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Biomechanical signals exert sustained attenuation of proinflammatory gene induction in articular chondrocytes

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Summary

Objectives: Physical therapies are commonly used for limiting joint inflammation. To gain insight into their mechanisms of actions for optimal usage, we examined persistence of mechanical signals generated by cyclic tensile strain (CTS) in chondrocytes, *in vitro*. We hypothesized that mechanical signals induce anti-inflammatory and anabolic responses that are sustained over extended periods.

Methods: Articular chondrocytes obtained from rats were subjected to CTS for various time intervals followed by a period of rest, in the presence of interleukin-1β (IL-1β). The induction for cyclooxygenase (COX-2), inducible nitric oxide synthase (iNOS), matrix metalloproteinase (MMP)-9, MMP-13 and aggrecan was analyzed by real-time polymerase chain reaction (PCR), Western blot analysis and immunofluorescence.

Results: Exposure of chondrocytes to constant CTS (3% CTS at 0.25 Hz) for 4–24 h blocked more than 90% ($P < 0.05$) of the IL-1β-induced transcriptional activation of proinflammatory genes, like iNOS, COX-2, MMP-9 and MMP-13, and abrogated inhibition of aggrecan synthesis. CTS exposure for 4, 8, 12, 16, or 20 h followed by a rest for 20, 16, 12, 8 or 4 h, respectively, revealed that 8 h of CTS optimally blocked ($P < 0.05$) IL-1β-induced proinflammatory gene induction for ensuing 16 h. However, CTS for 8 h was not sufficient to inhibit iNOS expression for ensuing 28 or 40 h.

Conclusions: Data suggest that constant application of CTS blocks IL-1β-induced proinflammatory genes at transcriptional level. The signals generated by CTS are sustained after its removal, and their persistence depends upon the length of CTS exposure. Furthermore, the sustained effects of mechanical signals are also reflected in their ability to induce aggrecan synthesis. These findings, once extrapolated to human chondrocytes, may provide insight in obtaining optimal sustained effects of physical therapies in the management of arthritic joints.
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Introduction

Pathophysiologies associated with joint inflammation impose significant impediments to normal joint function[1,2]. Because of their notable benefits, patients with arthritic diseases are invariably prescribed rehabilitative therapies for reducing inflammation and to improve joint function[3–7]. Duration of treatment for such therapies varies between continuous passive motion for several days to a few hours per day of assisted or voluntary joint movements[8]. However, no consensus has been achieved whether constant motion over long periods of time, or precise durations of such therapeutic interventions are required to achieve their optimal benefits. Despite our increasing understanding of the molecular mechanisms of cartilage development and repair, little is known about how mechanical signals regulate cartilage repair. For the optimal use of physical therapies to accelerate cartilage repair it is desirable to understand the mechanisms of actions of mechanical signals in cartilage repair.

Biomechanical forces are critical for the complex process of cartilage development, homeostasis, and functionality[9–11]. As mechanosensitive cells, articular chondrocytes perceive and respond to mechanical signals throughout life. Chondrocytes synthesize a unique extracellular matrix and their integrity, to a large extent, depends upon intracellular signals generated in response to biomechanical forces[2,3,12–15]. Chondrocytes maintain a functional balance between cartilage degradation and repair by production of various enzymes, cytokines, and matrix associated proteins. A loss of this functional balance is associated with changes in their phenotypic characteristics which invariably lead to joint degenerative disorders like osteoarthritis (OA) and rheumatoid arthritis (RA)[16,17].

Phenotypically, articular chondrocytes are characterized by their ability to synthesize a specific matrix consisting of type II collagen and glycosaminoglycans (GAGs) that allows them to withstand changes in their mechanical environment. However, this phenotype is pliable and in response to a pathological insult, is modulated to an inflammatory phenotype[1,18–20]. For example, exposure of articular chondrocytes to an inflammatory stimulus leads to their activation and production of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), prostaglandin E_2 (PGE_2), nitric oxide (NO), and matrix metalloproteinases (MMPs)[2,17,21].

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Simultaneously, these cells lose their chondrocytic phenotype and fail to synthesize GAGs and collagen type II. This inflammatory phenotype is responsible for amplification of the immune response, cartilage destruction, and pathologies associated with arthritic diseases[1,18–20].

The phenotypic characteristics of chondrocytes are also regulated by biomechanical forces. Both the magnitude and frequency of biomechanical signals are critical determinants in modulating chondrocytic gene expression[21–24]. Biomechanical forces of high (traumatic) magnitudes are proinflammatory and induce enhanced expression of inflammatory mediators. Proinflammatory alterations in gene expression lead to enzymatic breakdown of cartilage and inhibition of matrix synthesis[12,24,25]. Trauma caused by mechanical forces of high magnitudes induces rapid synthesis of proteases involved in GAG and collagen degradation[12,25].

Biomechanical signals of lower magnitudes act as potent anti-inflammatory signals that modulate the inflammatory phenotype to a chondrocytic phenotype by down-regulating proinflammatory gene expression and up-regulating matrix synthesis[15,21–23,27–30]. For example, activation of articular chondrocytes by IL-1β or TNF-α results in a marked upregulation of proinflammatory gene induction[1,2,17]. Signals generated by mechanical forces of low magnitudes inhibit IL-1β-induced mRNA transcription and synthesis of potent catabolic genes like inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), and MMPs. Simultaneously, these signals upregulate matrix synthesis by counteracting the proinflammatory inhibition of tissue inhibitors of matrix metalloproteinases (TIMP), GAG synthesis, and collagen II expression[13,14,21,22,27–29]. In the same context, dynamic motion ameliorates the effects of arthritis and alleviates the pain, by exerting potent anti-inflammatory responses generating reparative signals[7,8,14].

Mounting evidence indicates that biomechanical forces regulate two main categories of genes, those involved in inflammation and those involved in matrix synthesis[21,23–25,29,30]. Pharmacological therapies like nonsteroidal anti-inflammatory drugs suppress the inflammatory response, but are not known to alter the inflammatory phenotype or induce cartilage repair or regeneration. Furthermore, these therapeutics provide transient relief mostly by inhibiting the cyclooxygenase pathway. In comparison, biomechanical signals appear to be more potent in that they inhibit the induction of a plethora of proinflammatory genes, as well as upregulate synthesis of matrix associated molecules[13,21,22,28–30]. A major consideration for the efficacious therapeutic use of biomechanical signals is to know how long the anti-inflammatory and reparative effects are sustained following the removal of the biomechanical stimulus. In this report we have examined the time-dependent consequences of the removal of biomechanical signals on the suppression of proinflammatory gene induction. Our studies show that effects of biomechanical signals are sustained for prolonged periods of time even in a proinflammatory environment.

Materials and methods

ISOLATION OF ARTICULAR CHONDROCYTES

Articular chondrocytes were obtained from the cartilage of shoulder and knee joints from 14 to 16 weeks old healthy Sprague Dawley rats ($n = 5–8$)[13,21]. Approximately 200 microns of superficial cartilage was shaved from the heads of the bones and finely chopped. The chondrocytes were enzymatically released by digestion with 0.2% trypsin for 15 min, followed by a 3 h digestion with 0.15% collagenase I (Worthington Biochemical Corp., NJ) in a two-compartment digestion chamber kept at 37°C. Chondrocytes were cultured in Tissue culture medium (TCM) [50% Dulbecco's modified Eagle's medium and 50% Ham's F-12 medium (Mediatech, VA) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (100 U/100 μg/ml)] at 37°C, in an atmosphere of 5% CO_2 for 7 days. Subsequently, chondrocytes (10^5/well) were transferred to collagen-I coated six-well Bioflex plates (Flexcell International, NC), and grown for 5 days to attain 75–80% confluence. Chondrocytes from the shoulder and knee were found to respond to mechanical signals in an identical manner, and therefore were pooled for these studies. Chondrocytes in passages 2 and 3 were used for experimentation, where they exhibited typical phenotypic markers, i.e., aggrecan, type II collagen, and sox-9 expression[13,31].

APPLICATION AND EXPOSURE OF CELLS TO CYCLIC TENSILE STRAIN (CTS)

Chondrocytes (5×10^4 cells/well) were cultured in type I collagen coated, six-well Bioflex-II plates (Flexcell International, NC) for 5 to 6 days. Twenty four hours prior to initiating experiments, TCM of the 80–90% confluent monolayers of chondrocytes was replaced with TCM containing 1% FCS as described earlier[13]. The cells were subjected to equibiaxial tensile strain, by placing Bioflex-II plates on a loading platform equipped with round loading posts in a FX-4000T Flexercell Tension System (Flexcell International, NC). Initially, cells were subjected to CTS of various magnitudes and frequencies in the presence or absence of recombinant human interleukin-β (IL-1β). CTS at a magnitude of 3% and a frequency of 0.25 Hz, suppressed greater than 90% of the rhIL-1β-induced iNOS mRNA expression and NO production, reproducibly. Concurrently, cells were subjected to (1) no treatment (control), (2) rHuIL-1β (1 ng/ml; Calbiochem, CA), (3) CTS at a magnitude of 3% at 0.25 Hz, or (4) CTS and rHuIL-1β. Cells in groups 3 and 4 were subjected to CTS at the start of the experiment, and rHuIL-1β was immediately added to groups 2 and 4. Cells were subjected to CTS for various time intervals, harvested, and analyzed as required. No differences in the proliferation rate and cell viability after stretching were observed during 24 h of experimentation.

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

RNA was isolated from cells using the RNeasy kit (Qiagen, CA) after shredding through a Qiashredder (Qiagen, CA). The RNA was subjected to DNAse digestion, and stored in 40 μl of RNAse-free water. The concentration and purity of RNA were spectrophotometrically assessed at 260 nm. The abundance of mRNA encoding for aggrecan was examined as described earlier[13,21]. A total of 1 μg of RNA was mixed with 1 μg oligo-dT (12–18 oligomers) in RT buffer and incubated for 10 min at room temperature. This mixture was transcribed with 200 units of MULV reverse transcriptase for 30 min at 37°C. The cDNA was amplified with 0.1 μg of specific primers in a reaction mixture containing 200 μM dNTP and 0.1 units of Taq polymerase in PCR buffer. The three steps of PCR-denaturation, amplification, and extension were done in a thermal cycler (Eppendorf) for 30 cycles of 40 s at 94°C, 40 s at 62°C and 60 s at 72°C. Each sample that underwent RT was assessed

for glyceraldehyde phosphate dehydrogenase (GAPDH) as a standard. The sense and antisense sequences of rat primers used were as follows: GAPDH (323 bp) sense 5′-AGACAGCCGCATCTTCTTGT-3′, antisense 5′-TACT-CAGCACCAGCATCACC-3′; aggrecan (179 bp) sense 5′-CTACGACGCCATCTGCTACA-3′, antisense 5′-GCT TTG CAGTGAGGATCACA-3′. PCR products were separated on a 2%-agarose gel at 100 V/cm in Tris−acetate/ethylenediaminotetraacetic acid (EDTA) electrophoresis buffer. The gels were stained with ethidium bromide and then photographed in Kodak Image Station 440 CF. The intensity of the bands was analyzed by IMAGE J (NIH, MD) program.

REAL-TIME REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Gene specific primer sequences were selected using the TaqMan Probe and Primer Design function of the Primer Express v1.5 software (Applied Biosystems, CA). The sense and antisense sequences of rat primers used were as follows: MMP-13 sense 5′-GTTCAAGGAATCCAGTCTC TCTATGG-3′, antisense 5′-TGGGTCACACTTCTCTG-GT GTTT-3′, probe 6-FAMd (CCAAGGAGATGAAGACCC CAACCCTAAGC) BHQ-1 (XM343345); iNOS sense 5′-TTC TGTGCTAATGCGGAAGGT-3′, antisense 5′-GCTTCCGA CTTTCCTGTCTCA-3′, probe 6-FAMd (CCGCGTCAGAG-CCACAGTCCT) BHQ-1 (D44591); Cox-2 sense 5′-CTT TGGCAGGCTGGATTTTAA-3′, antisense 5′-AGAAGCC CACTGATACCTTTTGC-3′, probe 6-FAMd (TGCACAG TATGACACAACAGCCCATCTCTC) BHQ-1 (AF233596); MMP-9 sense 5′-AGCG-CCAGCCGACTTATGT-3′, antisense 5′-ACACAGCTGGCAGAGGATTACC-3′, probe 6-FAMd (TCTTCCCCCAGACCTGAAAACCTCC) BHQ-1 (NM_031055). Reverse transcription reactions were carried out using 2 μg RNA and TaqMan Reverse Transcription reagents, followed by real-time PCR using TaqMan® PCR Master Mix and ABI Prism 7700 Sequence Detection System® (Applied Biosystems, CA). Reactions were performed as follows: Cycle I (1×): 95°C for 3.0 min, Cycle II (50×): Step 1 at 95°C for 0.3 min, followed by Step 2 at 55°C for 0.3 min, and Step 3 at 72°C for 30 min, and Cycle III at 40°C hold. Following amplification, a melting curve was obtained to ensure that primer-dimers or non-specific products had been eliminated or minimized. The data, obtained by real-time PCR, were analyzed by the comparative threshold cycle (C_T) method. In this method, the amount of the target, normalized to GAPDH, and relative to a calibrator (either untreated sample or IL-1β-stimulated cells), is given by $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T$ (sample) $- \Delta C_T$ (calibrator), and ΔC_T is the C_T of the target gene subtracted from the C_T of GAPDH[27].

CELLULAR PROTEIN ANALYSIS

After exposure to various treatment regimens, Flexcell membranes from each well were removed and cut into four to six pie shaped pieces. The cells growing on the Bioflex membranes directly over the loading posts of the Flexcell plate were analyzed, while the marginal area of the well stretched by vacuum was excluded. The cellular expression of proteins was analyzed by immunofluorescence staining, using mouse anti-iNOS IgG (BD Bioscience 610431) and FITC conjugated donkey anti-mouse IgG (Jackson Lab715095151); rabbit anti-COX-2 (Cayman Chemicals, MI) and goat anti-rabbit-CY3; goat anti-MMP-9 (Santa Cruz, CA) IgG and donkey anti-goat-FITC (Jackson Lab, MN). Subsequently, cells were mounted on the membrane with Vectashield (Vector Labs, CA), and observed under an epifluoresence microscope (Ziess Axioimage) or by laser scanning cytometry (LSC). At least three membranes from separate experiments were analyzed. On each membrane, 8 to 10 areas of 50 cells each were counted to assess the number of fluorescence positive cells by LSC or intensity of fluorescence by Zeiss Axiovision software (Carl Zeiss Inc., Germany).

For GAG analysis sections of the Flexcell membranes exposed to various treatment regimens were fixed with ice cold methanol and stained with 0.1% Safranin-O for 10 min, and washed gently with water. Subsequently, synthesis of GAGs in chondrocytes and pericellular area was examined microscopically and analyzed semiquantitatively using the Zeiss Axiovision software (Carl Zeiss Inc., Germany). The Field Density Means were obtained from five different areas of each section of the membrane containing 80 to 100 chondrocytes. The mean values were calculated and presented as Field Density Means per 100 cells to obtain a comparative value for GAG production in the chondrocytes following various treatments.

WESTERN BLOT ANALYSIS

For semiquantitative measurements of proteins synthesis Western blot analysis was used as described earlier[21,27]. Briefly, cells were lysed in ice cold Tris buffered saline (TBS) containing protease inhibitor cocktail (Roche, IN), and the extracted proteins were loaded on the sodium dodecyl sulphate (SDS)-10% acrylamide gels. The proteins were electrophoretically transferred to nitrocellulose membranes (Bio-Rad, CA) and identified by monoclonal mouse anti-MMP-13 IgG (1:1000 dilution; Calbiochem, CA). Monoclonal mouse anti-β-actin IgG (1:20,000; Abcam, MA) was used to reprobe the same blots to equilibrate protein input in all lanes. Horseradish peroxidase (HRP)-labeled donkey anti-mouse IgG (1:10,000 dilution; Chemicon, CA) or HRP-labeled goat anti-rabbit antibody (1:10,000 dilution; Santa Cruz, CA) was used as a second antibody. The presence of HRP was detected by Luminol (Amersham, IL), and the semiquantitative analysis of luminescent bands was carried out with Kodak Image Station 1000®, and Kodak 1D image analysis software.

DATA ANALYSIS AND STATISTICS

The SPSS 13.0 software (SPSS Inc., Chicago, IL) was used for statistical analysis. Each experiment was performed at least three times. For quantitative analysis, means ± S.E.M. were calculated. To determine whether significant differences exist between groups, One-Way Analysis of Variance (ANOVA) and the *post-hoc* multiple comparison Tukey test were applied. To identify differences between IL-1β-treated cells in the absence or presence of CTS at various magnitudes and frequencies, One-Way ANOVA and the *post-hoc* multiple comparison Dunnett test were used. Differences were regarded as statistically significant at values of $P < 0.05$.

Results

INHIBITION OF IL-1β DEPENDENT INOS INDUCTION BY CTS IS LONG-LASTING

To gain insight into the effects of biomechanical stimulation that results in sustained anti-inflammatory effects, we first determined IL-1β-induced iNOS mRNA expression in

chondrocytes continuously exposed to CTS for various time intervals. As shown in Fig. 1(A) inset, IL-1β induced a marked upregulation of iNOS mRNA in chondrocytes. Simultaneous exposure of cells to CTS for 4, 8, or 16 h blocked more than 90% of IL-1β-induced iNOS expression at all time points tested. To further determine how long the signals generated by CTS persisted, chondrocytes after addition of IL-1β were immediately subjected to CTS. After various time intervals CTS was removed and cells allowed to rest in IL-1β containing medium (CTS/rest) for additional periods of time, and analyzed for IL-1β-induced iNOS expression. Figure 1(A) demonstrates that, cells exposed to CTS/rest for 4/20, 8/16, 12/12, 16/8, 20/4, or 24/0 h, suppressed IL-1β-induced iNOS expression by 54%, 93%, 90%, 84%, 80%, and 90%, respectively, despite the presence of IL-1β. This suggested that a 4 h exposure to CTS, that inhibits 97% of IL-1β-induced iNOS expression [inset Fig. 1(A)], was not sufficient to inhibit iNOS expression 20 h later. Nevertheless, maximal inhibition of iNOS expression was observed following an 8 h CTS exposure which persisted for 16 h [Fig. 1(A)]. LSC analysis of chondrocytes immunostained for iNOS revealed that the transcriptional downregulation of iNOS expression by CTS was also reflected in a significant reduction ($P < 0.05$) in the number of iNOS positive cells [Fig. 1(B)]. Specifically, cells exposed to 8, 12 or 16 h of CTS, followed by 16, 12 or 8 h of rest exhibited lowest iNOS synthesis as compared to IL-1β-treated controls.

To further determine how long the CTS-mediated suppression of IL-1β actions persists, we next exposed IL-1β-treated chondrocytes to CTS for 8 h followed by a rest for the ensuing 16, 28, or 40 h. The results demonstrated CTS exposure for 8 h caused a 90% inhibition of iNOS mRNA expression 16 h later, but the same exposure failed to suppress iNOS mRNA expression 28 or 40 h later [Fig. 1(C)]. Concomitant measurements of NO release in the culture supernatants, measured by Griess reaction, revealed that NO production was also inhibited by 64% in chondrocytes exposed to CTS for 8 h followed by a 16 h rest [Fig. 1(D)]. However, CTS exposure for 8 h was insufficient to block IL-1β-induced NO production 28 or 40 h later. This showed that 8 h of CTS is sufficient to inhibit proinflammatory gene induction for a limited period of time, beyond which effects of IL-1β become apparent again [Fig. 1(D)].

SUSTAINED DOWNREGULATION OF COX-2 INDUCTION BY CTS

To confirm that CTS regulates transcriptional activation of multiple proinflammatory genes upregulated in arthritic joints, we next determined the effects of CTS on COX-2 induction. The extent of the persistence of CTS signals on COX-2 mRNA expression was evaluated by subjecting the chondrocytes to CTS and IL-1β as described above. Quantitative analysis of PCR products showed a low level constitutive COX-2 mRNA expression in chondrocytes. As

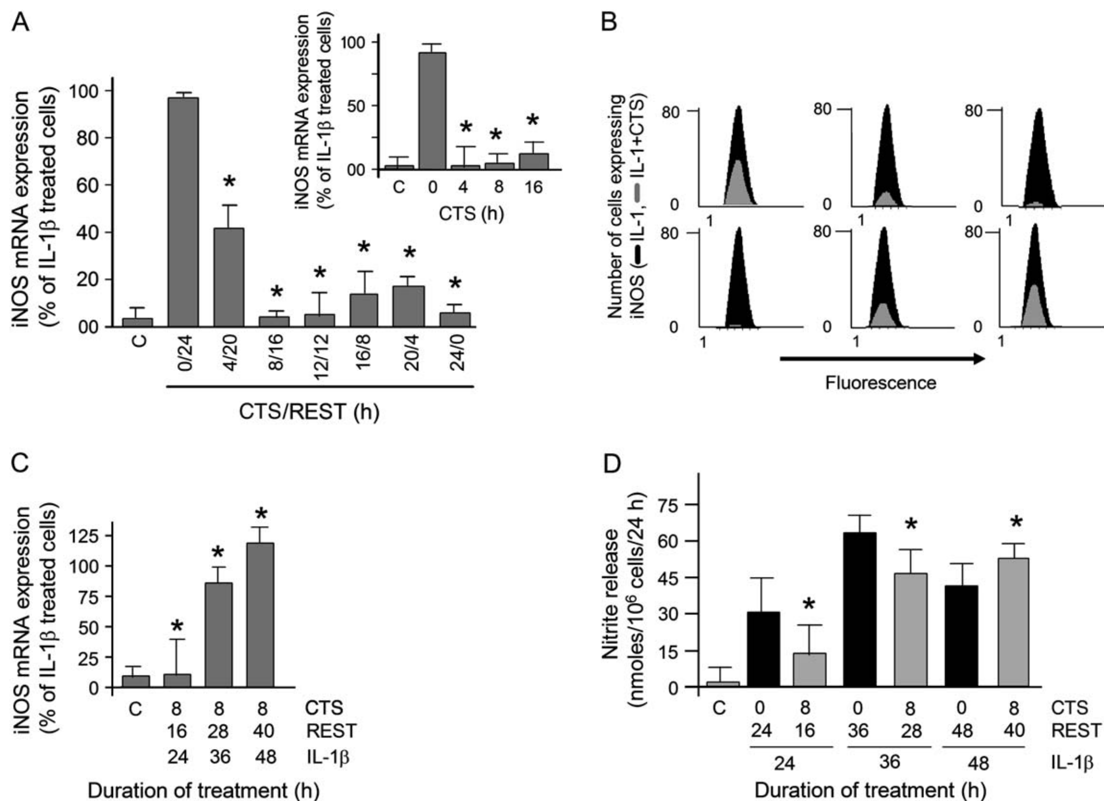

Fig. 1. Effect of CTS on iNOS induction in articular chondrocytes. Chondrocytes grown on Bioflex plates were exposed to CTS for 0, 4, 8 or 16 h, in the presence of IL-1β (inset A), or CTS followed by rest (CTS/rest) for 4/20, 8/16, 12/12, 16/8, 20/4 or 24/0 h, in the constant presence of IL-1β (A). The expression of iNOS mRNA was analyzed by real-time PCR (A, and inset A), and iNOS protein was assessed in immunostained chondrocytes by LSC (B). Analysis of iNOS mRNA expression in chondrocytes exposed to CTS/rest for 8/16, 8/28, or 8/40 h by real-time PCR (C), and total NO accumulation in the culture supernatants of chondrocytes exposed to CTS/rest for 8/16, 8/28, or 8/40 h (D). Data represent mean and s.e.m. of three separate experiments performed in triplicates. * indicates $P < 0.05$ as compared to IL-1β-treated cells.

shown in Fig. 2(A), the signals generated by CTS/rest for 4/20, 8/16, 12/12, 16/8, 20/4, or 24/0 h, significantly inhibited IL-1β induced COX-2 mRNA expression by 37%, 72%, 81%, 88%, and 83%, respectively [Fig. 2(A)]. Nevertheless, signals generated by CTS which could inhibit 96% of IL-1β-induced COX-2 mRNA expression [inset Fig. 2(A)] failed to inhibit more than 37% of COX-2 mRNA expression following a 20 h rest [Fig. 2(A)]. Quantitative analysis by LSC of cells immunostained for COX-2 revealed that CTS also inhibits its synthesis in a persistent manner in parallel to inhibition of COX-2 mRNA [Fig. 2(B)]. Similarly, despite the presence of IL-1β, signals generated by CTS/rest for 4/20, 8/16, 12/12, 16/8, or 20/4 h blocked COX-2 induction by a significant 68%, 63%, 67%, 76%, or 78%, respectively. Interestingly, exposure of cells to 24 h of continuous CTS without rest revealed more than 94% suppression of COX-2 induction, indicating that constant exposure to CTS is a persistent suppressor of COX-2 [Fig. 2(B and C)].

As evident in Fig. 2(C), COX-2 induction by IL-1β coincided with its nuclear translocation. Not only did CTS exposure down-regulate IL-1β-induced synthesis of COX-2, but also its nuclear translocation. Exposure of cells to CTS/rest for 8/16 to 16/8 h demonstrated a significant reduction in the presence of COX-2 in both the cytoplasmic and nuclear compartments [Fig. 2(C)]. Interestingly, while a complete inhibition of COX-2 mRNA expression was not observed at any time point tested, LSC analysis failed to detect the presence of COX-2 in cytoplasmic or nuclear compartment of untreated control chondrocytes.

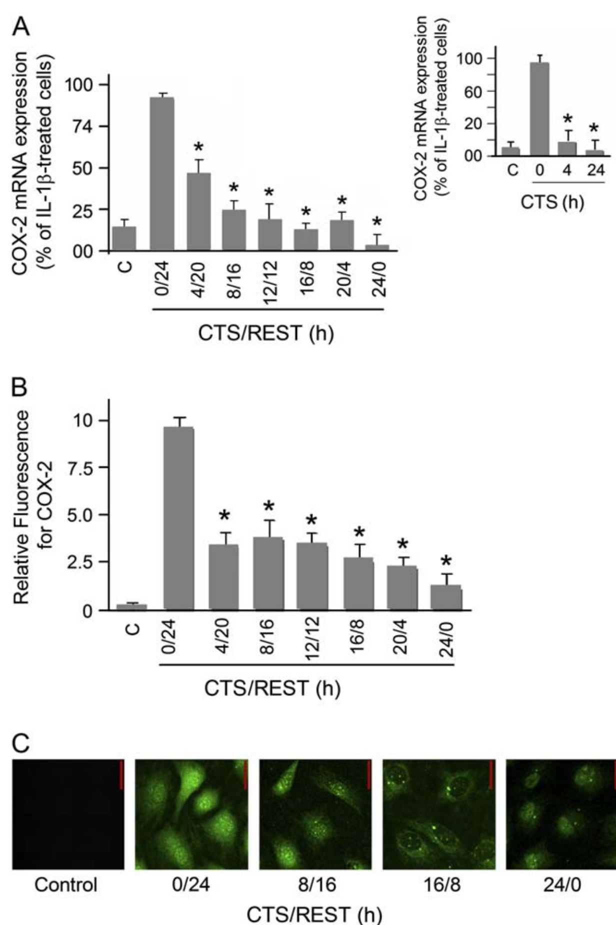

Fig. 2. Effect of CTS on IL-1β-dependent COX-2 induction in articular chondrocytes. Chondrocytes grown on Bioflex plates, were exposed to CTS for 4 or 24 h, in the presence of IL-1β (inset A), or CTS/rest for 4/20, 8/16, 12/12, 16/8, 20/4 or 24/0 h, in the constant presence of IL-1β (A). The expression of COX-2 mRNA was analyzed by real-time PCR (A, and inset A), and COX-2 protein was assessed in immunostained chondrocytes by LSC (B). Microscopic representation of chondrocytes treated with CTS/rest for 0/24, 8/16, 16/8, or 24/0 h in the presence of IL-1β showing nuclear localization of COX-2. Chondrocytes were stained with anti-COX-2 antibodies to show the sustained inhibition of COX-2 synthesis in cells treated with CTS/rest for various time intervals (C). Data in (A and B) represent mean and S.E.M. of three separate experiments performed in triplicates. Micrographs in (C) represent one of three separate experiments. * indicates $P < 0.05$ as compared to IL-1β-treated cells.

CTS-MEDIATED SUPPRESSION OF IL-1β-DEPENDENT MMP INDUCTION

To determine whether continuous application of CTS also results in the inhibition of IL-1β-induced MMP-9 and MMP-13 mRNA expression, chondrocytes were exposed to IL-1β alone for 4 or 24 h. As shown in Fig. 3(A) inset, IL-1β induced a marked upregulation of MMP-9 and MMP-13 mRNA in chondrocytes. Simultaneous exposure of cells to CTS for 4 or 24 h blocked more than 90% of IL-1β-induced MMP-9 and MMP-13 expression. To further determine whether CTS elicits sustained effects on the inhibition of MMP-9 and MMP-13 expression, cells were subjected to CTS/rest for 4/20, 8/16, 12/12, 16/8, 20/4, or 24/0 h, in the presence of IL-1β. Subsequent mRNA analysis demonstrated that like iNOS and COX-2, CTS continued to block both MMP-9 and MMP-13 mRNA expression persistently after its removal. An 8 h or longer exposure to CTS was required for its persistent effects. Again, constant application of CTS for 4 h which inhibited more than 90% of MMP-9 and MMP-13 mRNA expression [inset Fig. 3(A)], exhibited only a 52% (MMP-9) and 68% (MMP-13) inhibition following removal of CTS for 20 h, indicating that the signals generated by CTS persist for a limited time and the IL-1β-induced proinflammatory gene expression resumes after removal of CTS.

We further confirmed that CTS-mediated suppression of IL-1β induction of MMP-9 and MMP-13 mRNA was reflected in sustained inhibition of their synthesis [Fig. 3(B)]. Quantitative analysis of fluorescence by LSC in cells immunostained for MMP-9 and Western blot analysis for MMP-13 revealed that signals generated by CTS also blocked MMP-9 and MMP-13 synthesis in a sustained manner. As apparent in Fig. 3(B), protein synthesis in chondrocytes in response to CTS demonstrated a pattern similar to mRNA expression for both MMP-9 and MMP-13. Notably, a 4 h CTS exposure was insufficient to sustain the MMP-9 and MMP-13 inhibition more than 51% and 69%, respectively [Fig. 3(B)]. CTS/rest for 8/16 h exhibited maximal inhibition of both MMP-9 and MMP-13, 16 h later. On the contrary, examination of protein synthesis after CTS/rest for 12/12, 16/8, or 20/4 h revealed a suppression of 75%, 66%, or 77%, respectively, which is significantly less than that observed by CTS/rest for 8/16 h [Fig. 3(B and C)].

CTS-MEDIATED UPREGULATION OF AGGRECAN SYNTHESIS IS SUSTAINED FOLLOWING REMOVAL OF MECHANICAL SIGNALS

Since aggrecan constitutes the major protein in the GAGs, next experiments were designed to examine the effects of CTS on aggrecan gene transcription. Chondrocytes expressed high levels of aggrecan mRNA constitutively, and

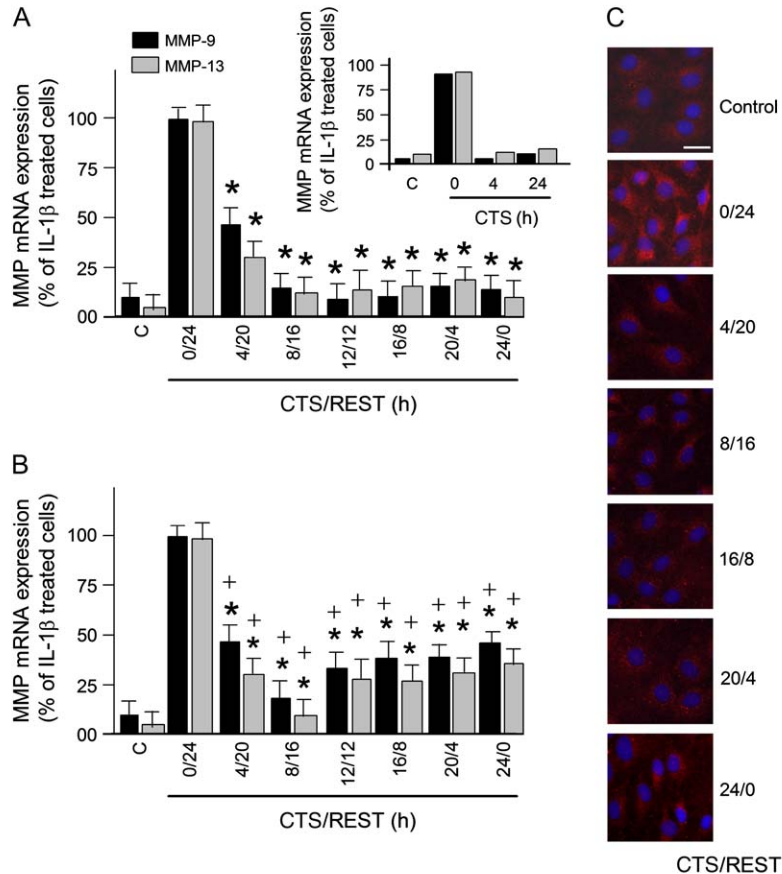

Fig. 3. CTS-induced blocking of IL-1β-dependent induction of MMP-9 and MMP-13. Articular chondrocytes grown on flexible bottom plates were exposed to various durations of CTS or CTS/rest. Chondrocytes exposed to CTS for 4 and 24 h in the presence of IL-1β and analyzed for MMP-9 and MMP-13 by real-time PCR [inset Fig. 1(A)]. Chondrocytes exposed to CTS/rest for 4/20, 8/16, 12/12, 16/8, 20/4 or 24/0 h were analyzed by real-time PCR for MMP-9 and MMP-13 mRNA expression (A). Synthesis of MMP-9 was assessed by LSC evaluation of immunostained cells (B and C) and synthesis of MMP-13 was determined by densitometric analysis of Western Blots (B). Data represent mean and S.E.M. of three separate experiments. * indicates $P < 0.05$ as compared to IL-1β-treated cells. ✣ indicates $P < 0.01$ as compared to chondrocytes treated with CTS/rest for 8/16 h.

this expression was markedly inhibited (95 ± 4%) by treatment of cells with IL-1β for 4 or 24 h. Simultaneous exposure of cells to CTS and IL-1β for 4 or 24 h rescued IL-1β-induced inhibition of aggrecan gene expression by 62% or 41%, respectively [Fig. 4(B)]. To determine whether the signals generated by CTS persistently upregulate aggrecan gene transcription, chondrocytes were subjected to the same regimen of CTS/rest as described above. Examination of cells exposed to CTS/rest for various time intervals, revealed that CTS consistently revoked IL-1β-dependent inhibition of aggrecan mRNA expression persistently at all time points tested, indicating an anti-catabolic role of CTS on chondrocytes. For example, a 4.57, 4.62, 5.71, 6.71, 12.28, or 9.57 fold increase in mRNA was observed in cells exposed to CTS/rest for 4/20, 8/16, 12/12, 16/8, 20/4, or 24/0 h, respectively [Fig. 4(A)]. Thus, cumulatively these data indicated that CTS exerts potent anabolic effects by mitigating the IL-1β-mediated inhibition of aggrecan transcriptional regulation.

The semiquantitative analysis of GAG contents in chondrocytes revealed a marked inhibition of total GAG contents in IL-1β-treated cells, as compared to untreated control cells. Exposure to CTS rescued GAG synthesis by counteracting IL-1β-induced GAG inhibition. The effects of CTS were persistent, and could be observed following removal of CTS. For example, chondrocytes exposed to CTS/rest for 4/20, 8/16, 12/12, 16/8, 20/4 and 24/0 h in the constant presence of IL-1β, respectively, exhibited a 245%, 252%, 287%, 330%, 370%, or 265% greater total GAG contents as compared to IL-1β-treated cells [Fig. 4(C)]. Nevertheless, GAG synthesis was below untreated control cells unless cells were treated with CTS/rest for 16/8 or 20/4 h.

Discussion

The present findings provide molecular basis for the persistent actions of biomechanical signals in chondrocytes. While earlier studies demonstrated that signals generated by both tensile and compressive forces of low magnitudes inhibit the IL-1β-dependent proinflammatory gene transcription[13,21,22,27–29], the persistence of these signals in a proinflammatory environment is as yet unclear. Studies directed toward arthritic joint healing *in vivo*, have demonstrated that controlled application of biomechanical stimulation reduces inflammation and enhances wound healing in inflamed or injured cartilage[7,8,33]. However, these reports do not address the persistence of biomechanical signals in relation to the duration of motion-based therapies. To answer such key questions, we have used primary cultures of chondrocytes to examine the long term effectiveness of biomechanical strain in attenuating inflammatory

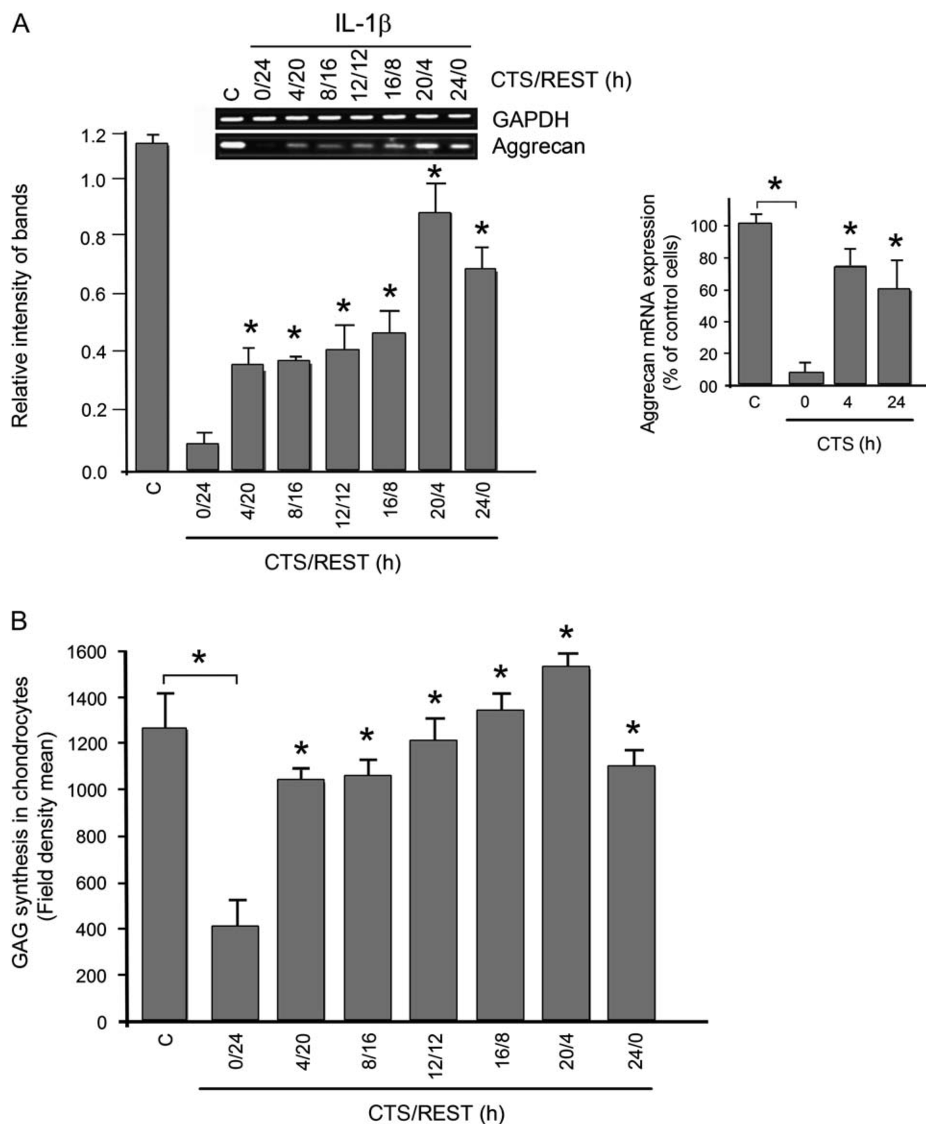

Fig. 4. Effect of CTS on IL-1β induced attenuation of aggrecan synthesis. Chondrocytes cultured on Bioflex plates were subjected to CTS or CTS/rest in the presence of IL-1β. Cells exposed to CTS/rest for 4/20, 8/16, 12/12, 16/8, 20/4, or 24/0, were analyzed for GAPDH and aggrecan mRNA expression by RT-PCR (inset A), and the intensity of each PCR product measured by densitometric analysis (A). RT-PCR analysis of chondrocytes exposed to CTS for 0, 4, or 24 h, in the presence of IL-1β (B). GAG synthesis in chondrocytes exposed to various periods of CTS/rest as shown in (A), was examined by Safranin-O staining, using dichromatic filter and Axioplan Software. The bars represent means and s.e.m. of Field Density Mean per 100 cells in five different areas of membrane, * represents $P < 0.05$ as compared to IL-1β-treated cells (C).

mediator production and augmenting the extracellular matrix synthesis *in vitro*. We show that signals generated by CTS are sustained for prolonged periods of time following the removal of mechanical stimulation, and continue to act as potent anti-inflammatory signals in a proinflammatory environment. We have used CTS at a magnitude of 3%, which inhibits proinflammatory gene induction. Since at these magnitudes CTS alone does not induce proinflammatory gene induction, these magnitudes do not appear to be hyper-physiologic, and hence may be at physiologic levels.

The examination of the sustained effectiveness of CTS as an anti-inflammatory signal is important to the application of biomechanical therapies on inflamed cartilage. Therefore, to gain broader understanding of the role of biomechanical signals in the preservation of cartilage, we have focused on the regulation of four major proinflammatory mediators with distinct functions in cartilage destruction[1,9,10,33–35], namely iNOS, COX-2, MMP-9 and MMP-13. All of these mediators are produced by chondrocytes and are elevated in arthritic joints. iNOS and COX-2 are involved in the amplification of proinflammatory responses. NO is cytotoxic to chondrocytes, damages cartilage, and is involved in the upregulation of MMPs[34,36,37]. COX-2 induces prostaglandins that in turn upregulate production of inflammatory mediators including cytokines to initiate cartilage destruction[35,38]. Chondrocytes synthesize a number of MMPs among which MMP-13 and MMP-9 are major mediators of matrix degradation in RA and OA[12,32]. MMP-9 and MMP-13 are directly responsible for proteolytic degradation of cartilage. In addition to collagen, MMP-13 also degrades the proteoglycans including aggrecan, giving it a dual role in matrix destruction. MMP-9 is

involved in degradation of non-collagen matrix components of the joints[16,33].

The present findings demonstrate a number of important points with regard to the biomechanical modulation of articular chondrocyte phenotype and functions. First, continuous exposure of chondrocytes to CTS induces a marked suppression of IL-1β-dependent proinflammatory gene induction, during the entire duration of exposure to CTS of low magnitude (3%) and frequency (0.25 Hz). However, these studies were limited to a 24–48 h window. Whether the effectiveness of biomechanical signals is reduced after longer durations is yet to be elucidated. Additionally, exposure of chondrocytes to continuous CTS from 4 to 24 h inhibits IL-1β-induced iNOS, COX-2, MMP-9, and MMP-13, mRNA expression and synthesis, suggesting that the effects of CTS are not limited to one or two genes, rather its effects are broad and modulate expression of several proinflammatory genes.

The second important point is that the effects of CTS are persistent and continue to attenuate IL-1β-induced proinflammatory gene transcription for hours after the removal of CTS even in an inflammatory environment. CTS-mediated modulation of proinflammatory gene expression is long-lasting, i.e., if chondrocytes are exposed to CTS for 8 h, the signals generated by CTS continue to block iNOS gene transcription and synthesis for the next 16 h. Furthermore, extending the time of exposure to CTS followed by a shorter interval of rest does not significantly alter the persistence of signals generated by CTS. Additionally, after removal of CTS the signals continue to suppress multiple proinflammatory genes like COX-2, MMP-9 and MMP-13 in a persistent manner, despite the presence of a proinflammatory environment. Both of the above observations suggest that the suppression of proinflammatory genes occurs at the transcriptional level. Previously it has been shown that CTS inhibits IL-1β-induced proinflammatory genes via suppression of nuclear translocation of NF-κB[21]. This may explain the CTS-mediated inhibition of multiple proinflammatory genes that are under the control of NF-κB[38]. It is also likely that the persistent effects of CTS in inhibiting proinflammatory gene induction are also controlled by NF-κB transcription factors. If so, whether this occurs via regulation of NF-κB gene transcription or regulation of its activation is as yet to be explored.

The third significant finding is that, during biomechanical modulation of chondrocyte functions, the duration of exposure to CTS is a critical determinant of the length of its persistent effects on cells. This is evidenced by the observations that constant CTS exposure for 4 h leads to greater than 90% inhibition of iNOS mRNA expression, whereas, a 4 h CTS exposure followed by a rest of 20 h results in the re-expression of all proinflammatory genes examined, i.e., iNOS, COX-2, MMP-9 and MMP-13. Similarly, constant CTS exposure for 8 h blocks more than 95% of iNOS gene transcription, and the level of inhibition of iNOS mRNA expression and synthesis is maintained following a 16 h rest. Nevertheless, an 8 h exposure of CTS was insufficient to block iNOS gene transcription and NO production 28 or 40 h later. These findings suggest that the extent of persistence of anti-inflammatory signals generated by CTS is dependent upon the duration of exposure of CTS to the chondrocytes. Additionally, CTS blocks expression of proinflammatory proteins transiently and these genes re-express following a defined period of rest in a proinflammatory environment. It is important to note that CTS alone as a constant signal, or after a period of rest, does not induce proinflammatory signals in chondrocytes. In this regard, the magnitudes of mechanical signals play an important role in inducing or attenuating proinflammatory signals[10,12,15,21–30].

LSC analysis in our studies revealed that IL-1β induces iNOS production in all chondrocytes. However, the re-expression of iNOS after removal of CTS, varied among chondrocytes. In cartilage, chondrocytes are shown to be of different types that differ in their responses to IL-1β[39]. Moreover, in our studies chondrocytes were obtained from two different sites, shoulder and knee. Therefore, it is feasible that CTS removal leads to iNOS induction in a certain population of cells in the presence of a proinflammatory stimulus. On the contrary, LSC analysis demonstrated that CTS blocked expression of COX-2, MMP-9 and MMP-13 to the same degree in all cells. Furthermore, re-expression of these proteins following removal of CTS was also similar in all cells. These findings again point to the fact that the sustained effects of CTS in blocking proinflammatory gene expression may be controlled at transcriptional level.

Finally, to be clinically effective as a therapeutic signal in joint repair, biomechanical signals must exhibit reparative actions. During biomechanical modulation of chondrocytes, signals generated by CTS are not only effective in suppressing proinflammatory gene induction, but also in unblocking the IL-1β-induced inhibition of aggrecan synthesis. The primary mechanisms for IL-1β-mediated cartilage destruction involve activation of proinflammatory genes on one side, and inhibition of matrix synthesis on the other side[7,13]. The above findings suggest that CTS not only limits the inflammation, but also augments repair of cartilage. For example, IL-1β-induced blockage of aggrecan mRNA expression is rapidly abrogated by CTS within 4 h, and these signals persist following removal of CTS for ensuing 20 h. Thus, biomechanical signals modulate proinflammatory genes and aggrecan transcription and synthesis in a diametrically opposite manner. These findings are of interest, because aggrecan gene expression is controlled by AP-1 transcription factors[40], and suggest that signals generated by CTS may simultaneously act on more than one signaling cascades to control inflammation and initiate repair of inflamed chondrocytes.

In conclusion, we have shown that signals generated by CTS are converted into potent biochemical events that block the synthesis of catabolic mediators and induce anabolic effects in a persistent manner, long after the removal of biomechanical stimuli. Within the constraints of these *in vitro* observations in a rodent model of chondrocyte activation, the clear evidence points to the fact that biomechanical forces may indeed induce sustained anabolic responses in inflamed cartilage. It is likely that human chondrocytes also respond to mechanical signals in a manner similar to rat chondrocytes. In such a case, our findings serve as a foundation for *in vivo* studies, and demonstrate the necessity of using adequate time intervals for sustained effects of motion-based therapies in the optimal management of acute/chronic inflammation of the joints.

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