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OXYGEN, NITRIC OXIDE AND ARTICULAR CARTILAGE

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Abstract

Molecular oxygen is required for the production of nitric oxide (NO), a pro-inflammatory mediator that is associated with osteoarthritis and rheumatoid arthritis. To date there has been little consideration of the role of oxygen tension in the regulation of nitric oxide production associated with arthritis. Oxygen tension may be particularly relevant to articular cartilage since it is avascular and therefore exists at a reduced oxygen tension. The superficial zone exists at approximately 6% O_2, while the deep zone exists at less than 1% O_2. Furthermore, oxygen tension can alter matrix synthesis, and the material properties of articular cartilage *in vitro*.

The increase in nitric oxide associated with arthritis can be caused by pro-inflammatory cytokines and mechanical stress. Oxygen tension significantly alters endogenous NO production in articular cartilage, as well as the stimulation of NO in response to both mechanical loading and pro-inflammatory cytokines. Mechanical loading and pro-inflammatory cytokines also increase the production of prostaglandin E2 (PGE_2). There is a complex interaction between NO and PGE_2, and oxygen tension can alter this interaction. These findings suggest that the relatively low levels of oxygen within the joint may have significant influences on the metabolic activity, and inflammatory response of cartilage as compared to ambient levels. A better understanding of the role of oxygen in the production of inflammatory mediators in response to mechanical loading, or pro-inflammatory cytokines, may aid in the development of strategies for therapeutic intervention in arthritis.

Key Words: Oxygen, nitric oxide, articular cartilage, prostaglandins, cytokines, mechanical loading.

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Introduction

Increased nitric oxide (NO) production is associated with both osteoarthritis and rheumatoid arthritis. Factors such as inflammation and mechanical loading can lead to increased production of inflammatory mediators such as nitric oxide and prostaglandins. There has been considerable interest in the use of inhibitors of NO production in the treatment of these diseases, but these inhibitors have not yet been used in the clinical setting. Since NO can interact with other inflammatory mediators such as prostaglandins and leukotrienes, recent research has pursued the idea of dual inhibitors of the inflammatory cascade. In this article we discuss how oxygen tension could be an important consideration in the biology of NO and the interaction of NO with other inflammatory mediators.

Articular cartilage is avascular

Skeletally mature articular cartilage is an avascular tissue. As a result, the oxygen tension of articular cartilage is reduced compared with vascularized tissues. Since oxygen and other nutrients must diffuse into the tissue from the synovial fluid surrounding the joint, an oxygen gradient is created in cartilage. Mathematical models suggest that oxygen levels may range from 5% on the surface to 1% in the deep zone (Zhou *et al.*, 2004), while measured 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 (Silver, 1975).

Information on the oxygen tension of synovial fluid is more abundant. The oxygen tension of synovial fluid in humans is 6.5 – 9.0% (50-70 mm Hg) (Falchuk *et al.*, 1970; Lund-Olesen, 1970). Mobilization of the joint can increase the oxygen supply to the joint. Inflammatory mediators are produced in response to inflammation or mechanical loading, which can also act to decrease the oxygen tension in the joint. In arthritic cartilage, oxygen delivery is compromised as a result of decreased capillary density and deep placement of capillaries within the synovial sac of arthritic joints (Stevens *et al.*, 1991). Therefore, it is likely that articular cartilage affected by rheumatoid arthritis (RA) and osteoarthritis (OA) is more hypoxic than normal cartilage (James *et al.*, 1990).

Effects of oxygen tension on articular cartilage

Oxygen tension can have significant effects on the metabolism of articular cartilage, including changes in proteoglycan synthesis (Fermor *et al.*, 2004; Grimshaw and Mason, 2000; Lee and Urban, 1997; Lee *et al.*, 2002; Ysart and Mason, 1994), expression of mRNA of β_1 integrin and integrin linked kinase (Grimshaw and Mason, 2001), growth factors (Etherington *et al.*, 2002), glucose

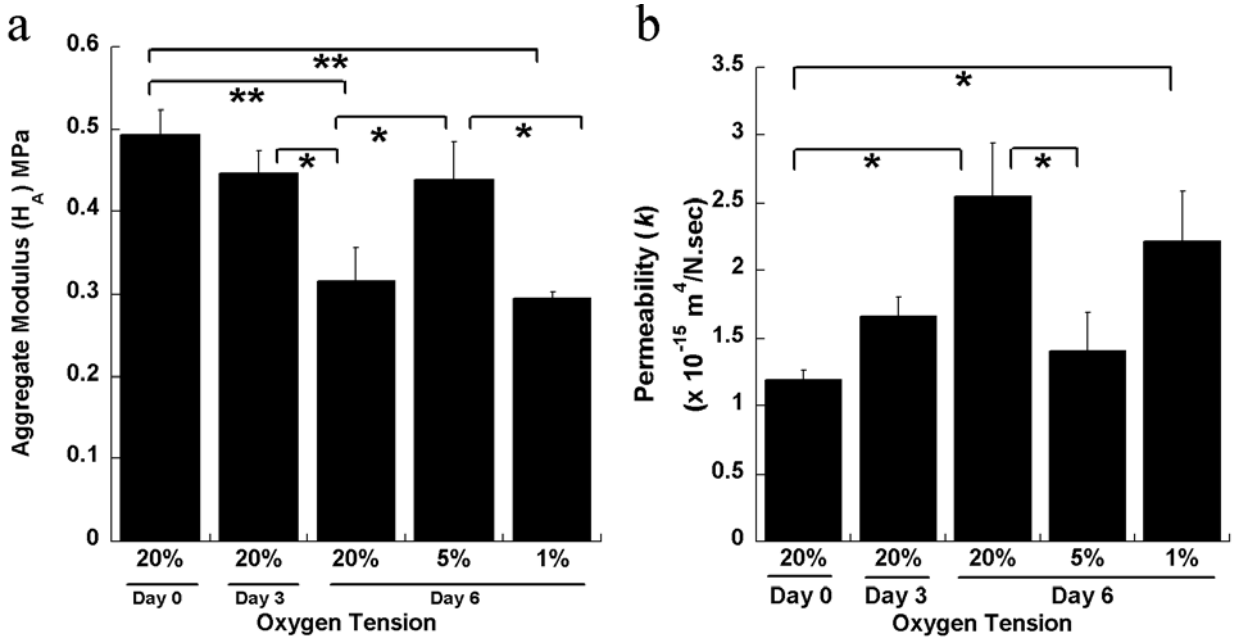

Figure 1. Full thickness explants of articular cartilage were harvested from the femoral condyles of 2-3 year old female pigs and cultured in Dulbecco's Modified Eagle Medium (DMEM), 10% FBS, 0.1mM non-essential amino acids, 37.5 µg/ml ascorbate-2-phosphate, 10 mM HEPES, 100U/ml penicillin and streptomycin, in a humidified environment at 37°C for 72 hours at 5% CO_2, 95% air. Explants were then cultured for a further 72 hrs at either 1% O_2, 5% CO_2, 94% N_2, or 5% O_2, 5% CO_2, 90% N_2 or 5% CO_2, 95% air. The aggregate modulus (H_A) and hydraulic permeability (k) were determined using a confined-compression creep test (Mow *et al.*, 1980) on a materials testing system (Enduratec ELF 3200, Minnetonka, MN), immediately after removal from the joint, after 3 days in culture at 20% O_2 and then after a further 3 days cultured at either 20, 5 or 1% O_2. The explant was placed in a chamber with the deep layer against a porous filter (pore size 50-100 µm, 50% porosity). A 20-gf-tare load was applied to the explants and allowed to equilibrate for an hour. A step load of 20 gf was then applied to the sample and displacement was monitored by LVDT until equilibrium. The resultant displacement history was fitted to the theoretical biphasic model by nonlinear regression to compute aggregate modulus (H_A) and permeability (k) of the cartilage (Mow *et al.*, 1980). Statistical analysis was performed by ANOVA using Duncan's post-hoc comparison on matched control and test samples, where * = $p < 0.05$ and ** = $p < 0.01$. Data are shown as Mean ± SEM, n = 6 from 3 pigs.

transporters (Mobasheri *et al.*, 2005), and ATP levels (Lee and Urban, 1997; Stefanovic-Racic *et al.*, 1994b) in chondrocytes. Different levels of oxygen tension within the physiological range for articular cartilage can have different effects. For example, culturing cartilage at 5% O_2 significantly increases proteoglycan and collagen synthesis compared with 20% O_2 (Fermor *et al.*, 2004), but culturing cartilage at 1% O_2 causes a significant decrease in proteoglycan and collagen synthesis compared with 20% O_2. Similarly, increased hyaluronan synthesis occurs after culturing for 12 hours at 5% O_2 compared to 20% O_2, but decreased hyaluronan synthesis occurs at 1% O_2 compared with 20% O_2 (Hashimoto *et al.*, 2006). These findings suggest that 5% O_2 is in some way "optimal" for articular cartilage matrix synthesis while 1% O_2 is not as good. Nevertheless, both 1% O_2 and 5% O_2 are often referred to as "hypoxic", which can be misleading in our understanding of the effects of oxygen tension in cartilage biology. No studies as yet have determined if there are zonal differences in the response of chondrocytes to oxygen tension.

If matrix turnover is altered by oxygen tension, it is possible that oxygen tension might also affect the material properties of articular cartilage, which in turn could affect the ability of the tissue to respond to mechanical stress. Removal of articular cartilage from the joint and culturing the cartilage under standard culture conditions of 5% CO_2, 95% air (equivalent to 20% O_2) for 3 days leads to a significant reduction in the aggregate modulus (H_A), a measure of the stiffness of the cartilage. H_A is decreased significantly further if the cartilage is incubated for a further 3 days at 20% O_2 (Fermor *et al.*, 2004). If however after 3 days of incubation at 20% O_2, the cartilage explants are incubated at 5% O_2 for a further 3 days, the H_A increases to a similar level to when it was first removed from the joint. Changing the cartilage to a 1% O_2 environment for a further 3 days, does not return the H_A to the level that it was when it was first removed from the joint (Fig. 1a). Changes in the permeability constant (k) also occur in response to changes in oxygen tension (Fig. 1b). The observed trends correlated with the changes that were observed in the H_A. Culturing cartilage at 20% O_2 led to a significant increase in the permeability constant k compared with the k value observed when the cartilage was first removed from the joint. Changing the cartilage to 1% O_2 after 3 days of culture at 20% O_2 did not cause any significant difference in the permeability constant k, compared with culturing cartilage at 20% O_2 for 6 days.

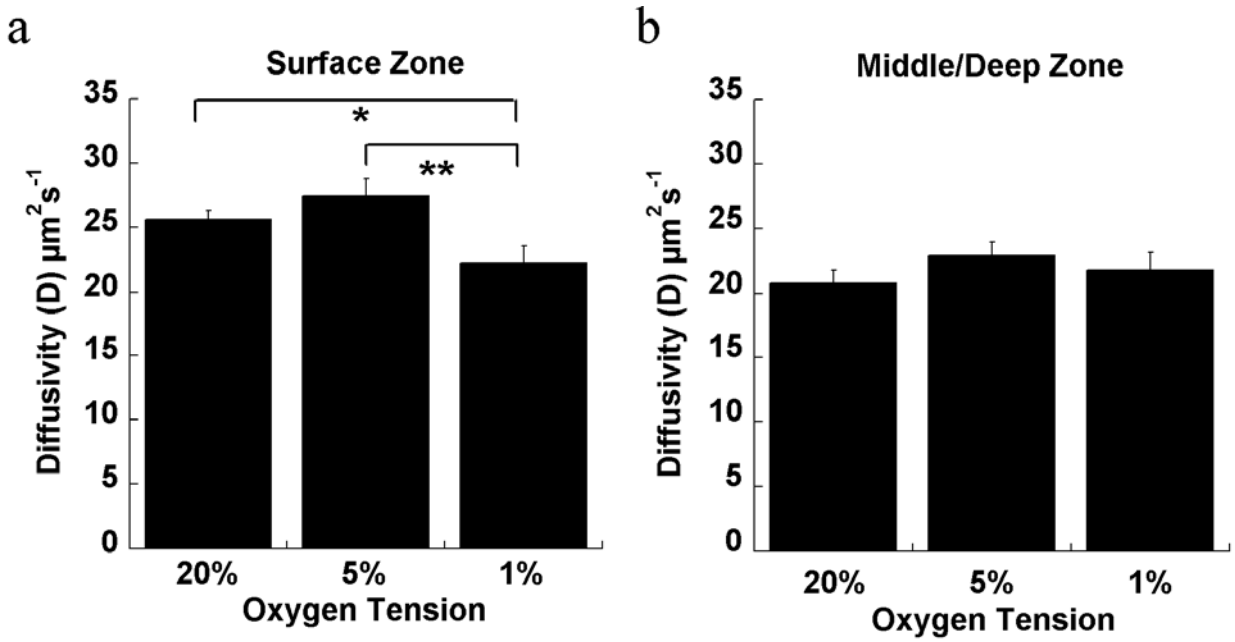

Figure 2. Full thickness explants of articular cartilage were harvested from the femoral condyles of 2-3 year old female pigs and cultured in Dulbecco's Modified Eagle Medium (DMEM), 10% FBS, 0.1mM non-essential amino acids, 37.5 μg/ml ascorbate-2-phosphate, 10 mM HEPES, 100U/ml penicillin and streptomycin, in a humidified environment at 37^{0}C for 72 hours at 5% CO_2, 95% air. Explants were then cultured for a further 72 hrs at either 1% O_2, 5% CO_2, 94% N_2 or 5% O_2, 5% CO_2, 90% N_2 or 5% CO_2, 95% air. Diffusion coefficients of uncharged dextran molecules in articular cartilage were measured using fluorescence recovery after photobleaching (FRAP). A full-depth slice of each explant was incubated at 4ºC for 24 hours in a PBS solution containing 2.27 mg/mL (0.032 mM) fluorescein-conjugated 70 kDa dextrans, then equilibrated to room temperature for imaging (Leddy and Guilak, 2003). FRAP was performed with a laser scanning confocal microscope (Zeiss LSM 510), using a 20x/0.5 NA Plan-Neofluar objective. Diffusivities (D) were calculated in the surface and middle/deep zones separately by curve-fitting the fractional fluorescence recovery of the bleached region versus the frequency-scaled time. This fractional recovery was measured by decay of the spatial Fourier transforms of the normalized images. Statistical analysis was performed by ANOVA using Duncan's post-hoc comparison on matched control and test samples, where * = $p < 0.05$ and ** = $p < 0.01$. Data are shown as Mean ± SEM, n = 12, from 2 pigs.

When the cartilage is in a 5% O_2 environment for 3 days after culturing at 20% O_2 for 3 days, the permeability constant *k* is returned close to the level that it was when it was first removed from the joint. So it appears that the increase in matrix synthesis that is observed at 5% O_2 is associated with an increase in the material properties of the cartilage.

Since changes occur in matrix synthesis with respect to oxygen tension, we would expect that altering oxygen tension might lead to changes in the ability of solutes to diffuse in articular cartilage. Diffusion is particularly important to this tissue, since articular cartilage is avascular, and so depends on diffusion to obtain nutrition and solutes. Diffusion coefficients of uncharged 70 kDa dextran molecules in articular cartilage were measured using the fluorescence recovery after photobleaching (FRAP) technique (Leddy and Guilak, 2003). This size (70 kDa) is representative of molecules equivalent to the size of decorin or biglycan, which are larger than most growth factors or cytokines but smaller than proteoglycan or cartilage oligomeric matrix protein (COMP). The diffusion coefficient (D) was significantly lower in the superficial zone at 1% O_2 than at either 5% or 20% O_2 (Fig. 2a). In the middle/deep zone, there was no significant difference in D with respect to oxygen tension (Fig. 2b).

These findings imply that the oxidation status of a chondrocyte can strongly influence net matrix synthesis and hence elastic properties of cartilage. Yet, an increase in matrix synthesis correlates with an increase in diffusivity, a counterintuitive trend given that increasing matrix proteins should inhibit the diffusion of the 70 kDa dextrans. However, the assembly and location of the newly synthesized matrix proteins within the cartilage have not been studied. Either of these characteristics could explain this increase in diffusion when matrix synthesis and material properties increase.

Since oxygen tension clearly can affect the metabolism of articular cartilage, we have been interested in determining if oxygen tension can have effects on the production of inflammatory mediators such as NO. These inflammatory mediators are present in diseased cartilage such as in arthritis, which will be discussed further.

Nitric oxide and arthritis

Increased levels of NO are associated with osteoarthritis (Amin *et al.*, 1997; Farrell *et al.*, 1992; McInnes *et al.*,

1996; Sakurai *et al.*, 1995) and rheumatoid arthritis (Ueki *et al.*, 1996; Grabowski *et al.*, 1997; McInnes *et al.*, 1996; St Clair *et al.*, 1996), which has led to considerable interest in therapeutic intervention using inhibitors of nitric oxide synthase (NOS) (Abramson *et al.*, 2001). NO is a gas that can readily interact with superoxide anions (O_2^-) to form peroxynitrite, which can nitrate certain proteins. Nitrated proteins can be detected using nitrotyrosine antibodies. Peroxynitrite formation can occur in arthritic cartilage, as shown by nitrotyrosine-positive chondrocytes detected in osteoarthritic tissues. Recently, elevated nitrated collagen peptides have been demonstrated in arthritic tissue (Deberg *et al.*, 2005).

The severity of arthritis *in vivo* is decreased by NOS inhibitors, suggesting that NO might play an important role in the onset and progression of arthritis in these models. L-NIL, an inhibitor of NOS2, can inhibit the progression of osteoarthritis in the canine ACL transection model (Pelletier *et al.*, 1998). Streptococcal cell wall-induced arthritis in rats is inhibited by arginine analogues such as the non-specific NOS inhibitors, L-NMMA (McCartney-Francis *et al.*, 1993; Stefanovic-Racic *et al.*, 1994a) and L-NAME (Ialenti *et al.*, 1993) and also by L-NIL (Connor *et al.*, 1995). Streptococcal cell wall-induced arthritis is not inhibited by aminoguanidine in rats (Stefanovic-Racic *et al.*, 1995) and osteoarthritic joint pathology is significantly inhibited in the murine collagen-induced arthritis model in NOS2 deficient mice (van den Berg *et al.*, 1999).

The use of superoxide dismutase has also been investigated in the treatment of arthritis for many years with limited success (Greenwald, 1991). Since the discovery of SOD, various SOD mimetics have been developed to enhance the ability of SOD to enter cells. Extracellular SOD (EC-SOD) knockout mice develop a more severe collagen-induced arthritis than wild type mice (Ross *et al.*, 2004). In addition, a synergistic beneficial interaction between methotrexate and a SOD mimetic occurs in the collagen-induced arthritis rat model (Cuzzocrea *et al.*, 2005). Recent observations show EC-SOD protein expression is decreased in humans with OA and in an animal model of OA (Regan *et al.*, 2005).

Inflammation and mechanical stress are risk factors for OA, and both are associated with up-regulation of NO (Guilak *et al.*, 2004). Although many of the physiological actions of NO are mediated through the activation of soluble guanylate cyclase, the mechanisms by which NO exerts its cytostatic/cytotoxic or tissue-damaging effects in any cell type are unclear. Upregulation of NO can lead to several different effects on chondrocytes, including inhibition of adhesion-modulating signal transduction, modulation of cytokine expression, suppression of matrix collagen and proteoglycan synthesis, activation of matrix metalloproteinases (MMPs), suppression of proliferation, and promotion of chondrocyte apoptosis (Lotz *et al.*, 1999; Pelletier *et al.*, 1999; Studer *et al.*, 2000). Further metabolism of NO can affect the biological response that NO elicits (Del Carlo and Loeser, 2002).

Biochemistry of nitric oxide

NO is formed by the conversion of L-arginine to citrulline, with oxygen serving as an electron acceptor (Fig. 3). The removal of the terminal guanidino nitrogen is catalyzed by the enzyme nitric oxide synthase (NOS) (Kwon *et al.*, 1990). NOS exists in three isoforms, NOS1 (nNOS), NOS2 (iNOS) and NOS3 (eNOS), and it is the inducible NOS2 isoform, which is predominantly responsible for NO production in articular cartilage (Sakurai *et al.*, 1995). The destructive effects of NO are linked to its ability to combine with superoxide anions (O_2^-) to generate reactive oxygen species. The unpaired radical electrons on NO and O_2^- join, to form the potentially more dangerous product

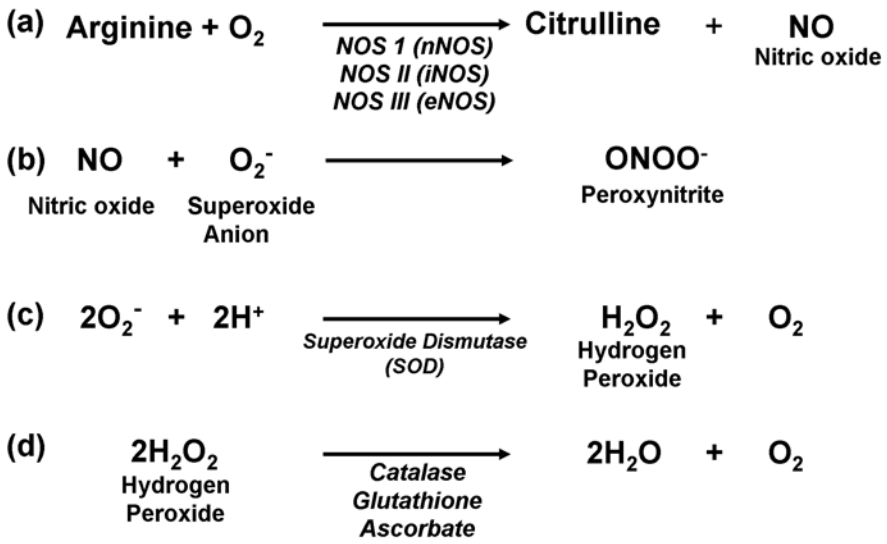

Figure 3. Biochemistry of nitric oxide. (a) Nitric oxide is formed by the conversion of arginine to citrulline. (b) Nitric oxide is a highly reactive gas that can interact with superoxide anions to form peroxynitrite. (c) Superoxide anions are dismutated to hydrogen peroxide by superoxide dismutase, which is present in articular cartilage. (d) Hydrogen peroxide is further metabolized by antioxidants to water and oxygen.

peroxynitrite ($ONOO^-$). NO can also bind to a reactive cysteine thiol to form S-nitrosothiols, which are very important regulators of physiology and pathology, playing roles in signalling and modulating of cellular and enzyme function (Hess *et al.*, 2005). Formation of ROS and the alteration of redox sensitive signalling pathways likely play important roles in the pathogenesis of osteoarthritis (Henrotin *et al.*, 2003).

NO can inhibit cytochrome oxidase in mitochondria, which results in a reduction of the electron transport chain, so favouring the formation of superoxide anions and altering the redox state of the cell. Superoxide anions and NO join to form peroxynitrite, a strong oxidant now thought to be responsible for some of the pathological actions of NO. Compounds such as peroxynitrite can promote formation of 3-nitrotyrosine. Peroxynitrite can also be detoxified depending on the microenvironment in which it is generated. Superoxide anions are converted to H_2O_2 by the antioxidant superoxide dismutase (SOD). H_2O_2 can then act as a second messenger that can mediate gene transcription, cell proliferation, necrosis or apoptosis depending on its concentration. Catalase can convert H_2O_2 to water and oxygen.

Antioxidants are molecules or compounds that act as free radical scavengers, binding and inactivating the free radicals by donating electrons. Antioxidants such as SOD can therefore protect against oxidative stress. There are three types of SOD. SOD1, or CuZnSOD, is localized primarily to the cytosol, SOD2 or MnSOD in mitochondria and SOD3 or extracellular SOD (EC-SOD). EC-SOD is a secreted tetrameric glycoprotein with a positively charged heparin-binding site (Fattman *et al.*, 2003). EC-SOD localizes to the extracellular matrix of tissues by binding to the negatively charged proteoglycans and collagen (Petersen *et al.*, 2004), which could be particularly important to cartilage homeostasis due to the large amounts of negatively charged extracellular matrix present in cartilage. The decreased level of EC-SOD found in osteoarthritic cartilage (Regan *et al.*, 2005) indicates the potential importance of EC-SOD to articular cartilage. In articular cartilage, there is a crucial balance between the cellular concentrations of NO, O_2^- and SOD. When an imbalance between the formation and neutralization of pro-oxidants or, when the critical ratio of NO and SOD, altered oxidative stress occurs.

Oxygen tension and inflammation in articular cartilage

Increased NO in articular cartilage can result from increased pro-inflammatory cytokines. Concentrations of proinflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) are increased in arthritic joints, and contribute to the increased production of NO in arthritic compared to healthy cartilage (Sakurai *et al.*, 1995; Stadler *et al.*, 1991). The mechanism of action of TNF-α has some similarities to IL-1 in terms of its involvement in the pathogenesis of osteoarthritis, as they both induce the formation of NO. IL-1 receptor antagonist or TNF-α antagonists are effective therapeutic agents in animal models of RA, OA and human RA (Evans *et al.*, 1998). Furthermore, the beneficial effects of the anti-TNF antibody infliximab in humans with RA is linked to NOS2. The effectiveness of this anti-TNF therapy closely correlates with the degree of reduction in the over expression of NOS2 in blood monocytes-lymphocytes from RA patients (Perkins *et al.*, 1998).

Molecular oxygen is involved in the formation of the inflammatory mediator NO. The level of inflammatory response of articular cartilage is dependent on the oxygen tension as well as other factors. Arthritic joints may be exposed to hypoxia-reoxygenation events with mobilization (Mapp *et al.*, 1995). Short-term exercise results in increased lactate production and increased pCO_2, suggesting decreased oxygen levels in the synovial fluid as pressure is applied to the arthritic joint (James *et al.*, 1990). At the start of motion, capillaries flatten as intra-articular pressure increases, decreasing blood flow and limiting the delivery of oxygen to the joint. Capillaries return to their open configuration as intra-articular pressure returns to normal levels. The synovium is then reperfused with blood, allowing the oxygen tension to rise above baseline for a short time after exercise (Mapp *et al.*, 1995). Studies show the production of NO in articular cartilage in response to cytokines is significantly altered with low oxygen or cycles of hypoxia/reoxygenation (Cernanec *et al.*, 2002; Martin *et al.*, 2004). Incubation of cartilage explants for 72 hours at 1% O_2 causes a significant reduction in the production of NO in response to 10 ng/ml IL-1α or 10 ng/ml TNF-α compared with incubation at 20% O_2 (Cernanec *et al.*, 2002). In contrast, incubation of isolated chondrocytes as a monolayer at 5% O_2 or 1% O_2 causes significantly more NO to be produced in response to 0.2-1 nM IL-β compared with incubation at 20% O_2 for a 48 hour time period (Mathy-Hartert *et al.*, 2005). Differences in the preparation of the cells, the amount of extracellular matrix and culture conditions might account for the differences in findings.

The reoxygenation to 20% O_2 of previously hypoxic (1% O_2) cartilage explants treated with IL-1α or TNF-α led to significantly increased NO production compared with those cultures that had remained at 20% O_2 (Cernanec *et al.*, 2002). A similar further increase in NO production after reoxygenation occurs in murine macrophages in response to interferon γ (Melillo *et al.*, 1996). This sustained NO production was not seen in cultures maintained under continuous ambient oxygen tension. Replenishment of cytokine after 72 hours of incubation in continuous ambient oxygen tension produced a much smaller increase in NO compared with that in replenished cartilage that was reoxygenated after hypoxia (1% O_2). This difference in NO production could be related to a hypoxia-induced increase in NOS2, which leads to increased protein translation after re-oxygenation. Decreased protein translation under hypoxic conditions (<1.5% O_2) has also been observed in other cell types (Liu *et al.*, 2006). When isolated articular chondrocytes are grown as a monolayer and subjected to 20% O_2 after incubation at 5% O_2 for 48 hours, there is no further increase in NO production (Mathy-Hartert *et al.*, 2005).

The mechanisms by which hypoxia influences NO

production are not fully understood. One study showed that hypoxia (5% O_2) increases IL-1α-induced p42/p44^{mapk} expression compared to 21% O_2 in human articular cartilage (Dudhia *et al.*, 2000). This difference could be attributed to differential phosphorylation of serine or threonine residues of these kinases, or to differential activation of upstream kinases in different oxygen tensions.

NO can play a protective role in cartilage *in vitro*, but can also cause damage (Evans *et al.*, 1996). The different effects of NO on chondrocytes may be influenced by oxygen tension. The ratio of NO to O_2 may 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, have different effects on biological events within chondrocytes (Del Carlo and Loeser, 2002; Mathy-Hartert *et al.*, 2003). For example it is suggested that NO alone cannot induce apoptosis in chondrocytes but requires the presence of reactive oxygen species (Del Carlo and Loeser, 2002). Similarly if lapine chondrocytes are transduced with an adenovirus carrying the human NOS2 gene, supra-physiologically high levels of NO are produced but no apoptosis is observed (Studer *et al.*, 1999). Furthermore, there are differences in the effects of NO and peroxynitrite on NFkB activation in cytokine stimulated bovine chondrocytes (Clancy *et al.*, 2004). Alterations in NFkB activation have important implications because of association with the catabolic effects of IL-1.

Therefore the balance between NO, O_2^-, and SOD may determine the biological effect. This balance could likely be affected by oxygen tension, or changes in oxygen tension.

Effects of oxygen on mechanically induced NO production

Biomechanical factors have a strong influence on the catabolic and anabolic events of the chondrocytes (Guilak *et al.*, 1997), which are responsible for maintaining the extracellular matrix of cartilage in a state of slow turnover. Mechanical loading is important for the normal homeostasis of articular cartilage. Alterations in the mechanical environment of the articular cartilage due to abnormal joint loading can lead to cellular and biochemical changes in the activity of the chondrocytes that are associated with cartilage degradation and the progression of osteoarthritis or rheumatoid arthritis (Helminen *et al.*, 1987; Howell *et al.*, 1992; Minor, 1999). Alterations in joint loading may be a result of joint pain, immobilization, instability, or deformity, and may be an important factor that further modifies joint physiology following the onset of disease. Furthermore, abnormal loading patterns may become more pronounced as the adjoining muscles weaken, a phenomenon often observed in arthritic patients. In particular, mechanical loading of articular cartilage can also increase the production of NO in response to physiological or hyperphysiological magnitudes of stress for articular cartilage. Intermittent mechanical compression (Fermor *et al.*, 2001b; Lee *et al.*, 1998) and shear stress (Lane Smith *et al.*, 2000) can stimulate NO production. Mechanically induced NO production is also dependent on the magnitude of stress applied (Agarwal *et al.*, 2004; Fermor *et al.*, 2005).

Oxygen tension can also influence the production of inflammatory mediators in response to mechanical compression in cartilage explants (Fermor *et al.*, 2005). NO production in response to mechanical compression is significantly reduced at 1% O_2 compared with 20% O_2, but is similar at 5% and 20% O_2. Cartilage cultured at 5% O_2 (with no mechanical compression) has an increased level of baseline NO production (Fermor *et al.*, 2005; Hashimoto *et al.*, 2006), compared with 20% or 1% O_2, and this stimulation is not further affected by mechanical compression. Therefore both the baseline and mechanically induced production of NO is dependent on the oxygen tension of the culture environment. Very low oxygen tension (i.e., 1% O_2) can inhibit the endogenous production of NO from articular cartilage (Fermor *et al.*, 2005).

It is not entirely known how cyclic mechanical compression may also alter the oxygen tension of cartilage. Although oxygen can readily diffuse through cartilage, larger solutes are unable to be transported as easily (Leddy and Guilak, 2003; O'Hara *et al.*, 1990). Cyclic loading could, however, enhance the diffusion and transport of larger molecules which might alter the nutrition and metabolism of cartilage, and hence oxygen consumption. The loading regimen that is described, however, contains a static component in addition to the dynamic component (Piscoya *et al.*, 2005), and static compression of articular cartilage can reduce solute diffusion (Quinn *et al.*, 2001).

Interactions of nitric oxide with prostaglandins

Addition of IL-1 or TNFα or mechanical stress to articular cartilage also leads to increased production of the pro-inflammatory mediator prostaglandin E2 (PGE_2). In a number of cell types, NO appears to suppress PGE_2 production, and inhibition of NO can lead to further increases in PGE_2 production, in response to mechanical stress or inflammatory cytokines, in articular cartilage, meniscus, or macrophages (Amin *et al.*, 1997; Cernanec *et al.*, 2002; Fermor *et al.*, 2002; LeGrand *et al.*, 2001; Murrell *et al.*, 1995). These findings have important implications with respect to the potential clinical use of NOS inhibitors for the treatment of joint disease, and suggest that the inhibition of NOS might influence the inflammatory response of different joint tissues due to a "super induction" of PGE_2. Although the mechanism of the interaction between NO and PGE_2 are not fully understood, it is hypothesized that the inhibition of PGE_2 by NO is due to the nitration of the tyrosine residue (*Tyr* ^{385}) leading to decreased COX2 activity (Amin *et al.*, 1997). In other cell types such as macrophages it is suggested NOS2 binds, S-nitrosylates and activates cyclooxygenase (Kim *et al.*, 2005). Indeed it may be the formation of different NO derivatives such as peroxynitrite or nitrosothiols that may determine the biological response to NO induction (Clancy *et al.*, 2004). Similarly interactions can also occur between the NOS and lipoxygenase pathways, in articular cartilage, in response to mechanical stress (Fermor *et al.*, 2001a). There is little

evidence of constitutive lipoxygenase activity in chondrocytes, but when mechanical compression is applied in the presence of the NOS2 selective inhibitor 1400W, lipoxygenase and leukotriene B4 (LTB_4) are increased (Fermor *et al.*, 2001a).

It appears likely that NO and oxygen tension may dually govern IL-1α-induced PGE_2 production in articular cartilage (Cernanec *et al.*, 2002). The NOS2-selective inhibitor 1400W causes a significant increase in IL-1α-induced PGE_2 production at 1% O_2, but not under normoxic conditions. This oxygen-dependent increase in PGE_2 suggests that NO has an inhibitory effect on PGE_2 production, and that this effect is marked under very hypoxic culture conditions. This PGE_2 super-induction could result from diminished IL-1α-induced NO production under hypoxic conditions. A significant increase in PGE_2 production occurred after replenishing TNFα following 72 hours incubation at 20% O_2. This increase in PGE_2 corresponds with reduced NO production after replenishing TNFα, again suggesting that decreased NO may increase PGE_2 production. The increase in PGE_2 at this time point was not seen in IL-1α-stimulated cartilage, suggesting that IL-1α and TNFα might function differently to produce pro-inflammatory mediators and influence their interplay. Similarly, both oxygen tension and NO could alter mechanically induced PGE_2 production (Fermor *et al.*, 2005). The complexities of events occurring in application of mechanical compression to cartilage explants make this a challenging system to better understand further interactions between oxygen, NO and other pro-inflammatory mediators.

Conclusions

Oxygen tension is an important factor in determining the biology of articular cartilage. In osteoarthritic cartilage the level of NO induced by pro-inflammatory cytokines or mechanical stress can be modified by oxygen tension. The production of prostaglandins in OA is dually governed by oxygen tension and nitric oxide. The balance between NO, superoxide anions O_2^- and anti-oxidants such as SOD are critical in determining the biological effects of mechanically or cytokine induced NO production. We suggest the role of oxygen tension should be considered more thoroughly in the development of therapeutic strategies for intervention in the pathogenesis of arthritis.

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Discussion with Reviewers

V. Duance: In the Introduction you mention that the oxygen tension in the joint cavity is higher in a mobilized joint when compared with a static one and in the same sentence claim that mechanical loading leads to hypoxia. Please explain?
Authors: Mobilization of the joint can increase the oxygen supply to the joint. Inflammatory mediators are produced in response to inflammation or mechanical loading, and these mediators can also act to decrease the oxygen tension in the joint.

V. Duance: Do chondrocytes come under shear stress *in vivo*?
Authors: Yes, chondrocytes are subject to a number of mechanical stimuli *in vivo*, including stress, strain, and pressurization. *In vitro*, shear stress can influence chondrocyte production of nitric oxide, matrix components (Lane Smith *et al*., 2000; text reference), and signalling molecules (Hung *et al*., 2000; Yellowley *et al*., 1999). This indicates that in joint loading the mechanotransduction of shear stress in chondrocytes may have a direct effect on cellular metabolism and matrix maintenance.

V. Duance: In what way do you imagine that NO and oxygen tension dually govern IL-1 induced PGE_2 production?
Authors: Different oxygen tensions alter IL-1-induced NO production. Since NO can inhibit PGE_2 production in articular cartilage, alterations in IL-1-induced NO production will further influence IL-1-induced PGE_2 production. The interaction between NOS and COX pathways appears to be cell-type specific. In some cell types, NO can stimulate the COX pathway *via* S-nitrosylation of COX2 (Kim *et al*., 2005; text reference). In articular chondrocytes, NO inhibits COX2 possibly by nitration of COX2 (Amin *et al*., 1997; text reference). Oxygen tension therefore could influence the production of peroxynitrite or nitrosothiols and hence its ability to nitrate or nitrosylate COX2.

C.W. Archer: What are the implications of these findings in relation to osteoarthritis and rheumatoid arthritis?
Authors: Anti-inflammatory and disease-modifying drugs are used in the treatment of both OA and RA. A better understanding of the molecular interactions between reactive species and PGE_2 with oxygen tension may aid in the development of drugs with more selectivity for these diseases, as suggested by the studies on Diacerein and Rhein (Martin *et al*., 2003).
C.W. Archer: Is there any evidence that oxygen tension regulates the chondrogenic phenotype?
Authors: Yes. Compared with 20% oxygen, 5% oxygen tension can enhance the re-differentiation of de-differentiated chondrocytes in alginate (Domm *et al*., 2004; Murphy and Sambanis, 2001). The effects of oxygen tension on chondrogenesis of mesenchymal stem cells, or adipose derived adult stem (ADAS) cells, has also been investigated by different groups. Some variation in findings occurs due to culture conditions and the matrix in which the stem cells are grown. 5% oxygen enhances chondrogenesis of ADAS cells compared with 20% oxygen (Wang *et al*., 2005), and 2% O_2 inhibits chondrogenesis compared with 20% O_2 (Malladi *et al*., 2006). Oxygen gradients in tissue-engineered constructs have been measured and modelled mathematically to give further insight into the relation between oxygen tension and chondrogenesis (Malda *et al*., 2004).

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