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Mechanical Stress and ATP Synthesis are coupled by Mitochondrial Oxidants in Articular Cartilage

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Abstract

Metabolic adaptation of articular cartilage under joint loading is evident and matrix synthesis seems to be critically tied to ATP. Chondrocytes utilize the glycolytic pathway for energy requirements but seem to require mitochondrial reactive oxygen species (ROS) to sustain ATP synthesis. The role of ROS in regulating ATP reserves under a mechanically active environment is not clear. It is believed that physiological strains cause deformation of the mitochondria, potentially releasing ROS for energy production. We hypothesized that mechanical loading stimulates ATP synthesis via mitochondrial release of ROS. Bovine osteochondral explants were dynamically loaded at 0.5Hz with amplitude of 0.25MPa for 1 Hour. Cartilage response to mechanical loading was assessed by imaging with dihydroethidium (ROS indicator) and a Luciferase based ATP assay. Electron transport inhibitor rotenone and mitochondrial ROS scavenger MitoQ significantly suppressed mechanically induced ROS production and ATP synthesis. Our findings indicate that mitochondrial ROS are produced as a result of physiological mechanical strains. Taken together with our previous findings of ROS involvement in blunt impact injuries, mitochondrial ROS are important contributors to cartilage metabolic adaptation and their precise role in the pathogenesis of osteoarthritis warrants further investigation.

Keywords

Articular Cartilage; Oxidants; Reactive Oxygen Species; Mechanical stress; Glycolysis

Introduction

There is overwhelming evidence to indicate that form and function of articular cartilage are susceptible to mechanical loads throughout a human life span. During development, the location of the subchondral growth front is believed to be influenced by local balance between intermittent shear stress and hydrostatic pressure within the deep zone cartilage¹. Clinical and animals studies provide definitive evidence on the causal relationship between joint loading and cartilage health and function. Increased joint loading by moderate exercise causes increase in cartilage thickness², proteoglycan content^{2,3}, and mechanical stiffness⁴

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whereas strenuous exercise may lead to cartilage lesions⁵. Decreased or complete unloading of joints leads to atrophy of the cartilage, characterized by cartilage thinning⁶ and decrease in proteoglycan content⁷. However, remobilization seems to partially restore most of the structural and biochemical changes associated with joint unloading^{8,9}. Review of numerous *in vitro* studies indicates that the underlying mechanotransduction involved in matrix synthesis is multifactorial and complex¹⁰. A better understanding of this process may enable pharmacologic manipulation to maintain cartilage health in scenarios when mechanical loading is undesirable such as joint injuries and fractures¹¹.

Matrix biosynthesis by chondrocytes is closely tied to ATP, which is produced primarily by glycolysis. However, work performed recently in our laboratory revealed that although respiratory activity in cartilage is minimal, mitochondria contribute to ATP production by providing oxidants that support glycolysis¹².

Deformation of cellular organelles during loading is one among many events that are likely to play roles in mechanotransduction^{13,14}. Of particular interest is mitochondrial distortion that may result in the release of reactive oxygen species (ROS)¹⁵. In previous studies from our lab, we showed that a blunt impact injury results in a burst of ROS indicating their potential role in cartilage mechanotransduction¹⁶. The mitochondrial origin of ROS and its relationship to mechanical deformation, was confirmed when mitochondrial electron transport inhibitor rotenone and cytoskeletal inhibitors suppressed the ROS generation after a single blunt impact load^{17,18}. While these findings indicate that mitochondria are the predominant source of superoxides ($O_2^{\bullet-}$) induced by impact loading it is not clear if mitochondrial ROS contribute to chondrocyte metabolism and matrix synthesis under physiological loading conditions.

In this study, we hypothesized that physiologic cyclic loading stimulates ATP synthesis in cartilage via mitochondrial ROS production. The effects of cyclic axial loading on superoxide and ATP generation were quantified in osteochondral explants. The mitochondrial electron transport inhibitor rotenone and the mitochondria-targeted free radical scavenger MitoQ® were used to probe for mitochondrial involvement in response to cyclic compression.

Methods

Explant Harvest and Culture

Mature bovine stifle joints were obtained after slaughter from a local abattoir (Bud's custom Meats, Riverside, Iowa) employing sterile techniques. Osteochondral explants (~12×12mm with >10mm subchondral bone) were harvested from the lateral tibial plateau and transported in sterile sample containers with HANKS salt solution (supplemented with Penicillin/Streptomycin and Amphotericin B). After multiple washes with the supplemented HANKS solution, the explants were allowed to equilibrate in phenol red free culture media containing 45% Dulbecco's Modified Essential Medium (DMEM), 45% F12, 10% Fetal Bovine Serum (Invitrogen®) (supplemented with Penicillin/streptomycin and Amphotericin B) in standard low oxygen conditions (5% O_2 , 5% CO_2). Explants were randomly divided in to groups of control, rotenone and MitoQ treated groups. Non-loaded explants were also included in each treatment group to assess basal effect of treatments employed.

Mechanical Loading

Compression Apparatus—Mechanical loading was achieved using a custom indentation device previously described¹⁹ (Figure 1A). Briefly, specimens were securely fixed in a custom jig interfaced with stainless steel screw fixators and the explant was completely

bathed in culture media. Contact with the cartilage surface was initiated with an 8mm-diameter rigid, smooth and impermeable plane-ended cylindrical indenter prior to load application (Figure 1B). The apparatus was interfaced with a custom LABVIEW application and housed inside a 5% O₂ controlled incubator.

Mechanical Loading protocol—After 2 days following explant harvest, specimens were either left untreated or pretreated with-1) 2.5 μ M Rotenone (Sigma-Aldrich®) or 2) 4 μ M MitoQ® (Generous gift from Dr. Robin Smith and Dr Michael Murphy of University of Otago, New Zealand via Dr. Douglas Spitz, University of Iowa) for 1 hour. Each explant to be loading was anchored inside a custom chamber using stainless steel screws and bathed with media that was pre-equilibrated in 5% O₂. Cartilage thickness was assessed with an ultrasound thickness gauge (Sonopen®, Olympus NDT) prior to loading. A load-controlled triangular waveform (0.25MPa amplitude; 0.5 Hz) for 1 hour (1800 cycles) was employed and displacement data were collected for each loading to determine cumulative strain that occurred during loading. Cartilage thickness information and the displacement data were utilized to determine maximum strain endured during compressive loading. Figure 2 illustrates the testing sequence performed in this study.

ROS detection

Immediately after treatment duration, the explants were placed in media containing 5 μ M of the superoxide probe dihydroethidium (DHE) (Invitrogen), along with 1 μ M of a live cell probe, Calcein AM (Invitrogen), for 30 minutes. Three sites within the loaded area on the loaded explant and three sites on the unloaded control explant were imaged on a BioRad 1024 Confocal Microscope equipped with a Krypton/Argon laser in a custom designed stage for live cell imaging (Central Microscopy Research Facility, University of Iowa). Imaging sites were selected at random but care was taken to identify the sites close to the radial center of the indented region in loaded explants. 20X image stacks were acquired and utilized for DHE quantification (Figure 3).

DHE and Cell count quantification

To determine the percentage of DHE-stained cells, confocal images were analyzed using a Custom MATLAB image analysis program. Briefly, the automated application is designed to employ image information such as fluorescence intensity, cell boundary and circularity information to reduce subjectivity in counting procedures. Programmed subroutines perform automatic thresholding of image slices and detect 'out of focus' cells to reduce counting redundancy. The program analyses each slice in a stack of images and outputs a final count of live cells and DHE positive cells. Data was reported as a ratio (%) of DHE positive cells and live cells.

ATP Content assay

Twenty-four hours after dynamic loading, 8mm-diameter full thickness biopsy was harvested from explants using a biopsy punch and ATP and analysis was performed using Sigma FLAA ATP Bioluminescent assay Kit following previously published methods²⁰. Briefly, cartilage biopsies were wet-weighed, and transferred in to a tube containing phosphatase inhibitor cocktail (Sigma Aldrich®). ATP was extracted from explants by boiling the explants in the inhibitor cocktail for 10 minutes. Immediately after boiling, a fraction of the extract was mixed with dilution buffer containing luciferase before transfer in to a 96 well scintillation microplate. Luminescence was detected using a Microbeta 1450 scintillation and bioluminescence detector (Perkin-Elmer Wallac® Trilux). Triplicate sampling was performed and data was calibrated with reference curves generated with serial

dilutions of ATP standard for every run. Luminescence data were collected and reported as ATP content (nmol) per unit wet weight of tissue (mg).

Statistical analyses

Using Sigma Plot® 11, two-way Analysis Of Variance (ANOVA) with post-hoc Tukey multiple comparison was employed to determine effects of loading and treatments on oxidant production (%) and ATP content (nmol/mg). Statistical significance (p) and confidence (α) were both set at 0.05.

Results

A minimum of 3 explants were utilized to test each condition in this study. Dynamic mechanical stress of 0.25MPa on osteochondral explants resulted in a mean maximum strain of 0.21 regardless of the treatments employed indicating that rotenone and MitoQ did not alter structural response of cartilage to mechanical loading. Live cell densities in explants treated with Rotenone or MitoQ were comparable to untreated explants. Under dynamic loading, oxidant production (Figure 4) observed in cartilage ($43.1\% \pm 18.8\%$) was significantly higher ($p < 0.001$) than non-loaded explants ($1.8\% \pm 2.2\%$). Significant decrease in oxidants in the dynamically loaded explants was observed with the pretreatment of rotenone ($6.0\% \pm 7.8\%$; $p < 0.001$) or MitoQ ($4.6\% \pm 4.3\%$; $p < 0.001$). Identical trends were observed in ATP measurements with the loaded group (0.45 ± 0.14 nmol/mg) showing significantly higher ($p = 0.001$) ATP content (Figure 5) than non-loaded counterpart (0.12 ± 0.07 nmol/mg). Pretreatment with rotenone or MitoQ depleted ATP content (Rotenone: 0.15 ± 0.07 nmol/mg, $p < 0.001$; MitoQ: 0.04 ± 0.003 nmol/mg, $p < 0.001$) in dynamically loaded explants.

Discussion

Our study demonstrates that reactive oxygen species (ROS) contribute to chondrocyte ATP synthesis and mechanical loading seems to be the primary stimulus for ROS release. The significant mitigation of ROS production in explants treated with rotenone and MitoQ supports our hypothesis that mechanically-induced ROS production is predominately mitochondrial in origin. Additionally, decrease in ATP content in the presence of both treatments provides direct evidence that mechanical load induced mitochondrial ROS drive the glycolytic activity in chondrocytes.

Rotenone elicits its action by binding to the ubiquinone binding site of mitochondrial Complex I thereby disrupting the transfer of electrons from iron-sulfur centers in complex I to ubiquinone. This binding of rotenone interferes with NADH during the creation of cellular energy. MitoQ® (MitoQuinone) is a mitochondrial antioxidant that is targeted to mitochondria²¹. Conjugated with a lipophilic cation, MitoQ accumulates within cellular mitochondria up to 1,000 fold, compared to other non-targeted antioxidants. Despite diverse mechanisms of action, our results indicate that cartilage metabolism is dictated by the availability of mitochondrial ROS. Our study did not specifically preclude extra-mitochondrial sources of superoxides such as cyclooxygenase and NADPH oxidase. The impetus for studying mitochondrial oxidants was based on previous work from our facility that indicated that mitochondrial ROS are major contributors to chondrocyte response to mechanical injury. Further study is warranted to better understand the roles of non-mitochondrial sources of ROS in mechanotransduction.

Prior evidence indicates that mitochondria are mechanically linked to the chondrocyte cell membrane via f-actin and this mechanical linkage ensures movement and distribution of mitochondria within the cytosol²². The interaction of the mitochondria and the cytoskeleton

influences energy, and ROS production¹⁷ along with calcium homeostasis²³ and mitochondrial apoptotic factors²⁴. Results of this study are consistent with our hypothesis that dynamic mechanical loading of cartilage results in oxidant release from mitochondria, possibly by distortion of the mitochondrial organelle¹⁵.

ROS elicit various functions in human physiology immune response against pathogens²⁵ and multiple intracellular processes regulating transcription factors^{26–28}. In chondrocytes, mitochondrial ROS production led to significant increase of cyclooxygenase, a potent catabolic inflammatory molecule²⁹ and MMP-13³⁰ indicating their degradative potential. We and others have shown that oxidants play an important role in regulating metabolic activity in cartilage^{31,12,17}. ATP production in response to dynamic loading could be explained by a known spontaneous non-enzymatic oxidation of NADH which is dependent on the availability of superoxides. Moreover, there is evidence to indicate that ROS may possess a more diverse role in regulating cellular glycolytic mechanisms. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an oxidoreductase primarily known for its role in glycolysis. We found in our previous study that that oxidative stress can stimulate glycolysis to boost intracellular ATP by regulating GAPDH³² expression in chondrocytes. As a secondary but equally important role, oxidative modification of GAPDH can cause a cell to temporarily shift to pentose phosphate shunt to augment nicotinamide adenine dinucleotide phosphate (NADPH) production in an attempt to boost a cell's antioxidant capability by recycling glutathione (GSH)³³. Stabilization of hypoxia inducible factor (HIF-1 α) as a response to ROS may also influence ATP synthesis by regulating transcription of glycolytic enzymes and boost glucose uptake in chondrocytes^{32,34}.

In vivo, cartilage thinning under normal physical activities is relatively small. For instance, maximum cartilage thickness loss observed after 30 knee bends was 2.8% and squatting at a knee angle of 90° for 20s lead to a loss of 4.9%³⁵. Maximum peak compartmental deformation of 30% was experienced when performing a weight bearing single leg lunge³⁶. Preliminary experiments suggested that the magnitude of mechanical stress selected in this study produced cumulative strains <30% of cartilage thickness so as to test our hypothesis under comparable tissue strains. At higher strains, mitochondria can be physically damaged, a process that would lead to increased leakage of oxidants from the electron transport chain. Mitochondrial damage^{37,38} and subsequent apoptosis of cells can result in decrease in matrix synthesis, and also initiate other degenerative cascades that are hallmarks of osteoarthritis. Further work is needed to better define the response to such super-physiologic strains.

The indentation method is a popular technique used to study cartilage response to mechanical loads³⁹. Under mechanical stress, intra-tissue strain distribution influenced by the apparent inhomogeneity and anisotropy of cartilage. Stress distribution across the surface of the cartilage is also subject to indentation parameters (rate of loading, duration, indenter geometry and size) adding to the complexity of the mechanical environment experienced by the resident chondrocytes^{40,41}. It is clear from our findings that mechanically-induced oxidants can support glycolysis and their origin seems to be mitochondrial in nature^{18,12,17}. However, it is uncertain as to how specific mechanical inputs (compressive strain, shear strain or hydrostatic pressure) interact to influence chondrocyte metabolism. DHE employed in this study is a widely-used fluorescent superoxide indicator. Recent studies have indicated that DHE can potentially react with other oxidants, rendering it less reliable as a superoxide indicator⁴². Although consistent with our previous work^{18,17}, it will be worthwhile to employ secondary analytical techniques such as High Pressure Liquid Chromatography (HPLC) to unequivocally conclude if superoxides are the major ROS in chondrocyte metabolism.

MitoQ's role as a mitochondria specific anti-oxidant is with regard to the lipophilic cation tethered to the ubiquinone moiety that facilitates its preferential accumulation inside the mitochondrial membrane by a membrane potential-driven mechanism⁴³. As such, MitoQ can potentially reduce oxidants anywhere inside the cell since it is unlikely that complete mitochondrial localization of MitoQ could be achieved even in the most ideal circumstances. In our study, decrease in DHE fluorescence observed along with a corresponding decrease in ATP content in the presence of either rotenone or MitoQ indicate that the majority of ROS produced are mitochondrial in origin and potentially superoxide in nature. However, the involvement of other extra-mitochondrial oxidants (albeit to a lesser extent) in ATP synthesis cannot be ruled out completely.

In conclusion, the results of this investigation show that dynamic loading of cartilage induces mitochondrial ROS release that supports chondrocyte ATP synthesis. We believe that mitochondrial oxidants are released as a result of mitochondrial distortion caused by gross cellular deformation. Due to its avascular nature, oxygen levels in articular cartilage are quite low (~ 6%)⁴⁴, but chondrocytes are metabolically adapted to hypoxia and generate most of their ATP from glycolysis⁴⁵. However, anoxic conditions (~ 1% O₂) result in a sharp decline of glycolytic activity in cartilage (referred to as *negative Pasteur effect*) that can be effectively reversed by exogenous oxidants³¹. Cartilage seems to be capable of metabolic adaptation to physiologic loading through the production of modest, sublethal levels of ROS. However, the results of this and previous studies suggest that ROS production in cartilage is strain-related and that super-physiologic loading results in excessive oxidative damage. Further studies are warranted to determine if this process contributes to the pathogenesis of OA.

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Figure 1. Mechanical loading device. (A) Device for axial compression shown in a low O₂ incubator. (B). The close-up shows an osteochondral explant submerged in culture medium in the housing under the compression platen with the shaded region denoting the area of contact with the platen.

Figure 2.
Schematic of experimental design.

Figure 3.

Representative confocal images showing live (Green) cells (A, D, G), ROS (Red) producing cells (B, E, H) and composite images (C, F, I) of dynamically loaded cartilage explants. Dynamic loading resulted in greater co-localization of ROS in live cells (C) whereas explants treated with either Rotenone (F) or MitoQ (I) showed minimal ROS production under identical loading conditions. Scale bar denotes 100 μ m.

Figure 4.

Effect of dynamic loading (0.25 MPa at 0.5 Hz) on reactive oxygen species production (stained with DHE) on chondrocyte treatment groups. Legend: **NT Dyn**: No chemical treatment and Dynamic loading, **Rot Dyn**: Rotenone treated and Dynamic loading, **MitoQ Dyn**: MitoQ treatment and Dynamic loading, **NT non-loaded**: No chemical treatment and no dynamic loading, **Rot non-loaded**: Rotenone treated and no dynamic loading, **MitoQ non-loaded**: MitoQ treated and no dynamic loading.

Figure 5.

Effect of dynamic loading (0.25 MPa at 0.5 Hz) on ATP production on chondrocyte treatment groups. Legend: **NT Dyn**: No chemical treatment and Dynamic loading, **Rot Dyn**: Rotenone treated and Dynamic loading, **MitoQ Dyn**: MitoQ treatment and Dynamic loading, **NT non-loaded**: No chemical treatment and no dynamic loading, **Rot non-loaded**: Rotenone treated and no dynamic loading, **MitoQ non-loaded**: MitoQ treated and no dynamic loading.