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Mechanotransduction in primary human osteoarthritic chondrocytes is mediated by metabolism of energy, lipids, and amino acids

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ABSTRACT

Chondrocytes are the sole cell type found in articular cartilage and are repeatedly subjected to mechanical loading *in vivo*. We hypothesized that physiological dynamic compression results in changes in energy metabolism to produce proteins for maintenance of the pericellular and extracellular matrices. The objective of this study was to develop an in-depth understanding for the short term (< 30 min) chondrocyte response to sub-injurious, physiological compression by analyzing metabolomic profiles for human chondrocytes harvested from femoral heads of osteoarthritic donors. Cell-seeded agarose constructs were randomly assigned to experimental groups, and dynamic compression was applied for 0, 15, or 30 min. Following dynamic compression, metabolites were extracted and detected by HPLC-MS. Untargeted analyzes examined changes in global metabolomics profiles and targeted analysis examined the expression of specific metabolites related to central energy metabolism. We identified hundreds of metabolites that were regulated by applied compression, and we report the detection of 16 molecules not found in existing metabolite databases. We observed patient-specific mechanotransduction with aging dependence. Targeted studies found a transient increase in the ratio of NADP+ to NADPH and an initial decrease in the ratio of GDP to GTP, suggesting a flux of energy into the TCA cycle. By characterizing metabolomics profiles of primary chondrocytes in response to applied dynamic compression, this study provides insight into how OA chondrocytes respond to mechanical load. These results are consistent with increases in glycolytic energy utilization by mechanically induced signaling, and add substantial new data to a complex picture of how chondrocytes transduce mechanical loads.

1. Introduction

Osteoarthritis (OA) is the most common joint disorder, affecting more than 40 million individuals in the United States (Cicuttini et al., 2011; Woolf et al., 2012). OA is characterized by deterioration of the protective, low-friction, load-bearing cartilage that surrounds the joint. The highly specialized chondrocyte plays an important metabolic role in synthesizing, maintaining and repairing the tissue and is the sole cell type in articular cartilage (Sophia Fox et al., 2009). At these joint surfaces (*e.g.* the femoral head), the articular chondrocyte is subjected to repeated mechanical loading (*e.g.* standing, walking, running, etc.). Chondrocytes and other mammalian cells sense and respond to these mechanical stimuli through biochemical and biological outputs, but the intracellular pathways behind chondrocyte mechanotransduction remain unclear (Haudenschild et al., 2008; Grygorczyk et al., 2013; Jaalouk and Lammerding, 2009).

Mechanical stimulation has both anabolic and catabolic effects on articular chondrocytes (Ruiz-Romero et al., 2008; Farnsworth et al., 2013). In chondrocytes, anabolic responses promote synthesis and production of extracellular matrix (ECM) and pericellular matrix (PCM) (Ruiz-Romero et al., 2008). Catabolic responses involve secretion of proteases (*e.g.* MMP-13) which results in the breakdown of ECM molecules. Dynamic loading has been shown to promote these anabolic responses in chondrocytes; whereas, static loading has been shown to inhibit them (Bougault et al., 2012; Buschmann et al., 1995). Dynamic compression has been shown to alter signal transduction including activation of GTPase signaling via the Rho-A and ROCK pathways, Erk-01 and -2, MAPK and SEK and Smad2 (Bougault et al., 2012; Haudenschild et al., 2010; De Croos et al., 2007; Fitzgerald et al., 2008).

In addition to signal transduction, chondrocytes can alter their energy metabolism in response to mechanical loading. The enzyme AKT is important in regulating FoxO signaling for energy

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homeostasis (Lehtinen et al., 2006), and cyclic loading has been shown to reduce phosphorylation of AKT in OA chondrocytes (Kawakita et al., 2012); whereas, mechanical stimulation induces AKT phosphorylation in healthy cells (Niehoff et al., 2008). These energy-related signaling responses may affect matrix synthesis because healthy chondrocytes show increases in sulfated glycosaminoglycans (sGAG) in response to mechanical loading in contrast to OA chondrocytes (Holledge et al., 2008). However, how loading might change chondrocyte metabolite levels, which can mark changes in biosynthetic activity, is unknown.

Previous research suggests that both inflammation and OA alter central energy metabolism, including the balance between glycolysis and oxidative phosphorylation (Nishida et al., 2013). One potential mechanism of energy-related mechanotransduction involves regulation of AMP-activated protein kinase which can prevent catabolism induced by mechanical injury (Petursson et al., 2013). Based on these and other data, we hypothesized that physiological dynamic compression increases chondrocyte glycolytic energy flux to promote the anabolic response to maintain the environment of the ECM and PCM. The objective of this study was to use both untargeted (global) and targeted metabolomics to identify candidate mediators of chondrocyte mechanotransduction in primary human OA chondrocytes. This study evaluates our hypothesis using primary human OA chondrocytes subjected to applied dynamic compression following encapsulation in physiologically stiff agarose.

Chondrocyte mechanotransduction happens on both short- and long- timescales. Because early responses have the potential to set the trajectory for longer-term behavior, in this study, we focus on short term (<30 min), loading-induced changes in small molecules (*i.e.* cellular metabolites smaller than $\sim$1000 Da). Building upon previous methodology to encapsulate chondrocytes in agarose similar in stiffness to the human PCM, we profiled the metabolomic responses of primary human OA chondrocytes in response to applied compression at physiological levels (Zignego et al., 2013; Jutila et al., 2014).

This study identified changes in 1421 untargeted metabolites and 48 metabolites pertinent to central energy metabolites and protein production. Metabolites were identified via database searches (METLIN, and HMDB), and 16 of the 1421 metabolites were not found in database searches, potentially representing novel mediators of chondrocyte mechanotransduction. Future research may build on these results to test mechanical loading as an OA therapy.

2. Materials and methods

2.1. Chondrocyte culture and encapsulation

Primary human chondrocytes were harvested from hip cartilage of five Grade IV OA patients undergoing joint replacement surgery (mean age: 63 years, age range: 54–80, mean weight: 80.4 kg, weight range: 56.09–99 kg). The cartilage was digested in Type IV collagenase (2 mg/mL for 12–14 h. at 37 °C), and then cultured in DMEM with 10% fetal bovine serum and antibiotics (10,000 I.U./mL penicillin and 10000 μg/mL streptomycin) in 5% atmospheric CO_2. Cells were encapsulated using previously optimized methods (Jutila et al., 2014) at a concentration of $\sim$500,000 cells/gel (gel diameter$=$7 mm, gel height$=$12.7 mm).

2.2. Mechanical stimulation

From each donor ($N=5$), cell-seeded agarose gels were randomly assigned to a loading group ($n=05$ biological replicates for each loading group). Loading groups consisted of unloaded controls (*i.e.* 0 min of loading), 15, or 30 min of dynamic, cyclic compression. A short loading timescale was used because initial early-time responses can set the trajectory for longer-term behavior. The loading protocol followed previously optimized methods (Jutila et al., 2014) in which homogeneous deformations (Zignego et al., 2013) were applied to the cell-seeded gels using a custom built bioreactor emulating physiological loading conditions: frequency of 1.1 Hz (Umberger and Martin, 2007) and average sinusoidal compressive strains of 5% with an amplitude of 1.9% based on initial gel height (Supplementary Fig. 1). The loading frequency was selected based on the preferred stride rate in humans (Umberger and Martin, 2007), and the applied strain profile was selected to be in the range of deformations measured in human patients using MRI (Sutter et al., 2015).

2.3. Metabolite extraction

Metabolite extraction was performed using identical methods from our previous study (Jutila et al., 2014). Gels were flash frozen, pulverized, and metabolites were extracted by adding 1 mL of a 70:30 Methanol:Acetone solution and vigorously vortexing the mixture every 5 min for 20 min. Samples were kept at -20 °C overnight for further metabolite extraction. Proteins were removed by centrifugation, the supernatant extracted, and the solvent removed via centrifugation under a vacuum for 6.5 h. The dried samples were then resuspended in 100 μL of mass spectrometry grade water and acetonitrile (50:50 v/v).

2.4. Untargeted and targeted metabolomic profiling

Metabolomics is an experimental technique for characterizing large numbers of small molecules (<1000 Da) in biological samples (Patti et al., 2012). Recent studies of joint tissues and fluids have used metabolomic analysis to examine OA phenotypes, identify candidate biomarkers and explore the inflammatory responses (Zhang et al., 2014; Mickiewicz et al., 2015; Attur et al., 2012; Mickiewicz et al., 2015). In this study, metabolites were extracted following dynamic compression and analyzed by nano-liquid chromatography and mass spectrometry (Supplementary methods, (Jutila et al., 2014). Untargeted metabolites were detected in positive mode on an Agilent 6538 Q-TOF spectrometer with a resolution of $\sim$20,000 and accuracy of $\sim$5 ppm. For the targeted approach, $\sim$50 metabolites known to be involved in central energy metabolism were analyzed using the Quantitative Analysis package within the Agilent MassHunter Workstation B.04.00 (Agilent Technologies) and a database of the calculated isotopic distributions (including H+ and Na+ adducts) of these targeted masses (Isotope Distribution Calculator, Agilent Technologies).

To assess the effects of physiological loading on chondrocyte biology, three experimental groups were used: unloaded control samples (UC) and samples that received either 15 (DL15) or 30 (DL30) minutes of dynamic compression. Principal components analysis (PCA) was utilized to assess metabolome-scale changes caused by mechanical loading. Pearson's correlation coefficients were used to estimate the flux of metabolite intensities over the timecourse of loading. To assess the differences in intensity distributions (m/z spectra plots for the various loading groups), two-sample Kolmogorov–Smirnov tests were implemented. Targeted metabolite profiles were analyzed by PCA, hierarchical agglomerative cluster analysis and correlation analyzes. Additionally, the median ratios of NADP+:NADPH, NAD+:NADH, ATP:ADP and GDP:GTP were calculated as a function of time to assess relative changes in energy metabolism.

2.5. Compound identification and enrichment analysis

To putatively identify compounds, a batch search of all of the untargeted metabolite mass to charge (m/z) values was performed in METLIN and HMDB. Both databases contain over 80,000 identifiable metabolites (Wishart et al., 2013; Zhu et al., 2013). Search parameters included using a mass tolerance of 20 ppm, and positively charged molecules with potential $+1H^+$ or $+1Na^+$ adducts. Compounds with LipidMAPS identifications (Fahy et al., 2009) were designated as human and not considered further if detected in a non-human species at the time of database search. Untargeted metabolites were then examined by unsupervised clustering to identify groups of co-regulated metabolites. Clusters of co-regulated metabolites were analyzed for pathway enrichment using IMPaLA (Cavill et al., 2011).

3. Results

The objective of this study was to characterize the cellular response to applied compression for primary human OA chondrocytes by examining changes in metabolomic profiles. Chondrocytes were harvested from donor joint tissue, grown in tissue culture, embedded in agarose with stiffness similar to the human PCM, and dynamically loaded in tissue culture. Samples were then flash frozen, pulverized, and the metabolites were extracted and analyzed using HPLC-MS. We analyzed untargeted metabolomics profiles to minimize bias from *a priori* selection of relevant pathways which inherently excludes potentially important data. To evaluate our hypothesis, we also analyzed metabolomics profiles