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Integrative transcriptomic and proteomic analysis of osteocytic cells exposed to fluid flow reveals novel mechano-sensitive signaling pathways

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ABSTRACT

Osteocytes, positioned within bone's porous structure, are subject to interstitial fluid flow upon whole bone loading. Such fluid flow is widely theorized to be a mechanical signal transduced by osteocytes, initiating a poorly understood cascade of signaling events mediating bone adaptation to mechanical load. The objective of this study was to examine the time course of flow-induced changes in osteocyte gene transcript and protein levels using high-throughput approaches. Osteocyte-like MLO-Y4 cells were subjected to 2 h of oscillating fluid flow (1 Pa peak shear stress) and analyzed following 0, 2, 8, and 24 h post-flow incubation. Transcriptomic microarray analysis, followed by gene ontology pathway analysis, demonstrated fluid flow regulation of genes consistent with both known and unknown metabolic and inflammatory responses in bone. Additionally, two of the more highly up-regulated gene products – chemokines *Cxcl*1 and *Cxcl*2, supported by qPCR – have not previously been reported as responsive to fluid flow. Proteomic analysis demonstrated greatest up-regulation of the ATP-producing enzyme NDK, calcium-binding Calcyclin, and G protein-coupled receptor kinase 6. Finally, an integrative pathway analysis merging fold changes in transcript and protein levels predicted signaling nodes not directly detected at the sampled time points, including transcription factors c-Myc, c-Jun, and RelA/NF-κB. These results extend our knowledge of the osteocytic response to fluid flow, most notably up-regulation of *Cxcl*1 and *Cxcl*2 as possible paracrine agents for osteoblastic and osteoclastic recruitment. Moreover, these results demonstrate the utility of integrative, high-throughput approaches in place of a traditional candidate approach for identifying novel mechano-sensitive signaling molecules.

1. Introduction

Mounting evidence suggests that osteocytes, positioned within bone mineral's interstitial space, coordinate cellular remodeling leading to functional adaptation in response to mechanically-induced stimuli (Bonewald, 2011; Schaffler et al., 2014). Interstitial fluid flow is one such stimulus (Kufahl and Saha, 1990; Weinbaum et al., 1994) observed upon cyclic whole bone loading (Knothe Tate and Knothe, 2000; Price et al., 2011). Fluid flow exposes osteocytes to enhanced solute transport (Price et al., 2011), streaming potentials (Cowin et al., 1995), and cyclic fluid shear stress transduced via cellular adhesion molecules, the cell membrane, the actin cytoskeleton, and possibly primary cilium (Bonewald and Johnson, 2008).

In vitro, specific responses of osteocytic cells to steady, pulsating, and oscillating fluid flow (OFF) have been widely studied. However, these studies are generally constrained to a limited set of candidate genes or proteins as part of known or suspected signaling pathways. Osteocytic cells elastically deform when subject to physiological peak fluid shear stresses up to 5 Pa (Kwon and Jacobs, 2007; Price et al., 2011), initiating the rapid release of adenosine triphosphate (ATP) and prostaglandin E_2 via gap junctions or hemichannels (Batra et al., 2012; Cherian et al., 2005; Genetos et al., 2007; Klein-Nulend et al., 1995). In turn, these factors contribute to cell network propagation of intracellular calcium waves in proportion to flow-induced shear stress in vitro (Huo et al., 2008; Lu et al., 2012) as well as during in situ dynamic bone loading (Jing et al., 2013). Subsequent to OFF, osteocytic cells demonstrate stress amplitude-, frequency-, and duration-dependent shifts in mRNA levels with

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increased prostaglandin E_2-synthesizing *Ptgs*2 (*Cox*-2) and a decreased *Rankl*/*Opg* mRNA ratio associated with reduced recruitment of bone-resorbing osteoclasts (Kim et al., 2006; Li et al., 2012; Xiong and O'Brien, 2012). Fluid flow also regulates molecules involved with Wnt/β-catenin pathway activation (Kamel et al., 2010; Santos et al., 2009) and protects against osteocyte apoptosis (Cheung et al., 2011; Kitase et al., 2010).

Related studies have broadened our knowledge of skeletal mechano-sensing through transcriptomic (Mantila Roosa et al., 2011; McKenzie et al., 2011; Reijnders et al., 2013; Rolfe et al., 2014; Xing et al., 2005) or proteomic investigations (Li et al., 2011; Zhang and Wang, 2009) of heterogeneous cell populations from whole bones subject to in vivo mechanical loading. Others have analyzed global gene expression specifically in osteocytes isolated from loaded rat trabeculae (Wasserman et al., 2013) and osteocyte-like MLO-Y4 cells subjected to cyclic compressive force stimulation (Chen et al., 2010). However, no single study has taken an integrated transcriptomic and proteomic approach.

In this study we evaluated the utility of two unbiased high-throughput approaches, gene transcript microarrays and protein mass spectrometry, to investigate the response of osteocytes exposed to fluid flow. We mapped a time course of flow-induced fluctuations in both gene transcript levels and protein abundances at corresponding time points. Additionally, in spite of various post-transcriptional modifications, we computationally predicted sequences of critical signaling nodes using an integrative bioinformatics approach. We examined the hypothesis that this broadened inquiry will reveal a mechano-sensitive shift in gene transcript and protein abundances in response to fluid flow, reflecting regulation of known mechano-sensitive signaling pathways as well as novel signaling networks. Our results demonstrate both individual and global shifts in signaling molecules consistent with known regulation of bone metabolism. More importantly, we identified signaling molecules and pathways not previously implicated in mechanotransduction in bone, most notably, up-regulation of *Cxcl*1 and *Cxcl*2.

2. Methods

2.1. Cell culture

MLO-Y4 osteocyte-like cells (Kato et al., 1997), courtesy of Dr. Lynda Bonewald (University of Missouri—Kansas City) were maintained in normal growth medium (α-MEM [Invitrogen, Grand Island, NY] with 2.5% CS [Hyclone, Logan, UT], 2.5% FBS [Lonza, Walkersville, MD], 1% Penicillin/Streptomycin) throughout all portions of the experiment. Cells were seeded 48 h prior to fluid flow on $75 \times 38 \times 1$ mm glass slides coated with 300 μg/ml Type I Collagen (BD Biosciences, Bedford, MA) for 1 h and washed. Cell seeding density was 1.35×10^4 cells/cm^2 so that upon flow exposure, cells were roughly 60% confluent and interconnected by dendritic processes.

2.2. Fluid flow stimulation

Samples were subjected to 2 h of fully-reversed sinusoidal OFF with a peak shear stress of 1 Pa (10 dyne/cm^2) at a frequency of 1 Hz. As previously described (Haut Donahue et al., 2004; Jacobs et al., 1998), glass slides were assembled within parallel plate flow chambers in sterile conditions and placed within an incubator at 37 °C and 5% CO_2. A rigid-walled inlet tube connected to a Hamilton glass syringe was actuated by a servo pneumatic loading device (EnduraTec, Eden Prairie, MN). Paired controls were maintained in identical, static chambers. After two hours treatment, flowed and static cells were either collected immediately (0 h time point) or glass slides were transferred to culture dishes and incubated for 2, 8, or 24 h post-flow in 10 ml fresh medium. When collecting samples, medium was aspirated and cells were lysed directly on glass slides using RLT buffer (Qiagen, Valencia, CA). For each time point, triplicate samples were collected on separate days.

2.3. RNA and protein isolation

Total RNA was isolated using an Rneasy Mini Kit (Qiagen, Valencia, CA) per manufacturer instructions. Protein was collected from the same samples according to Qiagen's supplementary protocol for acetone precipitation using buffer RLT lysate flow-through from RNeasy spin columns. Four volumes of acetone were added to flow-through followed by 30 min incubation at −20 °C to induce protein precipitation. Samples were centrifuged at 20,000*g* for 10 min, supernatant discarded, pellet washed with ice-cold ethanol, dried and resuspended in 8 M urea.

2.4. DNA microarray analysis

Analysis of total RNA was carried out as in Waters et al. (2011). First, quality was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Biotin-labeled cRNA was synthesized and fragmented using Affymetrix 3′ IVT Express reagents for hybridization to Mouse Genome 430A 2.0 GeneChips (Affymetrix, Santa Clara, CA). After hybridization, the arrays were washed and stained with streptavidin–phycoerythrin, and scanned at a resolution of 2.5 μm using an Affymetrix GeneChip Scanner 3000. Quality control parameters were assessed throughout to assure maximum efficiency of transcription, integrity of hybridization, and consistency of qualitative calls. Synthesis and fragmentation of cRNA were assessed using the Agilent 2100 Bioanalyzer. Spike-in control transcripts were monitored to verify hybridization integrity. Raw data files were normalized using the Robust Multi-Array Analysis (Irizarry et al., 2003) and significantly regulated genes identified by one-way ANOVA (unequal variance) with Benjamini Hochberg false discovery rate (FDR) multiple testing correction (Dudoit et al., 2004) and Tukey HSD post-hoc statistics at $p < 0.05$ or $p < 0.1$ using GeneSpring GX 12.5 software. Positive fold-changes were calculated as flow/non-flow quantities while negative fold-changes were calculated as −1/(flow/non-flow). Raw microarray data files have been submitted to the Gene Expression Omnibus under accession number GSE42874.

2.5. Real-time RT-PCR

Complimentary DNA was synthesized from total RNA from original samples using the iScript reverse transcriptase kit (Bio-Rad, Hercules, CA). Quantitative PCR was then carried out in triplicate with the QuantiTect SYBR-Green PCR kit (Qiagen, Valencia, CA) and normalized to β-actin. The following murine-specific primers were used (5′ to 3′ direction): *Cxcl*1 (forward) GCTTGTTCAGTTTAAAGATGGTAGGC, (reverse) CGTGTTGACCATACAATATGAAAGACG; *Cxcl*2 (forward) ACAGAAGTCATAGCCACTCTC, (reverse) GCCTTGCCTTTGTTCAGTATC; β-actin (forward) AGATGTGGATCAGCAAGCAG, (reverse) GCGCAAGTTAGGTTTTGTCA. Relative expression levels were calculated as 2^(Ct[*βactin*]-Ct[*Cxcl*1,2]) (Zhang and Chen, 2000). Average values ($n=3$) within each time point were compared with an unpaired Student's *t*-test using Graphpad Prism 5 (La Jolla, CA).

2.6. Gene ontology (GO) analysis

Gene set enrichment for gene ontology biological process annotation was analyzed using Ingenuity Pathway Analysis (IPA 9.0, Ingenuity Systems, www.ingenuity.com) to identify the most significant cellular processes affected by flow. Canonical pathway analysis identified pathways from the IPA library that were most significant to the data set of gene transcripts demonstrating at least ±1.25 fold-change ($p < 0.1$). The significance of the association between the data set and the canonical pathway was measured in two ways: (1) a ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway was determined. (2) Fisher's exact test was used to calculate a *p*-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone. The Functional Analysis identified associated biological functions and/or diseases. A right-tailed Fisher's exact test was used to calculate a *p*-value determining the probability that each biological function/disease assigned to that data set is due to chance alone. A denominator representing number of molecules associated with each function is not reported as these databases are rapidly changing.

2.7. Proteomic analysis

Total protein was subjected to high mass accuracy liquid chromatography–mass spectrometry (LC–MS) to resolve, detect, and identify individual peptide peaks for protein quantification. Biological and technical replicates ($n=3$ and 2, respectively) of tryptically digested and isolated peptides from each sample were prepared as previously described (Brown et al., 2012) and subjected to LC–MS analysis using a LTQ-Orbitrap mass spectrometer (MS; Thermo Scientific, Waltham, MA, USA) and an electrospray ionization source manufactured in-house, as previously reported (Brown et al., 2012; Livesay et al., 2008; Xie et al., 2011). The accurate mass and time tag approach (AMT) was utilized to identify and quantify detected peptide