Introduction

Under normal physiological circumstances, articular cartilage is exposed to a complex and diverse array of mechanical stresses and strains due to the diarthrodial joint. Mechanical stress and other biophysical factors are believed to act as 'signals' that regulate chondrocyte gene expression and metabolic activity, providing a mechanism for tissue adaptation to the functional demands of the body. However, abnormal joint loading (e.g., that associated with pain, immobilization, or joint instability) may lead to cellular and biochemical changes that are associated with cartilage degradation and the progression of arthritis.

The sequence of biomechanical and biochemical events regulating these processes in vivo is not well understood. The inflammatory characteristics of arthritis have been attributed in part to the actions of nitric oxide (NO) and prostanoids such as prostaglandin E₂ (PGE₂), products of NO synthase (NOS) and cyclo-oxygenase (COX) respectively. COX is a membrane bound heme protein which is localized to both the endoplasmic reticulum and the nuclear membrane. COX1 is constitutively expressed by many cells, whereas COX2 expression is induced by a range of stimuli such as cytokines, mitogens, hormones, and serum. Increased PGE₂ synthesis from COX2 in articular cartilage is a cellular response to activation by pro-inflammatory stimuli and an important component in the pathogenesis of arthritis. Osteoarthritic cartilage produces more PGE₂ than non-arthritic cartilage, and anti-inflammatory cytokines and glucocorticoids decrease prostanoid production. New COX2-specific inhibitors are rapidly replacing non-specific non-steroidal antiinflammatory drugs for the treatment of both rheumatoid arthritis (RA) and osteoarthritis (OA). These highly selective COX2 inhibitors exhibit potent antiinflammatory effects with significantly reduced gastric toxicity.

NO is implicated in the pathogenesis of RA and OA and increased production of NO occurs in response to physiological mechanical compression of articular cartilage and meniscus. NO is synthesized from L-arginine by the heme-containing enzyme NOS which requires nicotinamide–adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin, and molecular oxygen as cofactors to produce L-citrulline. Three isoforms of NOS have been identified by gene cloning (NOS1, NOS2 and NOS3).
Because of the short half-life of NO, its effects are primarily mediated locally. NO can inhibit or stimulate PGE₂ production due to interactions between NO and prostanoid production⁹⁻¹¹. Prostanoids can also reduce NOS2 expression and NO production²¹.

Cytokine stimulation of cartilage increases both COX2 and NOS2 activity¹⁶; however, the influence of physiologic and pathologic mechanical stress on the COX pathway, and the effects of COX pathway metabolites on the NOS pathway in articular cartilage is unknown. These two enzyme systems are believed to play a role in mechanical signal transduction in various cell types such as osteoblasts, osteocytes, and endothelial cells²²⁻²⁴. The goal of this study was to examine the hypothesis that intermittent mechanical compression influences NO and PGE₂ production by articular chondrocytes within their native extracellular matrix. Furthermore, we examined interactions between the NOS2/NO and COX2/PGE₂ systems to determine whether the activation of one enzyme pathway has significant influences on the other.

Materials and Methods

EXPLANT CULTURE

Full thickness explants of articular cartilage (5 mm diameter) were harvested from the femoral condyles of 2-year-old female pigs within 4 h of death. Explants were cultured in standard culture medium containing Dulbecco’s Modified Eagle Medium (DMEM) (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), 10 mM HEPES (Gibco, Gaithersburg, MD), 100 U/ml penicillin and streptomycin (Gibco, Gaithersburg, MD). Test and control explants were removed from adjacent sites on the joint surface and paired at harvest to account for site-dependent variations in the tissue. In order to determine the baseline rate of PGE₂ production into the culture medium, explants were cultured for 7 days in standard culture media; each day media were removed and replaced with standard culture medium. PGE₂ was measured and normalized to the wet weight of each explant.

COMPRESSION EXPERIMENTS

All compression experiments were performed after allowing explants to equilibrate in culture for 72 h after harvest. For each experiment, 12 pairs of explants were placed into individual compression wells in 1 ml of culture medium. Each specimen was subjected to a 10 gf tare load and allowed to equilibrate for 1 h. All experiments were performed at 37°C and 5% CO₂, 95% air. In certain experiments, inhibitors of COX2 or NOS2 were added to the culture medium before the 1 h equilibration time period to allow time for them to diffuse into the explants prior to beginning the loading regimen.

Compressive loads were applied to individual explants using a modified version of the Biopress system, using Biopress culture plates (Flexcell International, Hillsborough, NC), as described previously¹⁷. Mechanical loads were applied as a square waveform at 0.5 Hz (1 s on, 1 s off) corresponding to stress magnitudes of 0.05, 0.1, 0.5 or 1.0 MPa. The compressive stress was determined from the applied load and the initial cross-sectional area of the explant. All control specimens were cultured in an unloaded state. The duration of loading was one hour followed by 23 h recovery, at which time the PGE₂ level in the culture media was assessed. The time course of the response was determined by application of intermittent compression to the explants for 1 h at 0.1 MPa, 0.5 Hz in 1 ml of standard culture medium with or without the COX2-specific inhibitor NS398 (100 μM, [N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide], Cayman Chemical, Ann Arbor, MI). After 4 h and 8 h of recovery time, 200 μl of media were removed and replaced with 200 μl of stock medium. After 23 h of recovery time all media were removed.

To determine if PGE₂ induction by mechanical compression was dependent on COX2, explants were intermittently compressed for 24 h at 0.1 MPa, 0.5 Hz in the presence and absence of 100 μM NS398. The media were removed and explants and media frozen at −80°C until analysis.

To determine the potential role of NO on PGE₂ production, explants were intermittently compressed for 1 h at 0.1 MPa, 0.5 Hz in the presence or absence of the NOS2 selective inhibitor 1400W (3 mM, [N-{2-(cyclohexyloxy)-4-nitrophenyl}methanesulfonamide]. Alexis Chemical Co). After 24 h of recovery time media were removed and frozen at −80°C until analysis.

PGE₂ AND NO ASSAYS

PGE₂ production was measured using a commercially available ELISA kit (Prostaglandin E₂, Immunoassay, R&D Systems). The cross reactivity of the antibody with other prostanoids is 70% PGE₁, 16.3% PGE₂, 1.4% PGF₁α, 0.7% PGF₂α, 0.6%-keto-PGF₁α, 0.1% PGA₂, 0.1% PGB₁, and <0.1% 13,14-dihydroxy-PGF₁α, 6,15-keto-13,14-dihydro-PGF₂α and thromboxane B₂. PGE₂ concentrations were normalized to the wet weight of each explant measured prior to compression.

Nitric oxide production was assessed by measuring the concentration of nitrate and nitrite in the media by techniques previously described¹⁴. Briefly, nitrate was enzymatically reduced to nitrite by the addition of nitrate reductase (Boehringer Manneheim) 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% naphthylethylenediamine) (Sigma) were added followed by 10 min incubation at room temperature. Nitrite was determined spectrophotometrically with absorbance read at 540 nm and interpolated with a sodium nitrate (Sigma) standard curve. Results were expressed as NOₓ μmoles per gram wet weight per 24 h (μmole/g/24 h).

IMMUNOBLOTS

Immunoblots were performed using monoclonal antibodies; anti-COX1 or anti-COX2 (Transduction Laboratories, Lexington, KY) and detected using the enhanced chemiluminescence reagents from Amersham (Arlington Heights, IL) as previously described²⁵. For known negative and positive control extracts, untreated cells from the murine macrophage cell line J774 and cells of the murine macrophage cell lines J774 and RAW 264 were treated with murine IFN-γ (200 U/ml) and LPS (200 ng/ml) for 3 days. Twenty μg protein from the human and murine cells was used in the individual lanes.

FLUORESCENT CELL VIABILITY ASSAY

Cell viability was determined in cartilage explants following compression using fluorescence-based viability assay
Explants were washed in PBS and incubated for 20 min in 1 ml of PBS containing 2.5 µl of Calcein-AM as a label of viable cells and 2.5 µl of ethidium homodimer-1 as a label for dead cells. The cartilage slices were imaged using a confocal laser scanning microscope (LSM 510, Zeiss, Thornwood, NY).

STATISTICAL ANALYSIS

Statistical analysis was performed using a paired Student’s t-test for site-matched samples. For multiple comparisons, statistical differences were determined using an ANOVA with repeated measures and Duncan’s multiple range test. Statistical significance was reported at the 95% confidence level.

Results

ENDOGENOUS PRODUCTION OF PGE2 FROM ARTICULAR CARTILAGE

Uncompressed explants of articular cartilage exhibited significant production of PGE2 within the first 48 h following harvest (Fig. 1). PGE2 production decreased rapidly after 48 h in culture and remained constant for up to 7 days in culture. Therefore, all compression experiments were performed after explants were cultured for 72 h in DMEM plus 10% FBS after excision from the pig joint.

PGE2 PRODUCTION IN RESPONSE TO MECHANICAL COMPRESSION

Mechanical compression altered the rate of PGE2 production in a manner that was dependent on the magnitude of stress (Fig. 2). Compression at 0.1 MPa or 0.5 MPa for 1 h followed by 23 h recovery induced a significant increase in PGE2 production from articular cartilage explants relative to uncompressed controls. Significant changes in PGE2 production were not observed at 0.05 or 1.0 MPa. The increased production of PGE2 following 0.1 MPa compression for 1 h was observed as early as 4 h and continued to accumulate over the entire test period [Fig. 3(a)]. The COX2 inhibitor NS398 completely inhibited the mechanical induction of PGE2 at all time points [Fig. 3(b)].

Immunoblot analysis of the articular cartilage explants demonstrated that mechanical compression induced expression of COX2 (but not COX1) protein (Fig. 4). The relative optical density (arbitrary units) for each group was
11±1 (mean± S.E.M.) for 0 MPa control as compared to 234±6 for 0.1 MPa.

Mechanical compression for 24 h at 0.1 MPa significantly increased NO production. This increase was abolished in the presence of the NOS2 selective inhibitor, 1400W [Fig. 5(a)]. However, compression in the presence of 1400W significantly increased PGE2 production [Fig. 5(b)] with compression alone. The further increase in PGE2 production by the inhibition of NOS2 was paralleled by an increase in COX2 protein levels [Fig. 5(c)]. The relative optical density of COX2 (arbitrary units) for each group was 11±5 (0 MPa), 132±11 (0.1 MPa) and 177±9 (0.1 MPa+1400W).

Twenty-four hours of intermittent compression 0.1 MPa, 0.5 Hz caused a significant increase in PGE2 and NO. NS398 diminished both PGE2 and NO synthesis to the level of the non-compressed controls [Fig. 6(a,b)]. There was no loss in cell viability after any of the mechanical regimens tested in the presence or absence of the COX2 inhibitor, as determined by a fluorescent live/dead assay.

Discussion

Our findings demonstrate that mechanical compression of articular cartilage can significantly increase COX2 protein expression and PGE2 synthesis. Inhibition of the PGE2 response to compression with a COX2 specific inhibitor significantly decreased the production of NO, but inhibition of NO synthesis significantly enhanced COX2 synthesis and PGE2 production. These findings suggest PGE2 production by chondrocytes in vivo may be regulated in part by mechanical stress on the articular cartilage through a NO-dependent pathway. Mechanically induced PGE2 production may therefore play a role in the physiological or pathophysiological regulation of chondrocyte metabolism.

PGE2 is a pleiotropic bioregulator that can modulate expression of many target genes. The frequencies and magnitudes of mechanical stress used in our studies were selected to represent a physiologic range and are generally associated with increased proteoglycan synthesis in cartilage explants. Thus, the increased production of PGE2 (in the absence of NOS2 inhibitors) may act in a pro-anabolic capacity. This pro-anabolic effects of PGE2 on cartilage have previously been identified in vitro by demonstration of increased proteoglycan, DNA, and collagen synthesis.

PGE2 causes a biphasic response, with low concentrations of PGE2 increasing and high doses decreasing collagen synthesis. This biphasic effect has also been attributed to the ability of PGE2 to activate both the cAMP-protein kinase A and the Ca2+ and protein kinase C second messenger systems. These second messenger systems also mediate the response of chondrocytes to mechanical stress. One of the earliest events in the response of chondrocytes to mechanical stress may be an increase in the intracellular concentration of calcium ion as a result of cell deformation initiated through mechanosensitive ion channels. The increased Ca2+ may be responsible for the activation of phospholipase A2, an enzyme required for the release of arachidonic acid from the cell membrane to form PGE2.

PGE2 can also exert anticytotoxic and antiinflammatory effects by reducing the expression and synthesis of the inflammatory cytokines interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α), as well as NOS2 and metalloproteases (MMP1, MMP3). Previous studies...
have shown that cyclic tensile stretch of chondrocytes in monolayer reduces IL1-dependent activation of NOS2, COX2, MMP1, and the inhibition of tissue inhibitor of metalloprotease (TIMP)37. The activation of COX2 could also play an antiinflammatory role since COX2 mediates the synthesis of cyclopentenone prostaglandins such as 15d-PGJ238, lipid mediators that may exert anti-inflammatory activity through activation of peroxisome proliferator-activated receptor-γ (PPARγ)39,40. The activation of the PPARγ pathway inhibits mouse and human osteoclast differentiation, blocking the effects of M-CSF and osteoprotogerin ligand-induced osteoclast formation and activity41.

Our finding that a NOS2-specific inhibitor enhances PGE2 production in response to mechanical stress is consistent with previous studies showing that inhibition of NO-enhanced PGE2 production in human osteoarthritic articular cartilage and LPS-stimulated macrophages30,42. These findings have important implications with respect to the potential clinical use of NOS2 inhibitors for the treatment of joint disease46, and suggest that the inhibition of NOS2 could potentially increase the inflammatory response of the chondrocytes due to a ‘superinduction’ of PGE2. Although the mechanism of this interaction is not fully understood, the inhibition of PGE2 production by NO may be due to decreased expression and nitration of the tyrosine residue (Tyr385) of COX24. NO can also inhibit the translocation of COX2 to a cytosolic compartment that favors enzyme activity44.

A better understanding of such interactions between the NOS and COX pathways may provide important insights into the potentially ‘protective’ role of NO in arthritis. Previous studies have shown that the selective NOS2 inhibitor L-NIL can exacerbate joint disease in streptococcal cell wall-induced arthritis in rats, whereas L-NMMA, a non-selective inhibitor of both constitutive and inducible forms of NOS, prevented intraarticular accumulation of leukocytes, joint swelling, and bone erosion45. The route of administration of NOS2 inhibitors can result in different effects on the acute inflammatory response in vivo, suggesting that local production of NO has a protective role whereas systemically NO can be destructive46. In other studies, NOS inhibitors have also been shown to decrease PGE2 production19, or have no effect on the COX pathway47. Differences in concentration, timing and source (endogenous versus exogenous) of NO can also lead to different results in vitro.

In summary, our findings indicate that intermittent mechanical stress of articular cartilage significantly increases PGE2 production at certain magnitudes of stress. Inhibition of mechanically induced PGE2 and NO production by COX2 inhibitors suggests that the antiinflammatory effects of COX2 inhibitors may be attributed to their influences on the NO pathway as well as the prostaglandin pathway. Furthermore, our findings indicate that significant interactions exist between the NO and PGE2 pathways, suggesting that the clinical use of NOS2 or COX2 inhibitors for arthritis may have significant effects on the physiological response of chondrocytes to their mechanical environment.

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References


