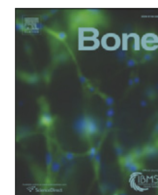
Bone 79 (2015) 71–78

Contents lists available at ScienceDirect

Bone

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



Original Full Length Article

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

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ARTICLE INFO

Article history:
Received 19 November 2014
Revised 16 April 2015
Accepted 12 May 2015
Available online 16 May 2015

Edited by: Mark Johnson

Keywords:
Osteocyte
Mechanotransduction
Sphingsosine-1-phosphate
Calcium
PGE_2
RANKL/OPG ratio

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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http://dx.doi.org/10.1016/j.bone.2015.05.017
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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-

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