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Increased sphingosine-1-phosphate production in response to osteocyte mechanotransduction

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ABSTRACT

Over the past few years interest has greatly increased in how the lipid mediator sphingosine-1-phosphate (S1P) influences bone homeostasis. Recent work has postulated multiple effects of S1P on osteoblasts and osteoclasts. Based on these findings, S1P has been proposed as a potential osteoporosis treatment. However, to date, there has been only a single study investigating S1P signalling in the cells that co-ordinate bone metabolism: osteocytes. This study aimed to elucidate the role of S1P signalling in osteocyte mechanotransduction.

Utilising 3D cell culture we established the expression profile of all genes related to the S1P signalling system in the Ocy454 osteocyte cell line. Exposure to mechanical loading resulted in a downregulation in *Sost*, *Spns2*, the S1P transporter, *Sgpl1* and *Sgppl1* the enzymes responsible for degradation and dephosphorylation of S1P. These findings, in conjunction with fluid-flow induced upregulation of *Sphk1*, the kinase responsible for phosphorylation of sphingosine, suggest that mechanical stimulation of osteocytes leads to an increase in intracellular S1P. This was confirmed with mechanical loading of Ocy454 cells rapidly increasing S1P production in conditioned media and protein lysates. These findings strongly suggest an important role for S1P in the response to mechanical loading of bone.

1. Introduction

Osteocytes form a cellular communication network throughout the bone matrix ideally suited for sensing the needs of the skeleton and responding to them. Indeed, targeted ablation of these cells demonstrated their central role in mechanosensing as this resulted in a diminished response of bone to mechanical unloading (Tatsumi et al., 2007). *If osteocytes orchestrate the adaptation of bone to mechanical loading the question arises: how is this biological action performed?*

Numerous studies have demonstrated that interstitial fluid flow (Piekarski and Munro, 1977; Weinbaum et al., 1994) stimulates osteocytic cell processes, leading to a cascade of intracellular events and production of local factors that regulate activities of osteoclasts and osteoblasts (Nakashima et al., 2011) to maintain adequate bone mass and architecture (Huiskes et al., 2000). However, stimuli of these signalling pathways remain to be determined. One significant candidate is sphingosine-1-phosphate (S1P) which has been shown to be potentially involved in bone mechanotransduction (Ishii et al., 2009; Karagiosis and Karin, 2007) with S1P receptor stimulation eliciting many of the same cell signalling responses that influence osteocyte mechanotransduction (Ishii et al., 2009; You et al., 2001; Alford et al., 2003).

Over the past few years interest has greatly increased in how S1P influences bone homeostasis. Early work postulated a role for S1P in bone remodelling as a coupling factor (a class of osteoclast-derived factors that stimulate osteoblast activity) (Ryu et al., 2006; Lotinun et al., 2013; Pederson et al., 2008). Subsequently multiple effects of S1P on osteoblasts and osteoclasts have been suggested (Ishii et al., 2009; Ryu et al., 2006; Lotinun et al., 2013). Based on these findings, S1P has been proposed as a potential osteoporosis treatment due to its anabolic effect on bone. Keller et al. proposed that increased bone formation seen in Calcr$^{-/-}$ mice was due to locally increased S1P concentrations resulting from increased Spns2-mediated transport (Keller et al., 2014). They also reported that S1PR3 null mice displayed an osteopenic (low bone mass) phenotype due to impaired bone formation, due to lack of osteoblast S1P responsiveness, further demonstrating a role for S1P in bone. Finally, a recent study (Zhang et al., 2015) utilising the MLO-Y4 osteocyte cell line suggested that treatment with S1P increased intracellular calcium and PGE2 release. Given the accepted central role for osteocyte control of bone formation, our focus on osteocyte-derived S1P is therefore all the more important.

This study aimed to elucidate the role of S1P signalling in osteocyte mechanotransduction. Here we report that exposure to mechanical loading significantly modifies osteocyte S1P signalling. These findings strongly suggest an important role for S1P production in the response to

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mechanical loading of bone.

2. Materials and methods

2.1. Materials

Phosphate buffered saline (PBS, Cat. No. 17-512F), Dulbecco's Modified Eagle Medium High Glucose (DMEM, Cat. No. 12-604F), Alpha modification of Eagle's medium (αMEM, Cat. No. 12-169F), and penicillin-streptomycin mixture (Cat. No. 17-602E) were obtained from Lonza (Mount Waverly, Australia). Fetal Bovine Serum was obtained from Bovogen (Keilor East, Australia). Trizol Reagent and TrypLE Express were purchased from Life Technologies (Thermo Fisher Scientific, MA, USA). IScript™ Reverse Transcription Super mix for RT-qPCR and iTaq™ Universal SYBR® Green Super mix were purchased from Bio-Rad (Gladesville, Australia). qRT-PCR primers purchased from Integrated DNA Technologies (Baulkham Hills, Australia).

2.2. Cell cultures

Ocy454 murine osteocytic cells were cultured as previously described (Wood et al., 2017). Briefly, for three dimensional cell cultures, 1.6×10^6 Ocy454 cells were plated on a 200 μm polystyrene Alvetex (Reinnervate) well insert scaffolds. Cells were allowed to grow at the permissive temperature (33 °C) for 2 days prior to transferring to (37 °C) for differentiation and fluid flow experiments.

2.2.1. 24 h fluid flow experiment

Ocy454 cells were differentiated for 14 days prior to transferring to the Reinnervate Perfusion Plate. The perfusion plates were attached to a Masterflex Peristaltic Pump (#7520-57) with a Masterflex Standard Pump Head (#7014-20) and were exposed to 2 dynes/cm^2 for 24 h prior to extraction for mRNA analysis. Conditioned media was collected for S1P ELISA.

2.2.2. Short-term fluid flow experiment

Ocy454 cells were differentiated for 14 days prior to transferring to the Reinnervate Perfusion Plate. The perfusion plates were attached to a Masterflex Peristaltic Pump (#7520-57) with a Masterflex Standard Pump Head (#7014-20) and were exposed to 5 dynes/cm^2 for 1 h. Trizol extraction was performed immediately or 3 h after fluid flow exposure for mRNA analysis.

2.3. S1P ELISA

S1P levels in conditioned media from either fluid flow stimulated Ocy454 cells or unstimulated control cells were analysed using a S1P competitive ELISA kit (Echelon Biosciences) according to the manufacturer's instructions. Cells were lysed in RIPA lysis buffer containing protease and phosphatase inhibitors and frozen at − 20 °C prior to analysis. Sensitivity of the assay was 30 nM. Either 20 μl of conditioned media or 50 μg protein of was added to the ELISA to assess S1P levels.

2.4. Real-time PCR (qPCR)

RNA was extracted from Ocy454 cells lysed in Trizol (Invitrogen, Australia), and cDNA was prepared using random hexamers (Promega, Australia) and Superscript III (Invitrogen, Australia) according to the manufacturer's protocol. Real-time RT-PCR was performed using the StratageneMX3000P (Agilent Technologies) as previously described (Wood et al., 2017; Gooi et al., 2014). Primers designed using Primer-BLAST are listed in Table 1. Primer sets were obtained from Integrated DNA Technologies (Coraville, IA). Post-run samples were analysed using Stratagene MxPro software and are expressed as linear ΔCT values normalized to Hypoxanthine Phosphoribosyltransferase 1 (HPRT1). The level of housekeeping gene did not vary significantly between treatment groups.

Table 1
Primer sequences for PCR analysis.

Primer	Forward sequence (5′ to 3′)	Reverse sequence (5′ to 3′)
SOST	GAGAACAACCAGACCATGAAC	GCTCGCGGCAGCTGTACT
SPHK1	TCCTGGAGGAGGCAGAGATA	GCTACACAGGGGTTTCTGGA
SPHK2	AAATCACCCCTGAATTGCTG	ATGCCTTCCCACTCACTCAG
SGPPL1	CCGCTGGCAGTATCCTCTTA	TTGTCAATCAGGTCCACCAA
SGPPL2	TGGCTGTGGTGTTCTCTACG	TGACACACACAGGGAAGAGG
SGPL1	TGAGCTTATCTTCCAGCCAGA	TACCCTGAGCAGGCAGAGTT
SPNS2	GGCATCTTCTTCTGGTCTGC	AGCATCAATGTGCGTGTGTT
S1PR1	ACCTAGCCCTCTCGGACCTATT	CCCAGACAACAGCAGGTTAGC
S1PR2	GCCATCGCCATCGAGAGA	TGTCACTGCCGTAGAGCTTGA
S1PR3	GTGTGTTCATTGCCTGTTGG	TTGACTAGACAGCCGCACAC
S1PR4	CAAGACCAGCCGTGTGTATG	AAGAGCACATAGCCCTTGGA
S1PR5	GCTGCTGAATCCCATCATCT	TAGAGCTGCGATCCAAGGTT
HPRT1	TGATTAGCGATGATGAACCAG	AGAGGGCCACAATGTGATG

2.5. Statistics

All experiments were performed a minimum of 3 times, using independent preparations of conditioned medium (n = 3 biological replicates). Data were analysed for statistical significance by Student's *t*-test or ANOVA as indicated in figure legends followed by Dunnett's multiple comparisons test, using Prism 6.0 (GraphPad). For all graphs, bars represent the mean/group and error bars indicate standard error of the mean (SEM).

3. Results

3.1. Expression of S1P signalling components in Ocy454 cells

RT-PCR gene expression analysis confirmed the presence of all S1P signalling components (S1P receptors, kinases, lyase and phosphatases) in the Ocy454 cell line (Fig. 1). Sphingosine-1-phosphate receptor 1 (*S1Pr1*) expression rose significantly at Day 3 and maintained a steady level of expression throughout differentiation and remained the most highly expressed receptor subtype. Sphingosine-1-phosphate receptor 2 (*S1Pr2*) showed steady, continual expression, 1.3 fold less than *S1Pr1*. Sphingosine-1-phosphate receptors 3–5 expression significantly increased throughout differentiation until Day 10 where they remained at a high level of expression. The expression of *S1Pr3*, *S1Pr4* and *S1Pr5* were 5, 4.2 and 17.8 fold lower than *S1Pr1* at peak levels respectively (Fig. 1A).

Sphingosine kinase 1 (*Sphk1*) and Sphingosine kinase 2 (*Sphk2*) expression was steady and high throughout differentiation. Expression of *Sphk2* was 1.1 fold higher than *Sphk1* at peak levels. Spinster homolog 2 (*Spns2*), expressed at significantly lower levels than both *Sphk1* and *Sphk2*, maintained a uniform level of expression throughout differentiation (Fig. 1B).

Sphingosine-1-phosphate lyase 1 (*Sgpl1*) expression was maintained at similar levels throughout differentiation. In contrast, S1P-specific phosphatase 1 (*Sgppl1*) showed significantly increased expression from Day 10 onwards. S1P-specific phosphatase 2 (*Sgppl2*) expression was an order of magnitude lower than *Sgpl1* and *Sgppl1*, 529 fold less than *Sgppl1*. Expression was significantly increased on Days 5,7 and 12 of differentiation compared to Day 0 (Fig. 1C).

3.2. Long-term fluid flow exposure modulates Sost, Sphk1, Sphk2 and Spns2 gene expression

Exposure of differentiated Ocy454 cells to 2 dynes/cm^2 of fluid flow for 24 h resulted in a significant downregulation of *Sost* mRNA expression (449 fold, $p = 0.0151$, Fig. 2A). *Sphk1* expression was significantly upregulated following exposure to fluid flow (2.8 fold,