Dynamic compression counteracts IL-1β-induced release of nitric oxide and PGE₂ by superficial zone chondrocytes cultured in agarose constructs

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Summary

Objective: To examine the effect of IL-1β-induced ·NO and PGE₂ release by stimulated superficial and deep chondrocyte/agarose constructs subjected to mechanical compression.

Design: Chondrocyte sub-populations were seeded separately in agarose constructs and cultured unstrained, within a 24-well tissue culture plate, for 48 h in medium supplemented with IL-1β and/or L-(1-iminoethyl)-ornithine (L-NIO). In a separate experiment, superficial and deep cell containing constructs were subjected to 15% dynamic compressive strain at 1 Hz, for 48 h, in the presence or absence of IL-1β and/or L-NIO. Nitrite was measured using the Griess assay, PGE₂ release was determined using an EIA kit and [3H]-thymidine and 35SO₄²⁻ incorporation were assessed by TCA and alcian blue precipitation, respectively.

Results: The current data reveal that IL-1β significantly enhanced ·NO and PGE₂ release for superficial chondrocytes, an effect reversed with L-NIO. ·NO and PGE₂ levels did not significantly change by deep cells in the presence of IL-1β and/or L-NIO. For both cell sub-populations, IL-1β inhibited cell proliferation whereas proteoglycan synthesis was not affected. Dynamic compression inhibited the release of ·NO and PGE₂ in the presence and absence of IL-1β, for cells from both sub-populations. L-NIO reduced ·NO and enhanced PGE₂ release for superficial zone chondrocytes, an effect not observed for deep cells in response to dynamic compression. The magnitude of stimulation of [3H]-thymidine incorporation was similar for both cell sub-populations and was not influenced by L-NIO, indicating an ·NO-independent pathway. The dynamic compression-induced stimulation of 35SO₄²⁻ incorporation was enhanced with L-NIO for IL-1β-stimulated deep cells, indicating an ·NO-dependent pathway.

Conclusion: The present findings suggest that dynamic compression inhibits ·NO and PGE₂ release in IL-1β-stimulated superficial cells via distinct pathways, a significant finding that may contribute to the development of intervention strategies for the treatment of inflammatory joint disorders.

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Introduction

The progressive deterioration and loss of articular cartilage during osteoarthritis (OA) and rheumatoid arthritis (RA) typically involves an imbalance between the anabolic and catabolic pathways and lead to an irreversible impairment of cartilage structure and function. Cartilage degradation is characterised by a focal loss of the load-bearing surface due to an upregulation of the catabolic pathways not involved in normal mechanotransduction processes. Many studies have demonstrated that pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor α (TNFα), act as key mediators of cartilage degradation. Associated events include suppression of matrix synthesis, activation of metalloproteinase (MMP) activity, free radical species production, inhibition of chondrocyte proliferation and induction of apoptotic cell death. These catabolic effects typically involve the sequential release of nitric oxide (·NO), a diatomic free radical, which can further stimulate other catabolic mediators such as prostaglandin E₂ (PGE₂) or reactive oxygen intermediates. ·NO is produced by a family of enzymes, collectively termed ·NO synthase (NOS), which includes both constitutively expressed isoforms (ecNOS and nNOS) and an inducible isoform (iNOS). The activity of all NOS isoforms can be inhibited by structural analogues of l-arginine, such as L-(1-iminoethyl)-ornithine (L-NIO). Various studies have demonstrated the overproduction of iNOS and ·NO in OA and RA cartilage compared with normal tissue. Furthermore, a number of studies have reported that the superficial chondrocytes synthesise ·NO at a greater rate than deep zone cells in response to IL-1β. Moreover, it has been demonstrated recently that ·NO mediates the inhibition of proteoglycan synthesis in chondrocyte sub-populations.

Several studies have examined the effects of physical forces on ·NO and PGE₂ release. It has been demonstrated that mechanical loading inhibits both ·NO and PGE₂ release, in the presence or absence of IL-1β, in full-depth chondrocytes. It is known, however, that superficial
and deep zone chondrocytes, seeded separately in agarose constructs, exhibit a differential response to mechanical stimulation. In the absence of IL-1β, dynamic compressive strain applied at a range of physiological frequencies altered proteoglycan synthesis and cell proliferation in a sub-population dependent manner. Using a well-characterised cell-straining system, the present study tests the hypothesis that dynamic compression counteracts the IL-1β-induced synthesis of both NO and PGE₂ by stimulated superficial and deep chondrocytes, seeded separately in agarose constructs.

Materials and methods

ISOLATION OF CHONDROCYTE SUB-POPULATIONS

Bovine articular chondrocytes were obtained from the metacarpalphalangeal joints of freshly slaughtered 18-month-old steers. Thin slices of cartilage representing the uppermost 15–20% of the total uncalcified tissue depth, as detailed by Lee et al., were removed from the proximal joint surface. The cells isolated from these slices were termed superficial cells. The residual cartilage/tissue was harvested taking care to avoid involvement of calcified cartilage. The cells isolated from these slices were termed deep cells. Representative slices of superficial and deep tissue were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.2, processed to wax, sectioned and stained with haematoxylin and safranin-O.

The cartilage slices were washed in 5 ml of Earle’s balanced salt solutions (EBSS; Sigma Chemical Co., Poole, UK) and transferred to a 35 mm tissue culture dish containing 5 ml of Dulbecco’s minimal essential medium supplemented with 20% (v/v) foetal calf serum (FCS), 2 mM L-glutamine, 20 mM HEPES, 100 unit ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 150 µg ml⁻¹ L-ascorbic acid (DMEM+20% FCS; Gibco, Paisley, UK). The tissue samples were diced finely using a scalpel and incubated at 37°C on rollers for 20 min. Using a sterile corer, cylindrical constructs, approximately 40×35×5 mm and allowed to gel at 4°C for 20 min. The tissue was poured into sterile Perspex moulds, measuring 5 mm in diameter and 5 mm in height, were cut and cultured in DMEM+20% FCS at 37°C in 5% CO₂ for 24 h. Superficial and deep cell containing constructs were subsequently cultured, unstrained, within a 24-well tissue culture plate, for 48 h in 1 ml of DMEM+20% FCS supplemented with 100 unit ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 2m ML-glutamine, 20 mM HEPES, 100 unit ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 150 µg ml⁻¹ L-ascorbic acid (DMEM+20% FCS; Gibco, Paisley, UK) and transferred to a 35 mm tissue culture dish (Costar, High Wycombe, UK) and mounted on a well-characterised cell-straining apparatus (Zwick Testing Machines Ltd, Leominster, UK) and subjected to 15% amplitude dynamic strain (0–15%) applied in a sinusoidal waveform at 1 Hz for 48 h.

APPLICATION OF MECHANICAL STRAIN

A well-characterised cell-straining apparatus (Zwick Testing Machines Ltd, Leominster, UK) was used to apply dynamic compression to chondrocyte/agarose constructs, as described previously. Superficial and deep cell containing constructs were transferred into a 24-well culture plate (Costar, High Wycombe, UK) and maintained within the apparatus. One millilitre of DMEM+20% FCS+1 µCi ml⁻¹ [³H]-thymidine and 10 µCi ml⁻¹ ³⁵SO₄, supplemented with 10 ng ml⁻¹ IL-1β and/or 1 mM L-NIO, was introduced into each well. One group of constructs was subjected to 15% amplitude dynamic strain (0–15%) applied in a sinusoidal waveform at 1 Hz for 48 h. A second group of constructs remained unstrained, but was maintained within the incubator of the cell-straining apparatus. All constructs were incubated at 37°C/5% CO₂ for 48 h.

BIOCHEMICAL ANALYSIS

Absolute concentrations of nitrite, a stable end-product of NO metabolism, were determined in the media of cultured cells using a spectrophotometric method based on the Griess assay, as previously described. Absorbance was measured at 550 nm and nitrite concentration was determined by comparison with standard solutions of sodium nitrite. PGE₂ release was determined in the culture supernatant using a high sensitivity commercially available EIA kit (Amersham Pharmacia Biotech, Buckinghamshire, UK), as previously described. Each sample was incubated with reconstituted antibody and peroxidase conjugate. The absorbance was measured at 450 nm, using PGE₂-diluted in assay buffer as a stock standard over the concentration range 20–640 pg ml⁻¹. The sensitivity of the assay is equivalent to 16 pg ml⁻¹. [³H]-thymidine incorporation was measured in the agarase/papain digests by 10% (v/v) trichloroacetic acid precipitation onto filters using the Millipore Multiscreen system (Millipore, Watford, UK), as described previously. Total DNA, determined using the alcian blue precipitation method, as described by the authors. Total DNA, determined using the Hoescht 33258 method, was used as a baseline for ³⁵SO₄ incorporation and [³H]-thymidine incorporation.

STATISTICAL ANALYSIS

Two-way ANOVA with post hoc Bonferroni-corrected t-tests was used to examine inter- and intra-group differences for cultured and absolute data. Unpaired Student’s t-tests were used to examine normalised data. In all cases, a level of 5% was considered statistically significant (P<0.05).
Results

CELL VIABILITY AFTER ENZYMATIC DIGESTION

Representative micrographs prepared from haematoxylin and safranin-O stained sections of superficial and deep cartilage slices are presented in Fig. 1. The total tissue thickness was approximately 300 µm. The superficial tissue slices were approximately 60 µm in thickness (20% of total tissue thickness) and the deep tissue slices were approximately 240 µm in thickness (80% of total tissue thickness). The pronase and collagenase isolation procedure resulted in complete digestion of both the superficial and deep tissue which yielded 31±4.1 and 69±4.4% of cells, respectively. The cell viability was 96±4.4% for the superficial cells and 98±0.7% for the deep cells, a difference which was not statistically significant (P>0.05).

INFLUENCE OF IL-1β ON CULTURED CELL SUB-PopULATIONS

Figure 2 illustrates the effects of IL-1β on nitrite release by superficial and deep cells, seeded separately in unstrained constructs cultured free-floating within a tissue culture plate. In the absence of IL-1β, nitrite release was significantly greater in the superficial cells than the deep cells (P<0.001) [Fig. 2(A)]. The cytokine induced a threefold increase (P<0.001) in the superficial cell constructs, compared to a non-significant increase for the deep cell constructs. This resulted in a 10-fold (P<0.001) difference in nitrite release between IL-1β-stimulated cell sub-populations. L-NIO significantly inhibited the IL-1β-induced nitrite release for both cell sub-populations (P<0.001 and P<0.01, for superficial and deep cells, respectively). PGE₂ release was significantly greater (P<0.05) by deep cells compared to superficial cells cultured in the absence of IL-1β, as illustrated in Fig. 2(B). However, PGE₂ levels for deep cells did not significantly change in the presence of IL-1β and/or L-NIO. By contrast, IL-1β induced a threefold increase (P<0.001) in PGE₂ release for superficial cells such that the levels measured were significantly greater (P<0.001) when compared to the deep cells. IL-1β-induced PGE₂ release was significantly reduced in the presence of L-NIO for the superficial cells, only (P<0.01). IL-1β inhibited [³H]-thymidine incorporation for superficial and deep cell constructs (P<0.001 and P<0.01, respectively) and this
effect could be partially reversed with L-NIO for both cell sub-populations [Fig. 2(C)]. By contrast, 35SO4 incorporation was not significantly affected by IL-1β, either in the presence or absence of L-NIO, for both cell sub-populations [Fig. 2(D)].

EFFECT OF DYNAMIC COMPRESSION ON IL-1β-STIMULATED CELL SUB-POPULATIONS

Absolute and normalised values for nitrite and PGE2 release, [3H]-thymidine incorporation and 35SO4 incorporation by cell sub-populations cultured within unstrained constructs and constructs subjected to dynamic strain are presented in Table I and Fig. 3, respectively. In unstrained superficial and deep cell constructs maintained within the cell-straining apparatus, IL-1β stimulated nitrite synthesis by 72% (P<0.001) and 30% (P<0.01), respectively. This effect was reversed by dynamic compression for both sub-populations (both P<0.01, Table I). The magnitude of the compression-induced inhibition of nitrate release was significantly reduced in the presence of L-NIO for IL-1β-stimulated superficial cells, only (P<0.05) [Fig. 3(A)]. IL-1β enhanced PGE2 release over twofold (P<0.001) in the unstrained superficial cell constructs compared to a minimal increase for unstrained deep cells (Table I). Dynamic compression reduced levels of PGE2 release both in the presence or absence of IL-1β, for superficial and deep cells, as presented in Table I and Fig. 3B. The presence of L-NIO enhanced compressive strain-induced inhibition of PGE2 release for superficial cells (P<0.05) compared to a reduction for deep cells [Fig. 3(B)].

In unstrained and strained constructs, [3H]-thymidine incorporation was significantly greater for deep cells (P<0.001) compared to superficial cells (P<0.01) in the presence and absence of IL-1β (Table I). IL-1β reduced [3H]-thymidine incorporation in unstrained constructs for both cell sub-populations (both P<0.001), although this effect was partially reversed by the addition of L-NIO (both P<0.01). For each test condition, dynamic compressive strain enhanced [3H]-thymidine incorporation for both cell sub-populations (Table I). However, with the exception of IL-1β-stimulated superficial cells, the magnitude of enhancement by dynamic compressive strain was not significant [Fig. 3(C)]. Furthermore, the addition of L-NIO did not influence the strain-induced stimulation of [3H]-thymidine incorporation. 35SO4 incorporation was greater in deep cells than in superficial cells for all test conditions (P<0.001, in all cases, Table I). With the exception of the results associated with IL-1β, the increases in 35SO4 incorporation due to dynamic stimulation of deep cells were statistically significant (P<0.05). By contrast, the increases due to dynamic stimulation of superficial cells were not found to be significant [Fig. 3(D)].

Discussion

The current study utilises the well-characterised chondrocyte/agarose model system and dynamic compression to investigate the mechanotransduction pathways that
Table I

Absolute values for nitrite production, PGE$_2$ synthesis, $[^3]$H-thymidine incorporation and $^{35}$SO$_4$ incorporation by chondrocyte sub-populations seeded in 3% agarose and subjected to 15% dynamic compressive strain, at 1 Hz for 48 h

<table>
<thead>
<tr>
<th></th>
<th>Unstrained</th>
<th>Dynamic compressive strain (1 Hz)</th>
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<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>10 ng ml$^{-1}$ IL-1β</td>
</tr>
<tr>
<td></td>
<td>Superficial</td>
<td>Deep</td>
</tr>
<tr>
<td>Nitrite release (µM)</td>
<td>18.9 (±0.97)</td>
<td>9.1 (±0.55)</td>
</tr>
<tr>
<td>PGE$_2$ release (pg.ml$^{-1}$)</td>
<td>109.2 (±11.4)</td>
<td>118.1 (±8.4)</td>
</tr>
<tr>
<td>$[^3]$H-Tdr incorporation (cpm per µg DNA)</td>
<td>45.0 (±3.7)</td>
<td>135.9 (±21.1)</td>
</tr>
<tr>
<td>$^{35}$SO$_4$ incorporation (µmole µMO$_4$/hour/µg DNA)</td>
<td>0.013 (±0.0019)</td>
<td>0.059 (±0.0052)</td>
</tr>
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</table>

Note: Each value represents the mean and s.e.m. of between 12 and 24 replicates from three separate experiments. ANOVA with post hoc Bonferroni-corrected t-test indicates significant differences between unstrained and strained values as follows: *P<0.05; **P<0.01; ***P<0.001.
involve \( \cdot \text{NO} \) and PGE\(_2\) in IL-1\(\beta\)-stimulated chondrocyte sub-populations isolated from distinct regions of articular cartilage. The tissue used in this study was approximately 300 \( \mu \)m in thickness and histological examination revealed that the superficial tissue removed from the joint contained zone I and a proportion of zone II cartilage, while the deep tissue represented zone III and a proportion of zone II cartilage (Fig. 1). The chondrocyte isolation protocol produced complete digestion of both superficial and deep tissue, and chondrocyte viability was over 96\% in both cases. There was no evidence, therefore, for selection of sub-populations of cells due to incomplete digestion or loss of viability.

L-NIO was used to inhibit the IL-1\(\beta\)-induced \( \cdot \text{NO} \) and PGE\(_2\) release in chondrocyte sub-populations. L-NIO is a non-specific inhibitor of all the NOS isoforms, with identical \( K_i \) values (3.9 \( \mu \)M) for ecNOS and iNOS\(^{38}\). L-NIO has been shown not to affect chondrocyte metabolism in the absence of IL-1\(\beta\)\(^{39}\).

The current data reveal significantly greater levels of \( \cdot \text{NO} \) release by IL-1\(\beta\)-stimulated superficial chondrocytes, compared to deep chondrocytes (Fig. 2(A)). The effects were reversed in the presence of the NOS inhibitor L-NIO, suggesting that \textit{de novo} synthesis of \( \cdot \text{NO} \) mediates the process. These findings are in agreement with a number of previous studies investigating cytokine stimulation of chondrocyte sub-populations \textit{in situ} or isolated and maintained in monolayer culture or in agarose or alginate gel culture\(^{20-24}\). PGE\(_2\) release was enhanced in IL-1\(\beta\)-stimulated superficial cells and the effect was partially reversed by L-NIO, indicating that the response is mediated by \( \cdot \text{NO} \) (Fig. 2(B)). By contrast, PGE\(_2\) release by deep zone chondrocytes was unaffected by either IL-1\(\beta\) or L-NIO. The differential effect may be due to a greater responsiveness to IL-1\(\beta\) by superficial cells, which are known to express a greater number of IL-1\(\beta\) receptors than deep zone chondrocytes\(^{20}\). Thus, the current data, together with the previous studies cited above, indicate that the superficial cells are a major source of \( \cdot \text{NO} \) and PGE\(_2\) when stimulated with IL-1\(\beta\). Accordingly, the cartilage surface may be more susceptible to degradation mediated by catabolic factors present within the synovial fluid during inflammatory joint disease.

The superficial cells exhibited reduced rates of proliferation and proteoglycan synthesis compared to deep cells (Fig. 2(C and D)), results of which are in agreement with previous studies\(^{35,40,41}\). Cell proliferation was inhibited by IL-1\(\beta\) for both cell sub-populations and the effect could be partially reversed in the presence of L-NIO, suggesting that this effect is mediated by \( \cdot \text{NO} \). These findings are in agreement with a previous study, which utilised full-depth chondrocytes\(^{13}\). By contrast, proteoglycan synthesis was not influenced by IL-1\(\beta\) or L-NIO in either sub-population. These data conflict with previous studies which reported an \( \cdot \text{NO} \)-mediated inhibition of proteoglycan synthesis in IL-1-stimulated bovine and human chondrocyte sub-populations cultured in monolayer or alginate gels\(^{22,23}\). However, such discrepancies may be due to differences in species or the model systems used. Indeed it has been reported previously that proteoglycan synthesis is not influenced by \( \cdot \text{NO} \) release in full-depth bovine chondrocytes cultured in agarose constructs\(^{13}\).
This study utilised a well-characterised cell-straining system for the application of 0–15% dynamic compressive strain to chondrocytes cultured in agarose. These values concur with previous studies which indicate that local strains range from 5 to 50% during prolonged static compression of bovine cartilage. Dynamic compression, however, these values will be reduced in both intact cartilage and agarose, due to viscoelastic nature of both systems. The nature of cell deformation is, however, known to be different in agarose and in intact cartilage due to differences in lateral confinement and osmolarity changes associated with the presence of a charged extracellular matrix (ECM), in situ. In a previous study, cell deformation on application of 15% dynamic compressive strain was identical for both sub-populations after 24 and 72 h in culture. Therefore, all dynamic experiments were performed between this time period. It could be argued that beyond this period, the increasing elaboration of ECM would lead to a changing mechanical environment associated with the cell.

Previous work has demonstrated that dynamic compression counteracted the effects of IL-1β on the release of NO and PGE2 by full-depth chondrocytes. A similar response was demonstrated for both superficial and deep cells whereby dynamic compression inhibited the release of NO and PGE2 in the presence and absence of IL-1β, as presented in Table I. Furthermore, the dynamic compression-induced inhibition of NO release by superficial zone cells was reduced with L-NIO, implicating NOS activity in the process. By contrast, the dynamic compression-induced inhibition of PGE2 release by IL-1β-stimulated superficial cells was further enhanced in the presence of L-NIO. A similar super-induction of PGE2 in the presence of NOS inhibitors has been reported previously in IL-1β-stimulated chondrocytes cultured in monolayer or osteoarthritic cartilage explants. The present findings suggest that IL-1β and dynamic compression influence PGE2 release in superficial cells via distinct pathways, which may exhibit a differential dependency on NO. L-NIO did not influence the magnitude of dynamic compression-induced inhibition of PGE2 release by IL-1β-stimulated deep cells, suggesting that the process is independent of NO. Accordingly, it appears that intrinsic differences in the behaviour of the cell sub-populations exist and their response to IL-1β and dynamic compression may occur via distinct pathways.

Dynamic compression stimulated [3H]-thymidine incorporation for both cell sub-populations under all test conditions although the differences were not statistically significant in the majority of cases (Table I). Although absolute levels of [3H]-thymidine incorporation were significantly greater for deep cells compared to superficial cells, the magnitude of stimulation was similar, suggesting that both sub-populations exhibit a similar response to dynamic loading. For both cell sub-populations the magnitude of dynamic compression-induced stimulation of [3H]-thymidine incorporation was not altered in the presence of L-NIO, indicating an NO-independent pathway. Incorporation data suggest that dynamic stimulation enhances proteoglycan synthesis in deep cells in the absence of IL-1β, which is in agreement with a previous study. The dynamic compression-induced stimulation was enhanced with L-NIO for IL-1β-stimulated deep cells, indicating an NO-dependent pathway. No significant differences in 35SO4 incorporation were detected in superficial cells when subjected to dynamic compression.

The results of the present study should be examined in conjunction with related previous studies, as summarised in Table II. It is evident that the nature of the response, in terms of NO, PGE2, cyclooxygenase-2 (COX-2) and glycosaminoglycan (GAG), is dependent on the cell type and mode of mechanical stimulation. It is also clear that further studies are required to elucidate the complexity of the intracellular signalling mechanisms. The present study demonstrates that the superficial cells have a greater capacity to synthesise NO and PGE2 and that dynamic compression, a physiologically relevant loading regime, can counteract these effects in IL-1β-stimulated chondrocytes. Thus, dynamic compression can act as an anti-inflammatory signal in IL-1β-stimulated chondrocyte sub-populations. As the release of NO and PGE2 and the influence of dynamic compression on the two mediators were less pronounced in deep cells, it would appear that different intracellular signalling mechanisms are occurring.
between superficial and deep chondrocytes. Since NO and PGE2 production by articular cartilage is implicated in the development and progression of OA, the inhibition of these inflammatory mediators by dynamic compression is a significant finding, which could have relevance in the treatment or prevention of joint disorders.

Acknowledgement
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