The influence of oxygen tension on the induction of nitric oxide and prostaglandin E2 by mechanical stress in articular cartilage

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Summary

Objectives: Articular cartilage is an avascular tissue that exists at low oxygen tension. Oxygen tension can influence the production of the pro-inflammatory mediators nitric oxide (NO) and prostaglandin E2 (PGE2) in cartilage, which are increased in osteoarthritis (OA). The synthesis of these molecules can be stimulated by mechanical stress, which is an important risk factor for OA. The objective of this study was to determine the influence of oxygen tension on the induction of NO and PGE2 production in articular cartilage in response to mechanical stress.

Design: Intermittent mechanical compression (0.05 MPa, 0.5 Hz for 24 h) was applied to full thickness skeletally mature porcine articular cartilage explants at either 20%, 5%, or 1% O2. NO, PGE2 and peroxynitrite formation were measured, and the effect of the selective nitric oxide synthase 2 inhibitor 1400 W was tested.

Results: Incubating articular cartilage at 5% O2 significantly increased (P < 0.001) baseline NO production, as compared with 1% or 20% O2. Peroxynitrite formation was lower at reduced oxygen tension. Mechanical compression significantly increased (P < 0.001) NO production at 20% O2 but not at 5% or 1% O2, and significantly increased (P < 0.001) PGE2 production at 20% O2 (50 fold) and 5% O2 (4 fold) but not at 1% O2. 1400 W blocked mechanically induced NO production and further increased PGE2 production at 5% O2 (P < 0.005).

Conclusions: Oxygen tension influences the endogenous production of NO and PGE2 in cartilage and can have a significant effect on the induction of these inflammatory mediators in response to mechanical compression.

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Key words: Cartilage, Oxygen, Mechanical loading, Nitric oxide, Prostaglandins.

Introduction

Skeletally mature articular cartilage is an avascular tissue. As a result, the oxygen tension of articular cartilage is likely to be reduced compared with vascularized tissues. Previous studies suggest that the oxygen tension in articular cartilage ranges from 7% (53 mm Hg) in the superficial layer to less than 1% (7.6 mm Hg) in the deep zone, while mathematical models suggest that oxygen levels may range from 5% O2 on the surface and 1% O2 in the deep zone.

The oxygen tension of synovial fluid in humans is 6.5–9.0% (50–70 mm Hg). Oxygen tension can have significant effects on the metabolism of articular cartilage, including proteoglycan synthesis, expression of messenger RNA for cytokines, integrins, and integrin linked kinase; and on the production of inflammatory mediators such as nitric oxide (NO) and prostaglandin E2 (PGE2).

Osteoarthritis (OA) is associated with the elevated production of NO and PGE2 in cartilage, meniscus, synovial fluid and peripheral blood. The production of NO is mediated by the enzyme nitric oxide synthase (NOS), and PGE2 production by cyclooxygenase (COX). COX inhibitors are currently used clinically for the treatment of the pain and inflammation associated with OA. The severity of OA in vivo, however, can be decreased by NOS inhibitors in experimental animals, suggesting that NO might play an important role in the onset and progression of arthritis.

In contrast, some evidence shows that NOS2 selective inhibitors can exacerbate the inflammatory response. Abnormal mechanical loading of the joint (e.g., due to injury or obesity) is a risk factor for OA. Biomechanical factors have a strong influence on the catabolic and anabolic events of the chondrocytes, the cells that maintain the extracellular matrix of cartilage in a state of slow turnover. Intermittent mechanical compression and shear stress can stimulate NO and PGE2 production. Mechanically induced NO can be inhibited by NOS2 selective inhibitors, but this phenomenon is also associated with an increase in PGE2 production. Conversely, mechanical compression in the presence of a COX2 inhibitor inhibits both NO and PGE2 production.

The inflammatory response of articular cartilage is dependent on the oxygen tension as well as other factors. The production of NO and PGE2 in response to cytokines is significantly altered with low oxygen (1%) or hypoxia/reoxygenation. NO can play a protective role in cartilage in vitro, but can also cause damage. The different effects of NO on chondrocytes may depend on the reaction of NO with superoxide to form other derivatives such as peroxynitrite, a process that may be influenced by oxygen tension. Peroxynitrite and NO both appear to modulate matrix degradation and articular calcification in...
tensions in the physiological range (1% or 5% O₂) can have different biological effects in articular cartilage.

Taken together, these findings suggest that oxygen tension could influence the effects of mechanical stress on chondrocyte physiology. We have hypothesized therefore that oxygen tension can affect NO and PGE₂ production in response to mechanical compression in articular cartilage, which in turn alters the interaction of NO with reactive oxygen species and the formation of peroxynitrite. The aim of this study was to determine how oxygen tensions in the physiological range (1% or 5% O₂) compared with ambient oxygen tension (20% O₂) affect the production of NO and PGE₂ in response to mechanical compression and the subsequent production of peroxynitrite, as measured by nitrotyrosine in cartilage explants.

Materials and methods

EXPLANT CULTURE

Full thickness explants of articular cartilage were harvested with a trephine (5 mm diameter) from the femoral condyles of 2-year-old female pigs within 4 h of death. The cartilage was separated from the subchondral bone using a scalpel, and explants were cultured in standard culture medium containing Dulbecco’s Modified Eagle Medium (Gibco, Gaithersburg, MD) with 10% heat inactivated fetal bovine serum (Sigma Chemicals, St. Louis, MO), 0.1 mM non-essential amino acids (Gibco, Gaithersburg, MD), 37.5 μg/mL ascorbate-2-phosphate, 10 mM Hepes (Gibco, Gaithersburg, MD), 100 U/ml penicillin and streptomycin (Gibco, Gaithersburg, MD). Test and control explants were paired at harvest and originated from adjacent sites on the joint surface.

COMPRESSION EXPERIMENTS

All compression experiments were performed after allowing explants to equilibrate in culture for 72 h after harvest in order to establish a stable baseline level of NO and PGE₂ production. Individual explants were dynamicaly loaded in unconfinned compression at stresses of 0.01, 0.006, 0.0125, 0.025 or 0.05 MPa using a modified version of the Biopress system, a computer-controlled instrument for compressing tissue explants (Flexcell International, Hillsborough, NC). Briefly, individual cartilage explants were placed in Biopress culture plates (Flexcell International) consisting of a Delrin chamber attached to the bottom of a flexible silicone rubber membrane. Each specimen was subjected to a 10-gf tare load and allowed to equilibrate for 1 h. A range of calibrated air pressures were applied to the membrane, and the corresponding compressive stress (σ) applied to the explant was determined from the applied force (F) and the initial cross-sectional area (A) of the explant using the equation σ = F/A. Unloaded (control) explants were incubated for 24 h under the same conditions. All experiments were carried out on explants derived from a minimum of N = 3 pig joints with 6 site-matched pairs of explants per animal for n = 18 specimens per experiment. To examine the role of ROS2 enzyme activity.

EFFECTS OF OXYGEN TENSION

To allow the cultures to equilibrate with the surrounding oxygen tension, wells containing explants were placed in media in a humidified incubator at 37°C with either 5% CO₂ and 95% air, or in an incubator supplied with 5% CO₂, 5% O₂ and 90% N₂, or cultured at 5% CO₂, 1% O₂ and 94% N₂ for 8 h prior to compression. Explants to be compressed were subjected to a 10-gf tare load during this 8 h incubation period. Compressive loads of 0.05 MPa, 0.5 Hz (square wave-form 1 s on, 1 s off) were applied for 24 h to individual explants using the Biopress system. This mechanical regime was selected since it resulted in significant elevation of both NO and PGE₂. This magnitude of compression causes 12% deformation, which is the upper physiological range for articular cartilage and is associated with decreased matrix synthesis. The Biopress system was housed in the incubator at the appropriate oxygen tension so that compression was applied at either 1%, 5% or 20% O₂. All explants were compressed in an unconfined state between two impermeable loading platens. All control specimens were cultured in an unloaded state. All experiments were done using explants derived from a minimum of N = 3 pig joints with 6 site-matched pairs of explants per animal for n = 18 specimens per experiment. To examine the role of OXY in NO production, the specific ROS2 inhibitor 1400 W (2 mM, [N-(3-aminomethyl) benzyl] acetamide·2HCl) Alexis Chemik, CA) was included in the culture media, which is an inhibitor of ROS2 enzyme activity.

NO ASSAY

NO production was assessed by measuring the concentration of total nitrate and nitrite (termed “NOx”) in the media by techniques previously described. Briefly, nitrate was enzymatically reduced to nitrite by adding 7 μL 1 M Tris, 10 μL 0.02 mM nicotinamide adenine dinucleotide phosphate (Sigma), 20 μL 5 mM glucose-6-phosphate (G6P) (Boehringer Mannheim), 3 μL 10.0 U/mL G6P-dehydrogenase (Boehringer Mannheim) and 10 μL 1.0 U/mL nitrate reductase (Boehringer Mannheim) to 50 μL of standard or sample. After a 30-min incubation at 37°C, 100 μL of Griess I (1% sulfanilamide) (Sigma) and 100 μL Griess II (0.1% naphthylethlenediamine) (Sigma) were added followed by 10-min incubation at room temperature. Nitrite was then determined spectrophotometrically at an absorbance of 540 nm and interpolated with a sodium nitrate (Sigma) standard curve. Results were expressed as NOx μmol per gram wet weight per 24 h (μmol/g wet weight).

PGE₂ ASSAY

PGE₂ production was assessed with the PGE₂ Immunoblot assay kit (R&D Systems, Minneapolis, MN). The optical densities of samples were read on a microplate reader at 405 nm and interpolated with a PGE₂ standard curve. The assay can accurately detect PGE₂ levels above 36.2 pg/mL. Results were expressed as ng/g wet weight.

NITROTYROSINE IMMUNOBLOTS

Protein extracts were made and 18 μg of protein separated by sodium dodecyl sulfate polyacrylamide gelelectrophoresis using a 4–15% Tris–HCl Ready gel (Bio-Rad). Protein was reacted with 1:2000 dilution monoclonal primary anti-nitrotyrosine antibody (Chemicon) and detected with goat anti-rabbit IgG dilution (1:2000) and Western Lightning Chemiluminescence Plus (Perkin Elmer). Specificity of the antibody was determined by blocking of binding with inclusion of 10 mM nitrotyrosine to the primary antibody. Immunoblot band densities levels were quantified using AlphaEaseFC image analysis software (Alpha Innotech Corp, CA).
STATISTICAL ANALYSIS

Statistical analysis was performed using analysis of variance with Duncan’s post hoc comparison on site-matched paired explants with significance reported at the 95% confidence level.

Results

MAGNITUDE OF MECHANICAL COMPRESSION AND NO PRODUCTION

Previous studies demonstrate that mechanical compression increased NO production at 0.1–1.0 MPa\(^2\) and PGE\(_2\) production at 0.1 MPa\(^3\) levels of stress which may exceed the physiological range\(^3\). In this study, we investigated lower levels of mechanical stress and found that NO production was significantly increased at all magnitudes of stress applied (Fig. 1). Increased PGE\(_2\) production was statistically significant at 0.025 and 0.05 MPa [Fig. 1(b)]. Compression at 0.05 MPa, 0.5 Hz for 24 h significantly increased NO and PGE\(_2\) production.

INFLUENCE OF OXYGEN TENSION ON NO PRODUCTION IN RESPONSE TO MECHANICAL COMPRESSION

We next investigated if oxygen tension can affect the level of NO produced in response to mechanical stress, selecting one mechanical regimen (0.05 MPa, 0.5 Hz, 24 h) for this investigation. Mechanical compression significantly increased NO production at 20% \(O_2\) but not at 5% or 1% \(O_2\). Basal NO production (no mechanical compression) was significantly elevated at 5% \(O_2\) but was not further increased by mechanical compression [Fig. 2(a)]. Mechanical compression of the explants in the presence of the NOS2 selective inhibitor 1400 W inhibited the mechanically

![Graphs showing NO and PGE\(_2\) production at different oxygen tensions and mechanical compression.](image-url-1)

Fig. 1. (a) NO and (b) PGE\(_2\) production in articular cartilage explants after mechanical compression at 0.001–0.05 MPa at 0.5 Hz for 24 h. Data are presented as mean ± S.E.M., where \(n = 18\) (\(n = 6\) from three pigs). *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\).

![Graphs showing NO production at different oxygen tensions and mechanical compression.](image-url-2)

Fig. 2. (a) NO production by articular cartilage explants incubated for 8 h at either 20% \(O_2\), 5% \(O_2\) or 1% \(O_2\), followed by 24 h mechanical compression at 0.05 MPa, 0.5 Hz at the same oxygen tension. (b) Represents the same experiments done in the presence of 2 mM 1400 W. Data are presented as mean ± S.E.M., where \(n = 18\) (\(n = 6\) from three pigs). *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\).
induced NO at 20% O₂, as well as the increased baseline level at 5% O₂ [Fig. 2(b)]. There was a significant increase in mechanically induced NO at 1% O₂ in the presence of the selective NOS2 inhibitor. At 1% O₂, the baseline level of NO production was lower than that at 20% O₂.

INFLUENCE OF OXYGEN TENSION ON PGE₂ PRODUCTION IN RESPONSE TO MECHANICAL COMPRESSION

As shown in Fig. 1(b), PGE₂ production is affected by the magnitude of mechanical compression applied. To assess further this response, we investigated if oxygen tension can affect the level of PGE₂ production when it is applied at one mechanical regimen (0.05 MPa, 0.5 Hz for 24 h). As shown in Fig. 3, mechanical compression applied at 20% O₂ significantly increased PGE₂ production by 20-fold compared with uncompressed control (Fig. 3). PGE₂ production, however, increased by only five-fold at 5% O₂, and there was no compression-induced increase in PGE₂ production at 1% O₂. However, the baseline production of PGE₂ was two-fold higher at 1% O₂, compared with culture at 5% or 20% O₂.

Endogenous PGE₂ production was higher in the presence of the NOS2 selective inhibitor 1400 W at either 5% O₂ or 20% O₂, but not at 1% O₂ [Fig. 3(b)]. Mechanical compression in the presence of 1400 W caused dramatically higher PGE₂ production at 5% O₂.

NITROTYROSINE IMMUNOBLOTS

Since NO production is affected by oxygen tension, we determined if oxygen tension can affect protein nitration. Nitrotyrosine immunoblots were performed to determine the amount of peroxynitrite that had been formed (Fig. 4). Multiple bands were detected and the most prominent band is shown. With decreasing oxygen tension, a decreased amount of nitrotyrosine was detected, indicative of less peroxynitrite formation. Incubation with 10 mM nitrotyrosine reduced the nitrotyrosine detected in the positive controls and caused partial reduction of the nitrotyrosine detected in the cartilage samples (Data not shown). Free nitrotyrosine binds antibody and thus inhibits nitrotyrosine detection by immunoblot analysis. This is a control that is used to show that the antibody is actually detecting nitrotyrosine in the immunoblots.

Fig. 3. (a) PGE₂ production by articular cartilage explants incubated for 8 h at either 20% O₂, 5% O₂ or 1% O₂, followed by 24 h mechanical compression at 0.05 MPa, 0.5 Hz at the same oxygen tension. (b) Represents the same experiments carried out in the presence of 2 mM 1400 W. Note the different y-axis scales in (a) and (b). Data represents mean ± S.E.M. where n = 18 (n = 6 from three pigs). *P < 0.05, ***P < 0.001.

Fig. 4. A representative nitrotyrosine immunoblot (a) of protein extracts from articular cartilage explants incubated for 32 h at either 20% O₂, 5% O₂, or 1% O₂. (b) Quantitation of immunoblots band densities of articular cartilage explants incubated for 32 h at either 20% O₂, 5% O₂, or 1% O₂. Blots were performed using protein extracted from cartilage cultured at 20%, 5%, and 1% on the same blot and expressed relative to the 20% control protein extract (N = 3, mean ± S.E.M.).
Discussion

The findings of this study support the hypothesis that oxygen tension can influence the production of inflammatory mediators in cartilage. Both the baseline and mechanically induced production of NO and PGE$_2$ were dependent on the oxygen tension of the culture environment. Of note, very low oxygen tensions (i.e., 1% O$_2$) (levels that are probably representative of those in the deep zone of articular cartilage in vivo) inhibited the endogenous production of NO from articular cartilage, but increased endogenous production of PGE$_2$. Cartilage cultured at 5% O$_2$ (a level of oxygen that chondrocytes are likely to be exposed to in vivo in the superficial zone of articular cartilage) was associated with increased production of NO.

The reduction in peroxynitrite formation, as measured by nitrotyrosine formation, with reduced oxygen tension suggests that oxygen tension can alter the interaction of NO with superoxide to form peroxynitrite. The ratio of NO to O$_2$ may therefore determine the amount of various NO derivatives (e.g., peroxynitrite and nitrosothiols) that are formed. Differences in the amounts of NO derivatives formed could have important biological implications since NO and peroxynitrite, and hence nitration of different proteins, have different effects on biological events within chondrocytes. Furthermore, there are differences in the effects of NO and peroxynitrite on nuclear factor $\kappa$B (NF$\kappa$B) activation in cytokine stimulated bovine chondrocytes. Alterations in NF$\kappa$B activation have important implications because of association with the catabolic effects of interleukin-1 (IL-1). In addition, cyclic mechanical stress can inhibit IL-1 induced NO formation via NF$\kappa$B activation. It is technically difficult to measure NF$\kappa$B activity in this explant culture system, but could be pursued further using chondrocytes cultured in alginate. It would however be important to first confirm that the effects we observe in cartilage explants are reproduced in chondrocytes grown in alginate.

The role of oxygen tension in articular cartilage metabolism was previously measured at a shorter incubation period of 4 h of incubation of explants of different species including porcine, bovine and human. The relatively short period of 4 h of incubation of explants of different species could be pursued further using chondrocytes cultured in alginate.

The range of magnitudes of compression which might alter the nutrition and metabolism of cartilage, and hence oxygen consumption. The loading regimen that we applied, however, contains a static component in addition to the dynamic component, and static compression of articular cartilage can reduce solute diffusion.

At 20% O$_2$, the production of inflammatory mediators increased with increasing magnitude of applied stress. NO production is also dependent on the magnitude of stress applied, when biaxial stress is applied to a monolayer of chondrocytes. The range of magnitudes of compression tested at 20% O$_2$, in this present study, corresponds approximately to the physiological range that articular cartilage is likely to be subjected to in vivo. In a previous study, the highest magnitude tested (0.05 MPa) results in a mean surface-to-surface deformation of 12% and is associated with a significant decrease in the rate of synthesis of proteoglycans and collagen, using the same model of dynamic compression on porcine articular cartilage explants. Compression at 0.006 MPa is associated with the greatest amount of proteoglycan release into the culture media, and also induces the highest amount of NO. PGE$_2$ production is several fold greater, on a molar basis, than that for NO production at all magnitudes of mechanical compression tested. This finding is in agreement with previous findings on articular cartilage explants mechanically compressed at 0.1 MPa at 20% O$_2$.

In our study, NO production in response to mechanical compression was significantly reduced at the lowest oxygen tension but was similar at 5% and 20% O$_2$. Cartilage cultured at 5% O$_2$ (with no mechanical compression) had the highest levels of baseline NO production, and this stimulation was not further affected by mechanical compression. PGE$_2$ production in response to mechanical compression, however, was clearly dependent on the oxygen tension. PGE$_2$ synthesis requires O$_2$, and oxygen availability may limit PGE$_2$ production when mechanical compression is applied at 1% O$_2$. The decrease in oxygen availability at 1% O$_2$ is supported by mathematical modeling which suggests that the oxygen consumption of articular cartilage is reduced at less than 5% O$_2$, however, there is little difference in the rate of oxygen consumption when articular cartilage is cultured at 5% or 20% O$_2$.

Oxygen availability may not fully explain the differences in inflammatory mediator production with oxygen tension. When mechanical compression was applied in the presence of the NOS2 selective inhibitor 1400 W, a further increase in PGE$_2$ production occurred, most prominently at 5% O$_2$. This finding suggests that the concentration of oxygen was not rate-limiting for PGE$_2$ production. There may be other unknown factors that reduce PGE$_2$ production in response to mechanical compression when it is applied at 5% O$_2$. This finding may be explained by interactions between NO and the COX enzyme activity. It has been suggested the interaction between the two pathways may be due to the nitration of tyrosine 134 of the COX enzyme by NO, a reaction that would inhibit PGE$_2$ production in articular cartilage. Similar interactions can also occur between the NOS and lipoxygenase pathways in articular cartilage in response to mechanical stress.

Future studies will delineate these interactions of the interplay of oxygen tension in the production of inflammatory mediators in response to mechanical stress.

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