NF-κB Signaling Pathway in Controlling Intervertebral Disk Cell Response to Inflammatory and Mechanical Stressors

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Background. Intervertebral disk degeneration (IDD) has a greater than 90% lifetime incidence and is one of the leading causes of chronic back pain in the United States. Despite the high societal cost of IDD, there is limited understanding of the biological effects of mechanical overloading on further degeneration. The transcription factor NF-κB (nuclear factor κB) has been implicated as a key mediator of disk cell response to inflammatory and mechanical stresses and represents a potential control point.

Objective. The study objective was to measure the effect of NF-κB signaling pathway inhibition on annulus fibrosus (AF) cell matrix synthesis and gene expression under conditions of combined inflammatory and mechanical stimulation.

Methods. Annulus fibrosus cells were harvested from rabbit intervertebral disks and grown in vitro on flexible plates. The cells were exposed to inflammatory and mechanical stimulation for 24 hours with and without NF-κB inhibition. Nuclear translocation of NF-κB was measured via immunofluorescent staining. Intervertebral disk cell homeostasis was assessed via inflammatory, anabolic, and catabolic gene expression and via matrix synthetic ability.

Results. NF-κB nuclear translocation in response to interleukin-1 beta (IL-1β) was reversed with exposure to NF-κB inhibition. NF-κB inhibition decreased matrix metalloproteinase-3, inducible nitric oxide synthase, and cyclooxygenase-2 gene expression and prostaglandin E_2 production response to combined inflammatory and mechanical stimulation. Proteoglycan and collagen synthesis were decreased by combined stimulation, but this effect was not reversed by NF-κB inhibition.

Limitations. In vitro modeling of conditions within the disk may not fully reflect the response that AF cells have in native matrix.

Conclusions. NF-κB signaling mediates catabolic and inflammatory responses to inflammatory and mechanical stimulation but does not mediate the decrease in matrix synthesis under combined harmful stimulation. Identification of key control points in the cellular responses to inflammatory and mechanical stimuli will facilitate rational design of exercise-based therapies and facilitate synergistic treatments of novel biochemical treatments with rehabilitation regimens.

Low back pain is a steadily increasing issue[1,2] affecting 80% to 90% of individuals worldwide at some point in their lives[3] and costing the United States alone more than $100 billion in health care costs and lost productivity.[4] Intervertebral disk degeneration (IDD) is widely acknowledged as one of the primary risk factors for low back pain.[5,6] Clinically, IDD is often associated with loading and inflammation[7]; however, the interplay between inflammation and mechanical signaling during the progression of IDD is poorly understood.

Despite the increasing costs resulting from disability due to progressive IDD, there are few biological or physical therapy treatments proven to prevent further degeneration.[8] Motion-based therapies have proven moderately effective in the management of low back pain.[9–12] Physical therapy and physical activity recommendations, however, cannot be properly targeted toward individual biology, or stage of degeneration, without understanding the biological mechanism behind the effects of motion on the intervertebral disk. Currently, the way in which physical therapy and physical activity recommendations can best be individualized to treat IDD-related ailments remains unknown.

Recently, regenerative rehabilitation, the process of enhancing cellular response to biological therapy through mechanical stimulation, has demonstrated positive results in both bone[13] and muscle[14,15] research, but has not yet been used for low back pain. Regenerative rehabilitation may be beneficial in the future to improve outcome in patients with IDD by combining targeted biologic therapy with physical therapy, to protect against any negative consequences of unintended excessive loading, and to change disk cell behavior and prevent further degeneration. At the cellular level, the response of intervertebral disk cells to mechanical loading has been shown to be highly dependent on age, frequency, and magnitude,[16–18] but the biological pathways mediating disk cell response to mechanical loading have not been elucidated. In previous research, disk cells under mechanical loading have shown both positive and negative responses with regard to matrix production, matrix degradation, proteoglycan synthesis, and inflammatory signaling.[16–18] Going forward, there is a need to determine which biological pathways are modified by mechanical loading to maximize beneficial and minimize negative responses in disk cells and best synergize with biologic therapy to achieve regenerative rehabilitation for low back pain.

The complex environment of the degenerated intervertebral disk contains high levels of inflammatory cytokines, specifically interleukin-1 beta (IL-1β) and TNF-α, which have been associated with pain[19] and increased production in catabolic matrix enzymes.[20] The nuclear factor κB (NF-κB) signaling pathway, one of the pathways responsible for regulating inflammatory cytokine response and catabolic matrix enzymes, has been shown previously to regulate integrin signaling and mechanotransduction[21] and modulate inflammatory signaling in annulus fibrosus (AF) cells,[22,23] making it a modifiable target in disk research.[22] Systemic inhibition of NF-κB in recent work showed increased proteoglycan and collagen synthesis within the aging intervertebral disk.[24] Conversely, prolonged loading and inflammation have been shown to have a synergistic negative effect on disk cell function, increasing catabolic enzymes and decreasing matrix synthesis,[25,26] thus making the NF-κB pathway an attractive biologic target, as it is involved in the response to both stimuli. Preventing the activation of the NF-κB pathway could potentially reduce the inflammatory response to proinflammatory cytokines within the intervertebral disk and prevent cellular response to mechanical overloading, which could decelerate the progression of IDD in patients. Therefore, study of the NF-κB pathway has the potential to help to unravel the complex interplay between inflammatory and mechanical signaling involved in the intervertebral disk response to traumatic loading that has the potential to hasten the degenerative cascade. Understanding the signaling pathways responsible for disk cell response to loading may allow clinicians to better prescribe regenerative rehabilitation protocols within a safe window of mechanical loading and potentially utilize the synergy between pharmacotherapy and motion-based treatments.

Our study aimed to evaluate the response of intervertebral disk AF cells to combined inflammatory and mechanical stimulation. We hypothesized that inhibition of NF-κB activation will decrease inflammatory and procatabolic response to levels of mechanical stimulation previously demonstrated to be detrimental in cultured AF cells, implicating the NF-κB pathway in this response to excessive loading.

Method

Cell Culture and Stimulation

Intervertebral disk AF cells were isolated from New Zealand white rabbits immediately after sacrifice and cultured in F-12, 10% fetal bovine serum, 1% penicillin-streptomycin at 37°C, and 5% CO_2 until 90% confluence, as previously described.[27,28] Passage 1 cells were transferred to flexible silicon plates coated with collagen I (BF-3001C, Bioflex, Flexcell International Corp, Hillsborough, North Carolina) at a concentration of 300,000 cells per well and allowed to proliferate for 3 days. Sixteen hours before experiments, media were changed to F-12, 1% fetal bovine serum, 1% penicillin-streptomycin to facilitate biological assays. Before starting the stretching regimens, cells were preincubated for 30 minutes with 1μg/mL recombinant human IL-1β (201-LB/CF, R&D Systems, Minneapolis, Minnesota) and for 1 hour with 1 nM ACHP (2-Amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-(4-piperidinyl)-3-pyridinecarbonitrile) (4547/10, R&D Systems), a selective chemical inhibitor of IKK-α/β over IKK3 to block activation of NF-κB. Cells were then exposed to 6% tensile strain at 0.1 Hz for 24 hours, shown previously to be a procatabolic and proinflammatory loading regimen using the Flexcell Tension Plus System (FX-3000, Flexcell International Corp).[17] Cyclic tensile strain at this level has been shown previously not to change AF cell adhesion or morphology.[28] Unstretched controls were maintained under identical conditions on unstretched flexible plates.

NF-κB Nuclear Translocation

NF-κB was visualized by immunofluorescent labeling using an anti-p65 antibody (SC372, Santa Cruz Biotechnology, Santa Cruz, California). Nuclear translocation of p65, a subunit of NF-κB, was quantified using a customized MATLAB (The MathWorks Inc, Natick, Massachusetts) program to analyze the images. Cytoplasmic and nuclear boundaries were determined by threshold detection using actin (for cytoplasmic boundary; Alexa Fluor 647 phalloidin, Thermo Fisher Scientific, Waltham, Massachusetts, which selectively stains F-actin) or DAPI (for nuclear boundary; 4′,6-diamidino-2-phenylindole)-stained images with manual boundary confirmation. The ratio of the summed intensity of the nuclear region to the summed intensity of the cytoplasmic region was used to determine NF-κB activation.

Gene Expression

Messenger RNA (mRNA) (n=3) was isolated using phosphate-buffered saline and adding RLT lysis buffer (79216, Qiagen, Valencia, California) containing 1% β-mercaptoethanol and detaching the cells from the membrane by mechanical disruption. The resultant solution was passed through a QIAshredder (Qiagen), and mRNA was isolated using an RNA extraction kit (74104, Qiagen) and a DNase I step to remove genomic material. The mRNA expression of matrix metalloproteinase-1 (MMP-1), matrix metalloproteinase-3 (MMP-3), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) was measured via reverse transcription-polymerase chain reaction (RT-PCR) using custom-designed and validated primers as previously described.[17] Relative changes in mRNA were quantified using the ΔΔCt method,[29] using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to normalize and compare with the untreated, unstretched control cells.

Matrix Synthesis

Total proteoglycan (n=6) and total collagen (n=3) synthesis were measured via incorporation of ^{35}S (20-μCi/mL media) and ^{3}H-proline (10-μCi/mL media), respectively, for 24 hours after treatment concluded. Media were removed and remaining cells and matrix were homogenized using 50-μM dithiotreitol and 1x protease inhibitor cocktail (Sigma-Aldrich, St Louis, Missouri). The resulting solution was filtered (0.45-μm pore size) and washed using 2 mL of wash buffer. Radio-labeled macromolecules were solubilized by gently shaking each filter in guanidine hydrochloride (4M) and isopropanol (33%) for 1 hour. Three milliliters of Hydrofluor scintillation fluid (National Diagnostics, Atlanta, Georgia) was added to each filter well, and radioactivity counts per minute were quantified using a liquid scintillation counter (Tri-Carb TR2100, PerkinElmer, Waltham, Massachusetts). Counts per minute were normalized to total DNA determined by PicoGreen assay (P7589, Life Technologies, Grand Island, New York) and reported as values normalized to untreated control cells.

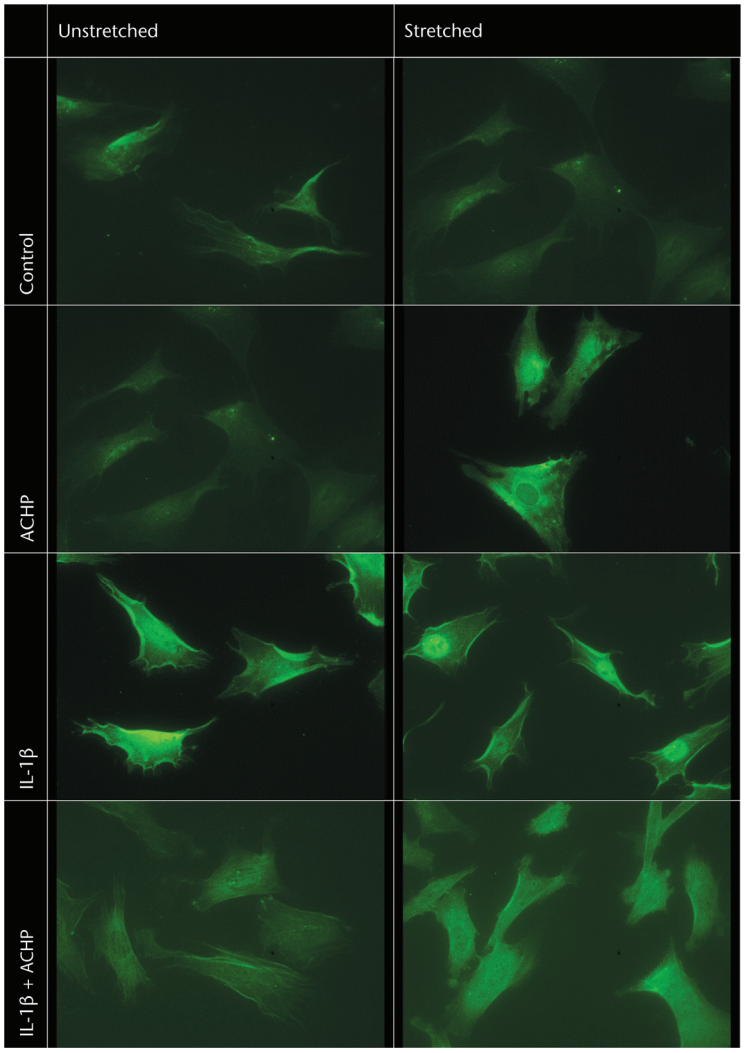

Figure 1.
Representative images of nuclear factor κB (NF-κB) staining for all conditions after 24 hours of cyclic tensile strain showing increased nuclear translocation of NF-κB after interleukin-1 beta (IL-1β) stimulation with and without stretching. Movement of NF-κB into the nucleus induced by IL-1β was diminished by ACHP preconditioning.

Prostaglandin E_2 Production

Conditioned media were removed from each well 24 hours after treatment. Fresh conditioned media were diluted 3-fold, and 24-hour total prostaglandin E_2 synthesis was measured using a competitive

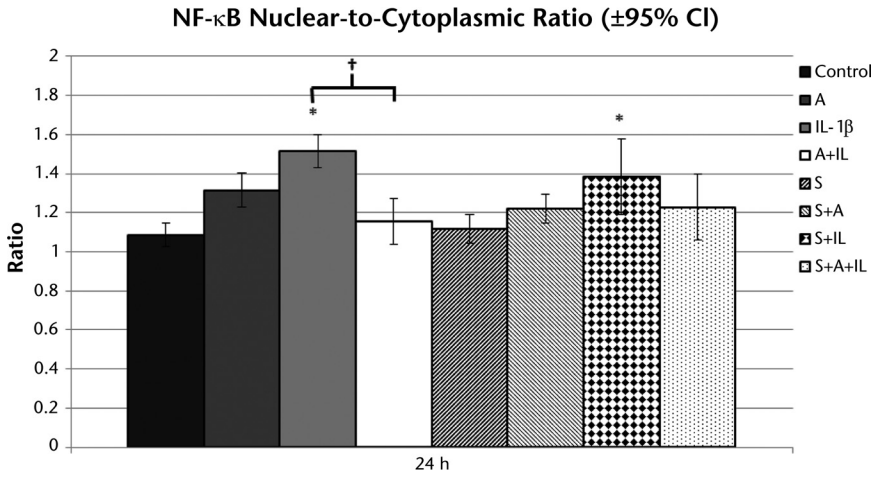

Figure 2.
Nuclear-to-cytoplasmic ratio of nuclear factor κB (NF-κB) in response to stretching and proinflammatory stimulation at 24 hours demonstrates a strong response to inflammatory (interleukin-1 beta [IL-1β]) signaling, but no further increase with the addition of mechanical stimulation. Three independent experiments were run, and a minimum of 3 cell images from each experiment were used. * Significantly greater than control ($P<.05$). † $P<.05$, N=6. A=ACHP, IL=IL-1β, S=stretch, CI=confidence interval.

prostaglandin ELISA assay (KGE004B, R&D Systems).

Values represent the average of 3 to 6 trials from independent cell isolations from separate rabbits. All values represent mean±95% confidence intervals based on the *t* distribution. Ninety-five percent confidence intervals were calculated from the percent change values to determine statistical significance at the $P<.05$ level compared with unstretched, unstimulated control cells. Post hoc comparisons of ACHP-treated states to inflammatory states were performed to specifically measure the effect of NF-κB inhibition relating to the primary hypothesis, and Bonferroni correction was made assuming 9 total comparisons. Computations were performed using Microsoft Excel (Microsoft Inc, Redmond, Washington) and IBM SPSS Statistics software, version 23 (IBM Corp, Armonk, New York).

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Results

NF-κB Activation

Activation of the NF-κB signal pathway was measured by nuclear translocation of NF-κB from the cytoplasm. Immunofluorescent staining of NF-κB (Fig. 1) showed significant increases in nuclear translocation of NF-κB under inflammatory (IL-1β) and inflammatory with stretching (S+IL) conditions (+51.5%±15.1% and +38.3%±15.0%, respectively). ACHP inhibited NF-κB nuclear translocation in response to IL-1β with stretching (S+A+IL) and without stretching (A+IL) (6.3%±7.3% and 13.3%±14.8%, respectively, over control cells), which was not statistically different from control cells, confirming NF-κB inhibition (Fig. 2).

Gene Expression Response to Inflammatory and Mechanical Stimulation

Relative gene expression demonstrated a significant increase in MMP-3, iNOS, and COX-2 mRNA with addition of IL-1β (Fig. 3) to AF cell cultures. Inhibition of NF-κB signaling by ACHP prevented the increase in MMP-3 and iNOS in response to IL-1β but had no significant effect on COX-2 gene response. IL-1β treatment and IL-1β + stretching increased anticatabolic gene expression (TIMP-1) similar to previous shorter-duration stretching experiments.[17] ACHP with IL-1β (IL+A) showed further increased TIMP-1 expression. The MMP-1 response, although statistically significant for IL+A, was small in magnitude compared with other tested genes.

Stretching alone for 24 hours had no significant effect on any of the measured gene expression, but stretching with inflammatory stimulation (S+IL) increased MMP-3, iNOS, and COX-2 expression more than inflammatory stimulation alone. ACHP was more effective at reducing iNOS and COX-2 response to inflammation in stretched cells (S+IL+A) compared with IL+A. ACHP significantly reduced MMP-3 response to stretching and inflammation. The TIMP-1 increased by 130%±97% over unstimulated control cells in response to combined stimulation (S+IL), and this effect was reversed by addition of ACHP.

Prostaglandin E_2 Response to Inflammatory/Mechanical Stimulation

The AF cells stimulated with IL-1β had a 5-fold and 7-fold increase in prostaglandin E_2 level (Fig. 4) with and without stretching, respectively. NF-κB inhibition by ACHP reduced prostaglandin production in response to inflammatory stimulation by 28.5% and response to combined stimulation by 58.5%.

Extracellular Matrix Production Response to Mechanical Overload Stimulation

Proteoglycan and collagen synthesis decreased significantly (−34.0%±15.6% and −42.9±23.8%, respectively) after 24 hours of exposure to 6% strain (Fig. 5). Inflammatory stimulation did not decrease proteoglycan and collagen synthesis as much as stretching and had no additive effect with stretching. The decreased proteoglycan and collagen synthesis with 24-hour strain was not changed by ACHP treatment (5.5%±20.1% and 2.3%±15.5%, respectively, with respect to stretching alone). ACHP did not rescue the loss of proteoglycan and collagen synthesis in response to mechanical or combined stimulation.

Discussion

The principal finding of this study was that prolonged mechanical stimulation exacerbates the effect of underlying inflammatory signaling on AF cells with

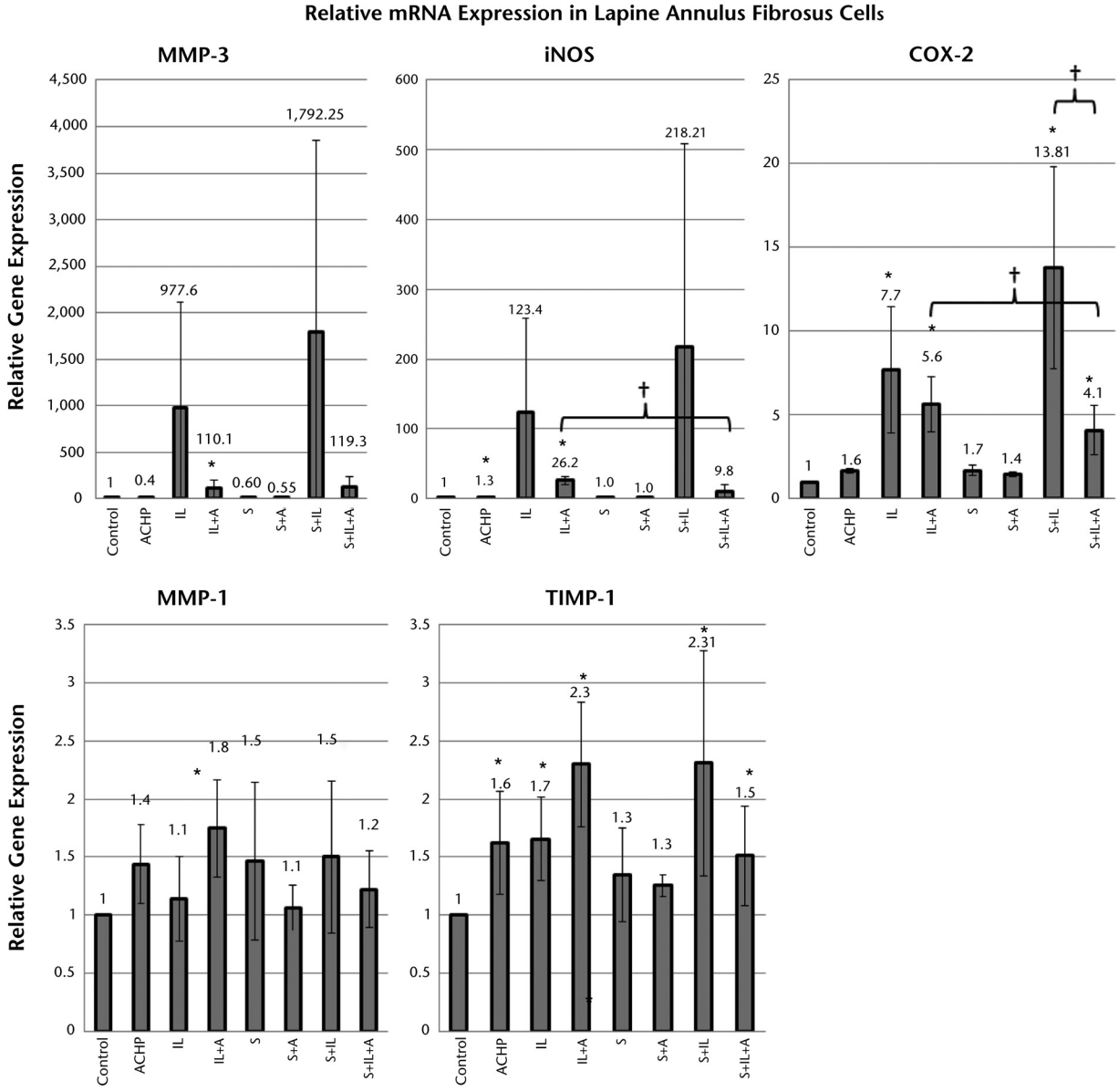

Figure 3.
Relative change in messenger RNA (mRNA) gene expression of matrix metalloproteinase-3 (MMP-3), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) as measured by real-time polymerase chain reaction shows increased matrix breakdown and inflammatory response with interleukin-1 beta (IL-1β) stimulation with and without stretching, which is reversed by addition of ACHP. Matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) show more modest changes in response to inflammatory and mechanical stimulation, although the increase in TIMP-1 in response to IL-1β was not reversed by the addition of ACHP. All values represent mean±95% confidence interval of n=3 samples. * Significantly greater than control ($P<.05$). † $P<.05$. A=ACHP, IL=IL-1β, S=stretch.

respect to catabolic or inflammatory response and prostaglandin synthesis, and this effect is largely reversed by blocking NF-κB signaling. To our knowledge, this study was the first to look at the effects of combined inflammatory stimulation and mechanical stretching on in vitro AF cells and assess the role of the NF-κB pathway in controlling the response of AF cells to a combination of mechanical and inflammatory stimuli.

We found that combined stimulation with mechanical stretching and IL-1β led to stronger gene expression changes than IL-1β or stretch alone and that all changes to iNOS, MMP-3, and activation of NF-κB were reversed with addition of the NF-κB inhibitor ACHP. We also found that 24-hour stretching decreased proteoglycan and collagen synthesis. However, this finding was not NF-κB dependent, as NF-κB inhibition did not reverse

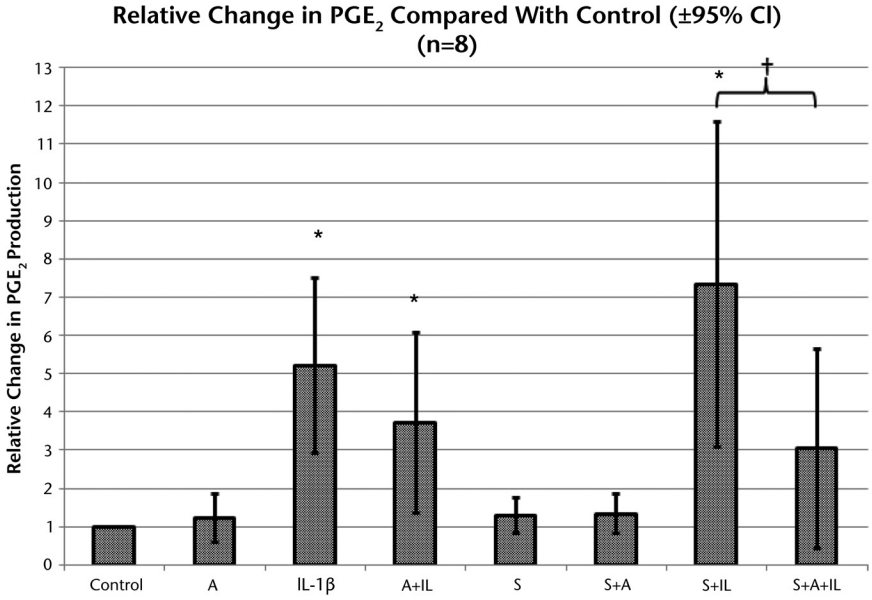

Figure 4.
Relative prostaglandin E_2 (PGE_2) levels at 24 hours (n=8) in response to interleukin-1 beta (IL-1β), stretching, and nuclear factor κB (NF-κB) inhibition demonstrates synergistic effect of inflammatory and mechanical stimulation to elicit an inflammatory response from annulus fibrosus cells. A=ACHP, IL=IL-1β, S=stretch, CI-confidence interval. * Significantly greater than control ($P<.05$). $^{\dagger}P<.05$.

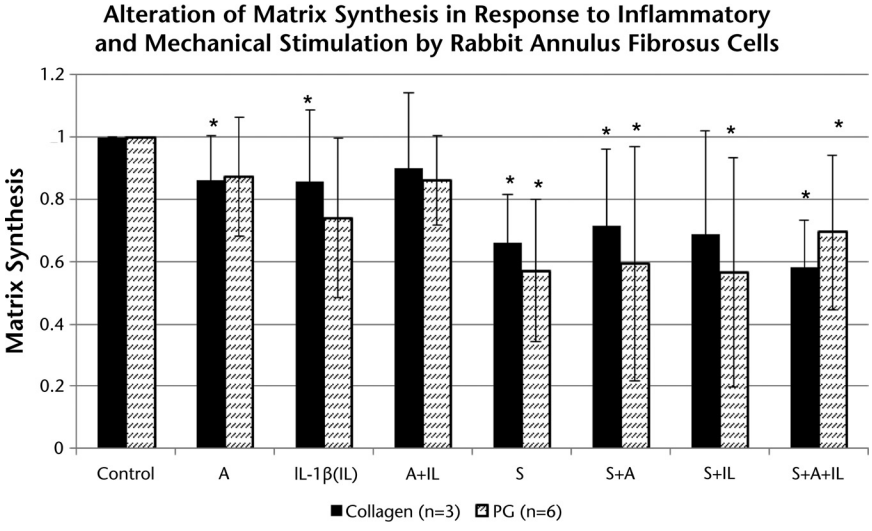

Figure 5.
Proteoglycan (PG) and total collagen synthesis normalized to noninflammatory nonstretched controls (n=3, mean±95% confidence interval) after 24 hours of strain measured by ^{35}S (20-μCi/mL media) and ^{3}H-proline (10-μCi/mL media) incorporation via pulse labeling shows increased extracellular matrix synthesis with short-duration stretching, which is reversed by nuclear factor κB (NF-κB) inhibition, and decreased synthesis with 24-hour stretching, which is unaffected by NF-κB inhibition.

this effect. This finding may indicate that a different pathway coordinates the overall anabolic response to stretching. Thus, it appears that the NF-κB pathway is involved in the inflammatory and procatabolic effects of prolonged mechanical stimulation but not in the antianabolic effects (Fig. 6). However, effects on net matrix homeostasis are needed to determine the overall impact on degeneration and is an important topic for future research.

One exception to this finding was that isolated inflammatory stimulation resulted in an increase in COX-2 production, which was not affected by NF-κB inhibition. Studer et al[30] showed that AF cell production of COX-2 in response to IL-1β was partially regulated by mitogen-activated protein kinase (p38). It is of interest that, in the setting of combined stimulation (IL-1β and stretching), COX-2 production was decreased by NF-κB inhibition, indicating that control of COX-2 production may shift more toward the NF-κB pathway under loading conditions. Prostaglandin E_2 production showed a similar pattern, with inhibition by ACHP being greater after combined stimulation compared with IL-1β alone.

The disk is a complicated structure, consisting of 4 distinct regions: outer AF, inner AF, nucleus pulposus, and vertebral end plate. A potential shortcoming of this study was that only rabbit AF cells were used. However, these cells were chosen to explore the mechanistic response to mechanical loading in vitro due to availability of consistent strain of cells, avoiding the biologic variability of human cells, as well as their phenotypic stability to mechanical loading in vitro.[28] In addition, the effects of NF-κB inhibition in nucleus pulposus and end-plate cells under compression were not measured. Recent research by Gawri et al[31] showed that nucleus pulposus cells may increase matrix synthesis in response to dynamic compressive loading. The remaining regions may affect overall disk health but, unlike the AF, do not undergo significant tensile strain, which was the focus of this study. In vivo, the AF matrix is composed of both collagen type I and II, but our in vitro system uses a coating comprising only collagen type I. How-

	Effect of NF-κB Inhibition on Inflammatory and Mechanical Stimulation	
	Inflammatory (IL-1β) + ACHP Relative to IL-1β	Stretch + IL-1β + ACHP Relative to Stretch + IL-1β
Nf-κB Activation	↓	↓
Catabolic RGE (MMP-1/3)	↓	↓
Anticatabolic RGE (TIMP-1)	↔	↓
Inflammatory RGE (iNOS, COX-2)	↓	↓
Inflammatory Cytokine Production (PGE_2)	↔	↓
Matrix Synthesis (PG, Collagen)	↔	↔

Figure 6.
Summary of annulus fibrosus response to inflammatory and mechanical stimulation. Mechanical stimulation increased all effects of inflammatory stimulation. Nuclear factor κB (NF-κB) inhibition reversed NF-κB activation and relative gene expression (RGE) response to both inflammatory and combined stimulation but did not reverse the decrease in matrix synthesis caused by inflammatory and combined stimulation. MMP-1=matrix metalloproteinase-1, MMP-3=matrix metalloproteinase-3, TIMP-1=tissue inhibitor of matrix metalloproteinase-1, iNOS=inducible nitric oxide synthase, COX-2=cyclooxygenase-2, PGE_2= prostaglandin E_2, PG=proteoglycan.

ever, this type was chosen to ensure appropriateness of the culture conditions in maintaining AF cell phenotype and attachment, as demonstrated previously.[28] In addition, the choice of IL-1β as the inflammatory stimulus represents a simplification of a complex inflammatory milieu in the intervertebral disk involving multiple cytokines, although it allows for testing of the effects of NF-κB inhibition because this pathway is stimulated by IL-1β. Although NF-κB has been shown to demonstrate phasic activation,[32] this could not be assessed using our outcome measures. Finally, we did not measure the effect of NF-κB inhibition on different loading intensities, longer loading durations, or durations less than 24 hours, all of which have been shown to change AF cell response in vivo,[33,34] but instead focused on a level of mechanical loading known to incite a catabolic response[17] to allow determination of the role of NF-κB activation in the cellular response to excessive loading.

The intervertebral disk in patients resides in a biomechanically active environment with limited blood flow and oxygenation, which makes the assessment of interventions in the in vitro setting more complicated. The use of combined stimulation more realistically mimics the complicated in vivo environment seen in patients with any inflammatory disease state and supports the use of this study's results in future translational applications. In the current study, we were unable to determine whether the observed effects were a result of ACHP's effect on the canonical or noncanonical NF-κB signaling pathways, as ACHP has been shown to disrupt both pathways through IKKα/β inhibition.[35] Systemic NF-κB inhibition has been shown to ameliorate age-related IDD in murine models, but it is still unknown whether the observed beneficial effect is due to local inhibition of NF-κB in disk tissue or to global effects of NF-κB inhibition on systemic inflammation.[24] Further research using local versus global delivery of NF-κB inhibitor is needed to resolve this question. Nevertheless, this study demonstrates that the NF-κB pathway may represent a target for future regenerative rehabilitation efforts in the intervertebral disk by minimizing the negative effects of inflammation and harmful mechanical overloading on the degenerating disk. Identification of the biomechanical pathways involved in mechanically induced disk damage and repair will facilitate the development of directed therapy for the treatment of IDD.

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