Real-time evaluation of chondrocyte glycolysis and oxidative phosphorylation

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INTRODUCTION: Cells derive ATP from two main aerobic processes: glycolysis, which occurs in the cytosol, and oxidative phosphorylation, which occurs in the mitochondria. While native cartilage has low metabolic activity and low oxygen concentration, mitochondrial function has recently been shown to play an important role in cartilage damage in response to mechanical loading (e.g., [1–3]). Reactive oxygen species (ROS), known to induce cell death when present in excess, are released by mitochondria in a strain-dependent manner [2,3]. Consistent overloading of cartilage leads to accumulation of ROS and eventual mitochondrial dysfunction, pointing to a likely role for metabolic dysregulation in the onset and progression of osteoarthritis [4].

Other methods have been established to evaluate chondrocyte metabolism. Synthesis of extracellular matrix proteins, enzymes, and growth factors and expression of associated genes have been evaluated via quantitative PCR, mass spectrometry, and RNA sequencing. These techniques have identified regulatory factors, age-dependent metabolic activity, and novel biomarkers of osteoarthritis [5–7]. Experimental metabolomic data has been combined with computational modeling to analyze and predict the metabolic response of chondrocytes to dynamic compression [8]. Effects of loading have also been evaluated by monitoring mitochondrial respiration, depolarization, and release of reactive oxygen species, all by commercially available kits [1,3,4]. Calcium signaling, a nonspecific indicator of metabolic activity, has been monitored in situ to gain further insight into the process of cartilage mechanotransduction [9].

Although insightful, previous methods of evaluating chondrocyte metabolism do not allow real-time, non-destructive evaluation of specific metabolic pathways. Conversely, imaging methods have been developed for use in other cell and tissue types that do allow real-time, non-destructive metabolic imaging [10–13]. Thus, the overall objective of this study was to apply an established method of evaluating cell metabolism to chondrocytes *ex vivo*. The two specific objectives were to demonstrate that metabolic imaging established in other cell sources would provide meaningful results in cartilage; and to evaluate the effect of a single mechanical load on chondrocyte metabolism.

METHODS: Porcine stifle cartilage was obtained immediately post-mortem. Cylindrical cartilage samples were obtained using a sterile 6 mm diameter biopsy punch and scalpel. Samples were cut in half to create hemicylinders for imaging and maintained at 37°C in chondrocyte culture media until imaging.

To observe glycolysis and oxidative phosphorylation, samples were imaged using an inverted epifluorescent microscope (Olympus IX-71) (Fig. 1). Autofluorescent NADH, a product of glycolysis, was captured using a DAPI filter cube (excitation 361-389 nm, emission 435-485 nm). Autofluorescent FAD, an early product of oxidative phosphorylation, was captured using a green filter cube (excitation 470-490 nm, emission 500-550 nm). Images were acquired at 20× at room temperature with samples immersed in media that had been warmed to 37°C. Average intensity from each image was obtained using ImageJ and used to calculated FAD, NADH and optical redox ratio defined as FAD/[NADH+FAD]. Intensities were normalized to control.

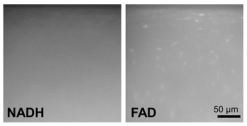

Fig 1. Example images.

To verify that imaging accurately captured chondrocyte metabolism, images were collected before and after treatment with metabolic inhibitors. 2-fluoro-2-deoxy-D-glucose (2-FG) (3mM) inhibited glycolysis. Oligomycin (10 μM) inhibited oxidative phosphorylation [14]. Control images were taken in chondrocyte media. Media was then replaced with the inhibitor of interest. Samples were allowed to equilibrate for 60 minutes before reimaging. Three to four samples from one 4 month old animal were used for each treatment. Two-tailed, paired t-tests were used to compare control and treated samples (significant at $p \leq 0.05$, trends at $0.05 < p \leq 0.10$).

To evaluate the effects of a single mechanical load on chondrocyte metabolism post-loading, a custom microscope-top loading apparatus was used. Three samples from one 2 year old animal were attached to a backplate using cyanoacrylate and loaded with a 1 mm diameter rod to 80% strain. Tile scans across the sample were acquired and analyzed before and after loading. Two-tailed, paired t-tests were used to compare all post-loading time points to control (significant at $p \leq 0.05$, trends at $0.05 < p \leq 0.10$). To ensure that the cyanoacrylate did not affect measured chondrocyte metabolism, a third set of samples was attached to the backplate with cyanoacrylate but not loaded. These samples showed no changes from the cyanoacrylate alone.

RESULTS: Metabolism was affected by inhibitors (Fig. 2). 2-FG significantly increased the redox ratio. Oligomycin decreased the redox ratio, but not significantly. Both significantly decreased NADH and FAD.

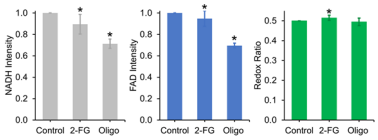

Fig 2. Inhibition response. *=significant.

NADH, FAD, and optical redox ratio exhibited time-dependent changes in response to a single mechanical load (Fig. 3). NADH intensity decreased throughout the post-loading period. FAD intensity showed a trend towards decreased intensity 10 minutes after loading, but recovered at 60 minutes (0.98 ± 0.06). Optical redox ratio was significantly increased 60 minutes after loading.

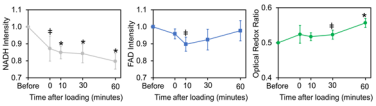

Fig 3. Loading response. *=significant, ǂ=trend.

DISCUSSION: Established methods of evaluating NADH and FAD autofluorescence in real-time can be applied to articular cartilage using an epifluorescent microscope. This technique surmounts the challenge of isolating cells from dense fibrous tissue and provides information on location-dependent metabolic activity, two main advantages over existing methods. Ultimately, this non-destructive, easily accessible technique can be used to evaluate the peracute response of cartilage to mechanical and chemical stimuli which can ultimately be applied to understanding biological underpinnings of osteoarthritis.

Given the interconnectedness of cellular respiration, it is unsurprising that 2-FG and oligomycin had significant inhibitory effects on both FAD and NADH. Evaluating the redox ratio of these metabolites sheds light on the differential activities of these inhibitors. 2-FG directly targets glycolysis, evidenced primarily by a decrease in NADH and, thus, an increase in redox ratio [14]. Oligomycin inhibits oxidative phosphorylation by targeting ATP synthase, directly causing a reduction in FAD and in the redox ratio [15,16].

The single mechanical load applied in this study caused short-term changes in chondrocyte metabolism. This is broadly consistent with previous studies that show metabolic changes in response to loading [1–3,8]. It is interesting to note the relatively minimal effect on oxidative phosphorylation (FAD) in comparison to recent studies that have shown an important role of mitochondria in cartilage response to load [1]. This is likely a result of the specific loads applied in this study; future studies can evaluate a broader range of mechanical and chemical stimuli.

SIGNIFICANCE: This study establishes methods to evaluate chondrocyte metabolism in real time, which has applications for understanding peracute responses to stimuli that lead to osteoarthritis.

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