiquitously expressed in all eukaryotic cells [44], the S1P signaling pathway has not previously been demonstrated in osteocytes and, specifically, the MLO-Y4 osteocyte-like cell line. We here first validated the presence of S1P signaling components in osteocytes, which is a prerequisite for S1P signaling components involvement in osteocyte mechanotransduction. Specifically, we have shown the existence of the primary S1P signaling pathway elements in terms of mRNA expression (i.e., sphingosine kinase 1, S1P phosphohydrolase 1 (SPP1) and the S1P receptors $S1P_1$, $S1P_2$ and $S1P_3$) in MLO-Y4 cells (Fig. 1(a)).

While osteocytes can potentially be the source of extracellular S1P in osteocytes immediate extracellular environment, no observation on this has been reported. Interestingly, osteoclasts have been shown to secrete S1P, which can induce activation of Wnt/BMP signaling in osteoblasts and osteoprogenitors [45]. We speculate that this osteoclast-secreted S1P can be transported to the extracellular environment of osteocytes by diffusion and/or convection. The mechanism of release of S1P is not yet fully understood. Since intracellular generated S1P possesses a polar head group, its transport through hydrophobic cell membranes has to be facilitated. Evidence from other cell type investigations have suggested that ATP-binding cassette (ABC) family of transporters are involved in this S1P facilitated release mechanism [44]. Nevertheless, it is not known whether this is also the mechanism of release of S1P by osteoclasts and/or osteocytes.

OFF and a moderate concentration of exogenous S1P trigger rapid and transient increases in intracellular Ca^{2+} [46–51] in MLO-Y4 cells (Figs. 1(b) and 2). This led us to hypothesize that the osteocyte Ca^{2+} response to OFF, which involves several mechanosensitive ion channels [49–51] and depends on gap junctional communication [11,17,38], might also be mediated by endogenously produced S1P. In this regard, inhibition of endogenous S1P production using di-methylsphingosine (DMS) failed to affect the amplitude of the Ca^{2+} increase, opposing the idea that S1P directly modulates the intracellular increase in Ca^{2+} (i.e., release from intracellular stores and/or transmembrane influx of Ca^{2+}). However, the number of MLO-Y4 cells responding to OFF (Fig. 3) was significantly reduced when sphingosine kinases were inhibited and hence, intra- and extracellular S1P levels were reduced. Our findings suggest that S1P coordinates the responses of several MLO-Y4 cells to OFF, presumably via routes of paracrine stimulation and/or intercellular communication [17,38,49,52]. Both mechanisms would be attenuated under the conditions of reduced endogenous S1P production. Of note, such a scenario would assume that not all cells are capable of responding to OFF with an enhanced production of S1P and that some cells would have a "pacemaker-like" function being essential for the response of the total MLO-Y4 cell layer.

Due to the small increase in the magnitude of Ca^{2+} response we observed in all flow/S1P treated experiments, we have measured the calcium response in each experimental set up repeatedly, and we are, therefore, confident in their statistical significance. The low calcium responses we observed in this study are within an order of magnitude of typical intracellular calcium response in osteocytes in response to fluid flow induced shear stress [13,53]. The reason for the lower magnitude of Ca^{2+} could be due to the application of the inhibitor DMS prior to the shear stress application since in cardiac tissue, $S1P_2$ could alter Rho A activation and have an impact in subsequent Ca^{2+} response [54]. And in cardiomyocytes Rho A affects calcium signaling, as well as ERK and AKT which are parts of the mechanotransduction pathway [55].

PKC plays an important part in osteocyte response to OFF [56]. Kim et al. showed that DMS inhibits PKC activity [57]. The DMS concentration we have used (3 μM) is low enough such that PKC activity is still around 40%–50% according to the study by Kim et al. [57]. Interestingly, there is contention in whether DMS actually affect PKC as shown by Edsall et al. [58], which had shown that DMS had no effect on PKC activity or translocation. The difference between these two studies may result from the different cell models used in these studies. The study by Edsall et al. [58] used 3T3 cell which is more closely related to osteocytes in terms of lineage. Therefore, it is unclear yet whether S1P acts via PKC to affect osteocyte calcium, PGE_2, and gene expressions in response to OFF. Based on the findings in this study and the above mentioned studies on PKC pathway involvement in S1P and OFF induced responses, we speculate that the effect of S1P in osteocyte mechanotransduction may be PKC dependent.

PGE_2 is an important signaling molecule in bone remodeling in that it has been shown to increase bone mass and activate bone remodeling [27], and increase osteogenic stromal cells [35]. The synthesis and release of PGE_2 by mechanically activated osteocytes are more intermediate responses, which, according to our data, are also subjected to the regulation by S1P. Exogenous S1P significantly increased the amount of PGE_2 released to the superfusate of MLO-Y4 cells, albeit to a lesser extent than OFF (Fig. 4(a)). Inhibition of endogenous S1P production reduced the amount of PGE_2 being released (Fig. 4(b)) by OFF by over 25%, indicating that endogenous release of S1P is partly involved in the up-regulation of PGE_2 release in mechanically stimulated osteocyte. Also, the correlated changes in COX-2 suggest that the increase in PGE_2

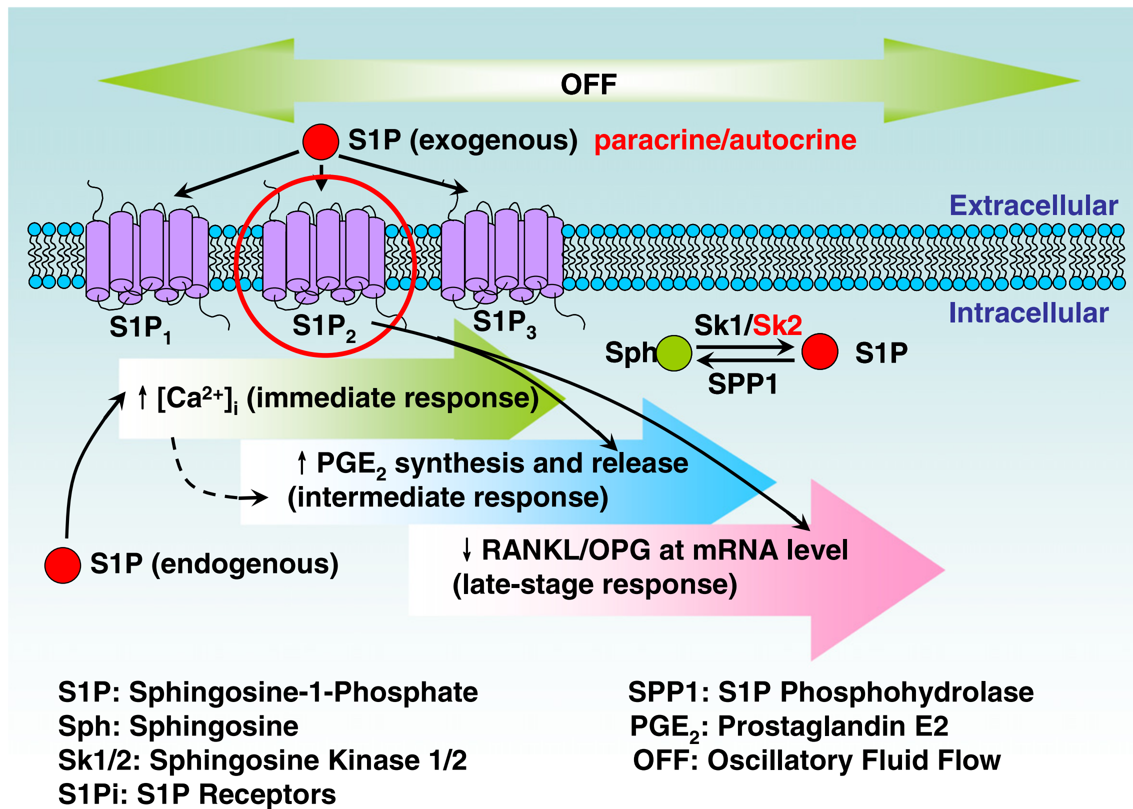

Fig. 6. The speculated involvement of S1P signaling in osteocytic mechanotransduction.

release incorporates the production of new PGE_2 instead of just induced release from existing intracellular PGE_2 store.

When OFF and exogenous S1P are applied together, PGE_2 release in response to the combination of both stimuli was higher than OFF or S1P alone, suggesting that the effect is additive in nature. However, the inhibition of the $S1P_2$ receptor reduced PGE_2 release to the level of applying only S1P, exceeding the effect seen with inhibition of endogenous S1P production (DMS treatment). Accordingly, JTE-013 greatly inhibited the increase in COX-2 mRNA following OFF. The amplitude of the effect was larger than the effect of exogenous S1P on COX-2 mRNA levels. This could either mean (i) that OFF strongly promotes the endogenous production of S1P, leading to local concentrations higher than 100 nM which we applied or (ii) that JTE-013 has unspecific effects in this experimental model that significantly affect COX-2 mRNA levels. From these results, $S1P_2$ is implicated in PGE_2 production through its regulation of COX-2 level in mechanically stimulated osteocytes.

The modulating effect of exogenous S1P on OFF-induced PGE_2 release in osteocytes, which acts partially through the $S1P_2$ receptor, might also overlap with the effect of PGE_2. As previously demonstrated in bone cells including osteocytes, PGE_2 not only elevates the sphingosine kinase activities [35], but also acts as a positive feedback stimulus via EP_2 receptors (autocrine/paracrine) on OFF-induced PGE_2 release [28,32]. These observations lead us to speculate that the modulating effect of S1P on OFF-induced PGE_2 release and synthesis could also be reciprocally amplified by altered PGE_2 level.

Finally, S1P also affected the RANKL/OPG mRNA ratio as an important late-stage anti-osteoclastogenic cellular response in OFF challenged osteocytes. The RANKL/OPG ratio was significantly reduced by the combined treatment with S1P and OFF; this effect was abolished following inhibition of endogenous S1P production with DMS and inhibition of $S1P_2$ receptor with JTE-013 (Fig. 5). Therefore our results point towards an important role of $S1P_2$ receptor-mediated signaling for the regulation of osteoclastogenesis, the survival of pre-existing osteoclasts, and consequently, the modulation of bone resorption [9,31–33]. Previous reports had suggested a role for mitogen-activated protein kinases (MAPK) in the down-regulation of the RANKL/OPG ratio [59]. However, our own preliminary data does not support this idea since they failed to show any activation (phosphorylation) of p42/p44 MAPK in MLO-Y4 cells in response to exogenous S1P or OFF (data not shown). We have chosen to determine activation of this particular kinase since it is known to be intimately linked to S1P signaling, located up- [60,61] and downstream [62,63] of Sk1.

In summary, our study suggests that sphingosine-1-phosphate (S1P) signaling modulates several steps of the osteocyte cellular responses to continuous oscillatory fluid flow (Fig. 6). This novel finding does not only highlight the S1P molecule as a potentially important player in osteocyte mechanotransduction and hence, the adaptation of the bone to mechanical load. It also introduces a new therapeutic target to the field and allows the utilization of already advanced strategies aiming to modify S1P signaling in other organs (i.e., the immune and cardiovascular system [20–22,47,49]).

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