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Anti-inflammatory effects of IL-4 and dynamic compression in IL-1β stimulated chondrocytes

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Abstract

Mechanical loading can counteract inflammatory pathways induced by IL-1β by inhibiting ·NO and PGE_2, catabolic mediators known to be involved in cartilage degradation. The current study investigates the potential of dynamic compression, in combination with the anti-inflammatory cytokine, IL-4, to further abrogate the IL-1β induced effects. The data presented demonstrate that IL-4 alone can inhibit nitrite release in the presence and absence of IL-1β and partially reverse the IL-1β induced PGE_2 release. When provided in combination, IL-4 and dynamic compression could further abrogate the IL-1β induced nitrite and PGE_2 release. IL-1β inhibited [^{3}H]thymidine incorporation and this effect could be reversed by IL-4 or dynamic strain alone or both in combination. By contrast, $^{35}SO_4$ incorporation was not influenced by IL-4 and/or dynamic strain in IL-1β stimulated constructs. IL-4 and mechanical loading may therefore provide a potential protective mechanism for cartilage destruction as observed in OA.

Keywords: IL-4; IL-1β; ·NO; PGE_2; Mechanotransduction; Dynamic compression; Anti-inflammatory; Osteoarthritis

The biomechanical environment of the chondrocyte is critical in maintaining normal joint function. However, under abnormal conditions, the induction of the pathophysiological pathways may result in the development of osteoarthritis (OA) and the destruction of articular cartilage [1–3]. OA is associated with an increased synthesis of interleukin-1 (IL-1), a pro-inflammatory cytokine responsible for mediating the breakdown of the cartilage extracellular matrix [4,5]. This process, which is known to inhibit proteoglycan synthesis and chondrocyte proliferation, involves the release of matrix metalloproteinases (MMPs) [6–8]. As a consequence, antagonists for IL-1 are prime targets for therapeutic approaches [9–12]. However, the mechanisms critical to OA pathology are complicated and, as such, require further examination.

It is well known that nitric oxide (·NO) and prostaglandin E_2 (PGE_2) are capable of mediating the pathological components associated with OA [6,7,13]. Overexpression of the nitric oxide synthase (NOS) and cyclooxygenase (COX) enzymes has resulted in the development of specific COX inhibitors, which inhibit the key enzymes that metabolise arachidonic acid to prostaglandins [14,15]. These inhibitors have been used clinically in the treatment of OA [16,17]. However, pleiotropic effects of the different eicosanoids on chondrocyte function in vitro have led to unwanted side effects as demonstrated in recent FDA approved clinical trials [18,19].

A number of key targets for alternative drug strategies for the treatment of OA have recently been identified. For instance, interleukin-4 (IL-4), interleukin-10 (IL-10), and interleukin-13 (IL-13) have been shown to reduce pro-inflammatory cytokine production and/or activity [20–22]. This protection against cartilage destruction has resulted in the development of IL-4 treatment for collagen-induced arthritis in the knee joint of mice [23–25]. Mechanical loading at physiological frequencies has been shown to counteract the IL-1β mediated inhibitory effects on cell metabolism [26,27]. Mechanical stimulation has also

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been shown to induce the paracrine/autocrine secretion of IL-4, a process mediated by the α5β1-integrin [28–30]. However, it is unclear whether these anti-inflammatory stimuli in combination would act to further abrogate IL-1β-induced ·NO and PGE_2 release, in an additive or synergistic manner. Accordingly, the present study tests the hypothesis that IL-4 in combination with dynamic compression can alter the synthesis of ·NO and PGE_2 release by IL-1β stimulated bovine chondrocytes cultured in agarose constructs.

Materials and methods

Preparation of chondrocyte/agarose constructs. Full-depth slices of cartilage were removed from the metacarpalphalangeal joints of 18-month-old cattle (Dawn Cardington, Bedfordshire, UK). The cartilage slices were washed, diced finely using a scalpel, and incubated at 37 °C on rollers for 1 h in Dulbecco's modified Eagle's medium supplemented with 20% (v/v) foetal calf serum, 2 μM L-glutamine, 5 μg ml^{-1} penicillin, 5 μg ml^{-1} streptomycin, 20 mM Hepes buffer, and 0.85 μM L-ascorbic acid (DMEM + 20% FCS) + 700 U ml^{-1} pronase, and for a further 16 h at 37 °C in DMEM + 20% FCS supplemented with 100 U ml^{-1} collagenase type XI (All Sigma Chemical, Poole, UK). The released chondrocytes were passed through a 70-μm pore size cell sieve (Falcon, Oxford, UK), washed twice in DMEM + 20% FCS, and finally resuspended in medium to give a cell concentration of 8×10^6 cells ml^{-1}. The chondrocyte suspension was added to an equal volume of molten 6% (w/v) agarose type VII (Sigma Chemical, Poole, UK) in Earle's Balanced Salt Solution (EBSS, Sigma Chemical, Poole, UK) to yield a final cell concentration of 4×10^6 cells ml^{-1} in 3% (w/v) agarose. The chondrocyte/agarose suspension was transferred into a sterile stainless steel mould, containing holes 5 mm in diameter and 5 mm in height. The chondrocyte/agarose suspension was allowed to gel at 4 °C for 20 min to yield cylindrical constructs, which were subsequently cultured in DMEM + 20% FCS at 37 °C in 5% CO_2 for 24 h.

Effect of IL-4 in free-swelling constructs. Chondrocyte/agarose constructs were cultured under free-swelling conditions for a further 48 h in DMEM + 20% FCS supplemented with 0, 0.1, 1, 10, and 100 ng ml^{-1} human recombinant IL-4 (Calbiochem, Nottingham, UK), either in the presence or absence of 10 ng ml^{-1} human recombinant IL-1β (PeproTech EC, London, UK) and/or 1 mM L-*N*-(1-iminoethyl)-ornithine (L-NIO) (inhibits all isoforms of NOS) (Calbiochem, Nottingham, UK). All constructs were additionally incubated in radiolabelled medium containing 1 μCi ml^{-1} [^{3}H]thymidine and 10 μCi ml^{-1} $^{35}SO_4$ (both Amersham Biosciences, Bucks, UK) for the assessment of chondrocyte proliferation and proteoglycan synthesis, respectively.

Application of mechanical compression. A fully characterised cell-straining system (Zwick Testing Machines, Leominster, UK) was used to apply dynamic compression to chondrocyte/agarose constructs, as detailed previously [31–33]. Constructs were equilibrated in culture for 24 h, transferred into a 24-well culture plate (Costar, High Wycombe, UK), and mounted within the apparatus. One millilitre of radiolabelled medium containing 0 or 10 ng ml^{-1} IL-4, either in the presence or absence of 10 ng ml^{-1} IL-1β or 1 mM L-NIO, was introduced into each well. Strained constructs were subjected to a maximum strain amplitude of 15%, with a range between 0 and 15%, and a sinusoidal waveform and frequency of 1 Hz for 48 h. Unstrained control constructs, subjected to a tare strain of approximately 0.8% and identical boundary conditions to the strained constructs, were maintained within the cell-straining apparatus [31,33]. Both groups of constructs were incubated for 48 h at 37 °C/5% CO_2.

Biochemical analysis. At the end of the culture period, the constructs and corresponding media were removed and frozen at −20 °C. The subsequent biochemical analysis has been previously detailed by the authors [26,27,31–33]. To review briefly, constructs were digested overnight at 37 °C with 10 U ml^{-1} agarose and for 1 h at 60 °C with 2.8 U ml^{-1} papain (both Sigma Chemical, Poole, UK). Absolute concentrations of nitrite (μM), a stable end-product of ·NO metabolism, were measured in the media using a spectrophotometric method based on the Griess assay [26,27,32]. PGE_2 production was measured in the culture supernatant, using a high sensitivity enzyme immunoassay kit (Amersham Biosciences, Bucks, UK) [26,27]. [^{3}H]thymidine and $^{35}SO_4$ incorporation was determined using the trichloroacetic acid and Alcian blue precipitation method [31,33]. Total DNA, determined using the Hoecsht 33258 method, was used as a baseline for [^{3}H]thymidine and $^{35}SO_4$ incorporation [34].

Statistical analysis. Unless indicated, all data represent means and SEM values of 12 replicates from two separate experiments. Two-way ANOVA with post hoc Bonferroni-corrected *t* tests were used to examine differences between unsupplemented, IL-1β, and IL-1β + L-NIO constructs at all IL-4 concentrations. post hoc Bonferroni-corrected *t* tests comparisons were made between unstrained and strained constructs for the different test conditions examined. In addition, Unpaired Student's *t* tests were used to examine normalised data between unsupplemented and treated constructs. In all cases, a level of 5% was considered statistically significant ($*p < 0.05$).

Results

Effect of IL-4 in free-swelling constructs cultured in the presence and absence of IL-1β

In the absence of IL-1β, IL-4 significantly inhibited nitrite release over concentrations ranging from 0.1 to 100 ng ml^{-1} IL-4, as illustrated in Fig. 1A ($p < 0.05$ and $p < 0.001$). Supplementing the culture media with IL-1β significantly increased nitrite release (all $p < 0.001$). This response was inhibited by the presence of IL-4 (all $p < 0.001$). At all IL-4 concentrations tested, the IL-1β-induced nitrite release was abolished with L-NIO (all $p < 0.001$).

In the absence of IL-1β, no significant differences in PGE_2 levels were found for constructs cultured with different concentrations of IL-4, as illustrated in Fig. 1B. The presence of IL-1β significantly induced PGE_2 release (all $p < 0.001$). This effect was partially reversed with IL-4, in a dose-dependent manner at concentrations ranging from 1 to 100 ng ml^{-1} ($p < 0.05$ and $p < 0.01$). L-NIO abolished the IL-1β-induced PGE_2 release at all IL-4 concentrations examined (all $p < 0.001$).

IL-4 (0–100 ng ml^{-1}) significantly enhanced [^{3}H]thymidine in the absence of IL-1β and L-NIO when compared to unsupplemented constructs, as illustrated in Fig. 1C (all $p < 0.05$). IL-1β inhibited [^{3}H]thymidine incorporation in the absence of IL-4 when compared to unsupplemented constructs ($p < 0.001$) but this effect could be partially reversed by co-incubation with IL-4 at all concentrations tested ($p < 0.01$ and $p < 0.001$, Fig. 1C). The presence of L-NIO further reversed the IL-1β induced inhibition of [^{3}H]thymidine incorporation at all IL-4 concentrations examined ($p < 0.05$, Fig. 1C).

$^{35}SO_4$ incorporation was enhanced by the presence of IL-4 at concentrations of 1 ($p < 0.05$), 10 or 100 ng ml^{-1} (both $p < 0.001$), when compared to unsupplemented constructs. IL-1β did not significantly influence $^{35}SO_4$ incorporation for constructs stimulated with 0 or 0.1 ng ml^{-1} IL-4

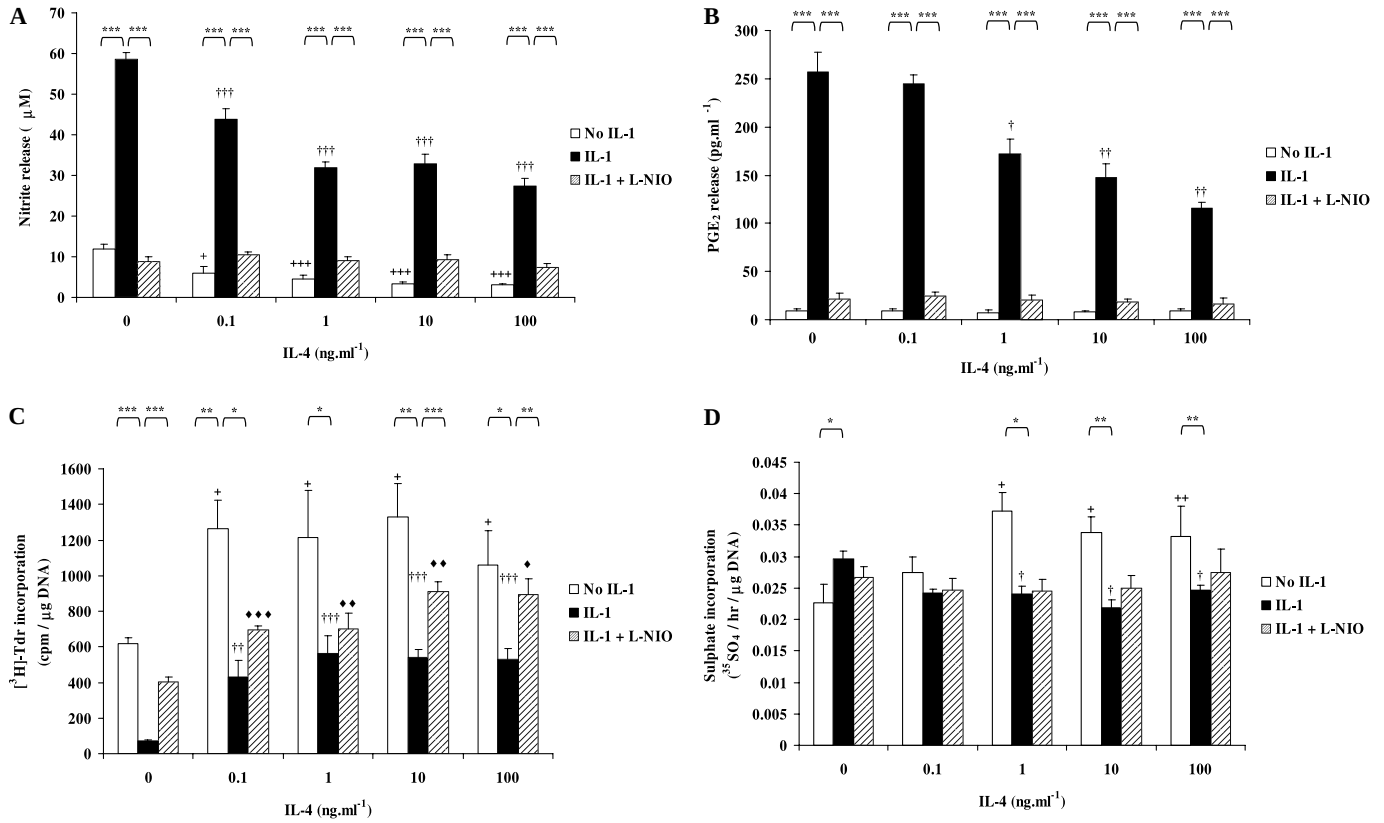

Fig. 1. Nitrite release (A), PGE_2 production (B), $^{35}SO_4$ incorporation (C), and [^{3}H]thymidine incorporation (D) by chondrocytes cultured in agarose constructs for 48 h in medium containing 0–100 ng ml^{-1} IL-4 and/or 10 ng ml^{-1} IL-1β or 1 mM L-NIO. Bars represent the mean and SEM of 6–11 replicates from two separate experiments. Two-way ANOVA with post hoc Bonferroni-corrected t tests was used to examine all data. Differences between unsupplemented, IL-1β, and IL-1β + L-NIO are indicated by an asterisk (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). Comparisons between unsupplemented and IL-4 stimulated constructs at concentrations ranging from 0 to 100 ng ml^{-1} are indicated by a cross (+) ($^{+}p < 0.05$, $^{++}p < 0.01$, and $^{+++}p < 0.001$). Comparisons between IL-1β stimulated constructs and constructs cultured with 0–100 ng ml^{-1} IL-4 are indicated with a dagger (†) ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, and $^{\dagger\dagger\dagger}p < 0.001$). Comparisons between IL-1β + L-NIO and constructs cultured with 0–100 ng ml^{-1} IL-4 are indicated by a diamond (♦) ($^{\blacklozenge}p < 0.05$, $^{\blacklozenge\blacklozenge}p < 0.01$ or $^{\blacklozenge\blacklozenge\blacklozenge}p < 0.001$). All other comparisons were not significant (not indicated).

in the presence and absence of L-NIO (Fig. 1D). However, at IL-4 concentrations of 1–100 ng ml^{-1}, there was marginal downregulation of $^{35}SO_4$ incorporation when compared to IL-1β stimulated constructs cultured in the absence of IL-4 and this effect was not significantly influenced by the presence of L-NIO.

Effect of IL-4 in mechanically stimulated constructs cultured in the presence and absence of IL-1β

In unstrained constructs, the presence of IL-4 significantly reduced nitrite release when compared to unsupplemented constructs, as illustrated in Fig. 2A ($p < 0.01$). In addition, nitrite release was reduced with the application of dynamic compression in both the presence and absence of IL-4 (both $p < 0.001$), resulting in similar levels in the magnitude of strain-induced inhibition of approximately 32%. In unstrained constructs, IL-1β significantly stimulated nitrite levels by approximately threefold, when compared to unsupplemented or IL-4 treated constructs (both $p < 0.001$). The application of dynamic compression inhibited nitrite release in IL-1β stimulated constructs ($p < 0.001$), resulting in a strain-induced inhibition of approximately 50.9%. A similar inhibition was achieved for unstrained constructs cultured in the presence of IL-4 and IL-1β, when compared to constructs subjected to dynamic strain in the presence of IL-1β. The magnitude of the strain-induced inhibition of nitrite release was greater in IL-1β stimulated constructs (−50.9%) compared to constructs cultured in unsupplemented (−32.5%) or IL-4 treated conditions (−31.9%). Co-incubation with IL-4 significantly reduced the magnitude of strain-induced nitrite release from −50.9% to −38.4% compared to IL-1β alone ($p < 0.001$). L-NIO reduced absolute levels of nitrite in unstrained constructs cultured in the presence of IL-4 and IL-1β ($p < 0.001$). In addition, the strain-induced inhibition of nitrite release was abolished by L-NIO.

In the absence of IL-1β and L-NIO, dynamic compression did not significantly influence PGE_2 release in the presence and absence of IL-4, as illustrated in Fig. 2B. In unstrained constructs, IL-1β enhanced PGE_2 levels when compared to unsupplemented or IL-4 treated constructs

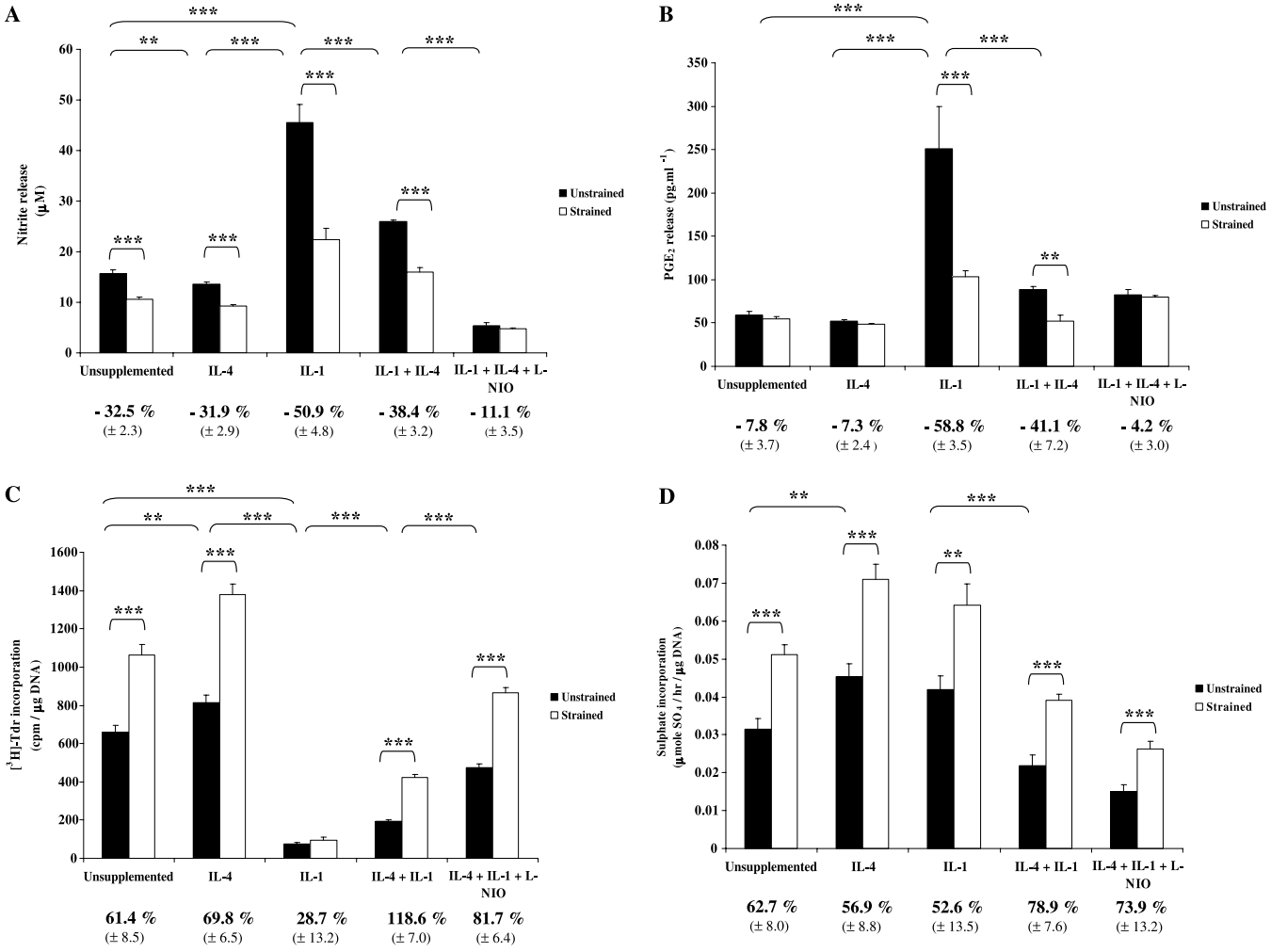

Fig. 2. Absolute values for nitrite (A), and PGE_2 release (B), [^{3}H]thymidine (C) and $^{35}SO_4$ incorporation (D) by chondrocyte/agarose constructs subjected to 15% dynamic compressive strain at 1 Hz, for 48 h in medium supplemented with 0 or 10 ng ml^{-1} IL-4 and/or 10 ng ml^{-1} IL-1β or 1 mM L-NIO. Bars represent the mean and SEM of 12 replicates from two separate experiments. Two-way ANOVA with post hoc Bonferroni-corrected *t* tests was used to examine all data, where values are as follows: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. All other comparisons were not significant (not indicated). In addition, the corresponding normalised strained values, presented as a percentage change from the unstrained controls, are highlighted in bold. Respective SEM values are shown in brackets.

(both $p < 0.001$). This response was inhibited with the application of dynamic compression ($p < 0.001$). In unstrained constructs, the presence of IL-4 and IL-1β downregulated PGE_2 levels when compared to IL-1β stimulated constructs ($p < 0.001$). However, a combination of IL-4 and dynamic compression reduced PGE_2 release to levels similar to those of constructs cultured in the absence of IL-1β ($p < 0.01$). Strain-induced inhibition of PGE_2 release was abolished by L-NIO.

IL-4 significantly increased [^{3}H]thymidine incorporation in unstrained constructs, as illustrated in Fig. 2C ($p < 0.01$). [^{3}H]thymidine incorporation was enhanced by the application of dynamic compression in the presence and absence of IL-4 (both $p < 0.001$). The compression-induced stimulation of [^{3}H]thymidine incorporation was significantly inhibited in the presence of IL-1β ($p < 0.001$). However, IL-4 partially reversed the IL-1β-induced inhibition of [^{3}H]thymidine incorporation in unstrained constructs and this effect was enhanced with the additional application of dynamic compression (both $p < 0.001$). Furthermore, the presence of L-NIO further enhanced [^{3}H]thymidine incorporation levels in unstrained constructs cultured in the presence of IL-4 and IL-1β ($p < 0.001$), resulting in a strain-induced stimulation of approximately 81.7% ($p < 0.001$).

As illustrated in Fig. 2D, the presence of IL-4 significantly enhanced $^{35}SO_4$ incorporation levels in unstrained constructs ($p < 0.01$). $^{35}SO_4$ incorporation was enhanced with the application of dynamic compression in the presence and absence of IL-4 (both $p < 0.001$). In addition, dynamic compression significantly stimulated $^{35}SO_4$ incorporation in IL-1β stimulated constructs ($p < 0.01$), resulting in a strain-induced stimulation of approximately 52.6%. In unstrained constructs, the presence of IL-1β and IL-4 downregulated $^{35}SO_4$ incorporation levels ($p < 0.001$). However, this effect could be reversed with

dynamic compression resulting in a strain-induced stimulation of approximately 78.9% ($p < 0.001$). Interestingly, in the presence of IL-4 and IL-1β, compression-induced stimulation of $^{35}SO_4$ incorporation was not significantly influenced by the presence of L-NIO (73.9%).

Discussion

In recent years, a clearer understanding of the fundamental pathways associated with the pathophysiology of OA has led to the development of new approaches for treatments aimed at specifically retarding the disease process. Compelling evidence implicates IL-4 as an important cytokine with potent anti-inflammatory properties [23–25]. This pleiotropic T-cell derived cytokine has been shown to suppress the release of ·NO, inhibit MMP activity and/or gene expression, and downregulate the production of pro-inflammatory cytokines such as IL-1β [35–38]. This pathway is thought to provide a protective mechanism to the inflammatory response in cartilage joint disease. Interestingly, new insights into chondrocyte mechanotransduction pathways have also identified IL-4 as part of the signalling cascade induced by mechanical stimulation [2,29,39]. Accordingly, the aim of the current study was to examine the effects of IL-4 in combination with mechanical load on the release of ·NO and PGE_2 by chondrocytes seeded in agarose constructs and cultured with IL-1β.

The current findings support previous studies which show the inhibitory effects of IL-4 on ·NO and PGE_2 release in cytokine stimulated chondrocytes (Figs. 1A and B, respectively) [38,40]. For example, IL-4 has been shown to reverse the IL-1β mediated upregulation of ·NO release in cartilage explants and monolayer cultures of chondrocyte sub-populations [38,40]. These studies suggest that IL-4 may act by reducing the IL-1β mediated effect on ·NO and PGE_2 release. The present data further demonstrate that the NOS inhibitor, L-NIO, abolished the IL-1β induced ·NO and PGE_2 release in the presence of IL-4, suggesting ·NO dependent mechanisms. IL-1β inhibited chondrocyte proliferation and this could be partially reversed with IL-4 and L-NIO, suggesting that the effect on cell proliferation was ·NO mediated (Fig. 1C). By contrast, proteoglycan synthesis was largely unaffected by a combination of IL-4 and L-NIO for IL-1β stimulated cells, suggesting that the response is mediated by ·NO independent mechanisms (Fig. 1D). The authors have consistently failed to show an inhibition of proteoglycan synthesis by IL-1β in bovine chondrocytes [26,27,32]. The effect appears to be species specific as studies using model systems incorporating chondrocytes from other species have shown different effects to those previously reported with bovine chondrocytes cultured in agarose gel [7,8,41,42]. Moreover, the effect may be concentration dependent. In a dose dependent study ranging from 0 to 100 ng ml^{-1} IL-1β, the authors observed that a concentration of 100 ng ml^{-1} IL-1β induced a significant upregulation of ·NO and PGE_2, and inhibition of proteoglycan synthesis [26].

Table 1 summarizes the effect of IL-4 and dynamic loading, alone or in combination, for constructs stimulated with IL-1β. Analysis of the mechanical loading data shows that in the presence of IL-1β, IL-4 or dynamic compression inhibited ·NO and release but a combination of both stimuli resulted in a greater level of strain-induced inhibition of ·NO. Similarly, the decrease in the levels of PGE_2 by a combination of IL-4 and dynamic strain was greater than either stimulus alone (Table 1). These data demonstrate that the inhibitory effects of IL-4 and dynamic strain are partially additive. Furthermore, the dynamic strain-induced inhibition of ·NO and PGE_2 release was abolished with L-NIO, confirming the de novo synthesis of ·NO which is required to mediate the mechanotransduction process (Figs. 2A and B). These data are consistent with a hypothesis suggesting that the downregulation of ·NO and PGE_2 release may be partially mediated by autocrine or paracrine derived IL-4. Thus, IL-4 in combination with dynamic compression could partially abrogate the IL-1β induced upregulation of ·NO and PGE_2 release, indicating anti-inflammatory effects for both stimuli. The signal transduction pathways may involve direct effects of exogenous IL-4 or mechanically induced integrin-mediated IL-4 release. However, a further study utilising IL-4 receptor antagonists and function blocking integrin antibodies is necessary to elucidate these pathways.

In a previous study, mechanical stimulation has been shown to induce the secretion of substance P, a neuropeptide which may has anti-inflammatory effects in conjunction with IL-4 [43–45]. It has been suggested that cross-talk with other integrin-mediated pathways activated by mechanical stimulation may also be necessary in the production of these autocrine/paracrine signalling molecules. However, such mechanisms remain to be defined.

Table 1
Percentage change from values for constructs cultured with IL-1β when constructs are additionally incubated with IL-4 alone, dynamic compression alone or both in combination

	IL-4	Dynamic compression	IL-4 and dynamic compression
Nitrite release (%)	−42.9 (±12.4)	−50.9 (±4.8)	-64.9^{**} (±1.8)
PGE_2 release (%)	−59.6 (±0.9)	−58.8 (±3.5)	-76.2^{***} (±2.2)
[^{3}H]thymidine incorporation (%)	160.7 (±22.5)	$28.7^{\dagger\dagger\dagger}$ (±13.2)	470.2^{***} (±18.4)
$^{35}SO_4$ incorporation (%)	48.0 (±16.7)	52.6 (±13.5)	25.1 (±7.3)

Note. Values in table represent means and SEM values of 12 replicates from two separate experiments. Unpaired Student's t tests were used to examine percentage change data, where values are as follows: $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate comparisons between dynamic compression and IL-4 + dynamic compression; $^{\dagger\dagger\dagger}p < 0.001$ indicates comparisons between IL-4 and dynamic compression. All other comparisons were not significant.

Recent work by the authors have demonstrated that RGD peptides, which act competitively with ligands for the α5β1-integrin, reversed the anti-inflammatory effects of dynamic compression for bovine chondrocytes cultured in agarose constructs in the presence of IL-1β, demonstrating cross-talk between integrin-mediated signalling and mechanical stimulation [46]. It should also be recognised that dynamic strain may influence the provision of cytokine to cells within 3D constructs, therefore affecting a response via a concentration-dependent mechanism. When provided in combination, dynamic strain and IL-4 enhanced cell proliferation by 470% for IL-1β stimulated constructs (Table 1). These findings indicate a synergistic action of IL-4 and dynamic strain on cell proliferation. The magnitude of stimulation was partially downregulated with L-NIO (Fig. 2C), suggesting that the mechanism for cell proliferation exhibits a partial dependency on ·NO. This process may involve the release of IL-4 via an integrin-dependent autocrine/paracrine signalling cascade activated by mechanical stimulation and involving neurotransmitters [28,29,43]. The magnitude of strain-induced stimulation of proteoglycan synthesis was not significantly influenced by IL-1β, IL-4 or L-NIO suggesting ·NO independent mechanisms (Fig. 2D). Therefore, the regulation of cell proliferation and proteoglycan synthesis by IL-4 and dynamic compression in the presence of IL-1β are not achieved through common pathways.

The current study demonstrates that IL-4 and dynamic compression, alone or in combination, counteracts IL-1β-induced stimulation of ·NO and PGE_2 release, and inhibition of cell proliferation. The combined effects of IL-4 and dynamic strain were partially additive for ·NO and PGE_2 release and synergistic for cell proliferation. Interestingly, IL-4 is thought to play a role in the signalling cascade induced by mechanical stimulation, suggesting that the combined effect of IL-4 and dynamic strain may be mediated by a combination of exogenous and autocrine or paracrine derived IL-4. The upregulation of ·NO has been implicated in the development and progression of OA. The suppression of ·NO by IL-4 and dynamic compression is a significant finding, which may provide a potential protective mechanism for cartilage in disorders such as OA.

Acknowledgments

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