BIOMECHANICAL SIGNALS BLOCK I-KAPPA B KINASE ACTIVATION FOR THEIR SUSTAINED ANTI-INFLAMMATORY EFFECTS ON ARTICULAR CHONDROCYTES

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Introduction:
Although the precise signaling mechanism underlying mechanotransduction is not completely understood, there is little doubt that mechanical stimulation plays a significant role in limiting joint inflammation. Our previous studies have suggested that mechanical signals induce anti-inflammatory and anabolic responses that are sustained over extended periods. We tested the hypothesis that biomechanical strain uses NF-κB signaling pathway to sustain anti-inflammatory responses.

Material and Methods:
Isolation of articular chondrocytes. Articular chondrocytes were obtained from knee joints cartilage from healthy Sprague Dawley rats and used between 2nd and 3rd passages, where they exhibited constant expression of typical phenotypic markers, aggrecan, type II collagen. Application of cells to equibiaxial dynamic tensile strain (DTF). Chondrocytes seeded on collagen-coated BioFlex-1 plates were grown to 70-80% confluence in 5% CO2. Cells were subjected to DTF using a FX-4000T Flexcell Int, NC). Cells were subjected to (A) control, (B) rhHuIL-1β 1ng/ml (C) DTF at a magnitude of 3% at 0.25 Hz, or (D) DTF and rhHuIL-1β at 1 ng/ml. Cells in groups C and D were subjected to DTF at the start of the experiment, and rhHuIL-1β was immediately added to groups B and D. The cells were subject to DTF for 4, 8, 12, 16, 20 or 24 hr, followed by a rest period of 20, 16, 12, 8, 4, or 0 h, respectively. At the end of 24 h, cells were harvested, and analyzed as described below.

Real Time Polymerase Chain Reaction (PCR). Messenger RNA expression for TNFα, NF-κB, IκBα, IκBβ, and GAPDH was measured by Real Time PCR. To analyze the data the comparative threshold cycle (CT) method was applied. ELISA. Chondrocytes TNF-α secretion in culture medium was determined using commercially available ELISA kit (R&D Systems).

Immunofluorescence. The nuclear translocation of NF-κB p65 was analyzed by immunofluorescence staining, using rabbit anti-p65 IgG and the cellular expression of IκBα and IκBβ was analyzed by using rabbit anti-IκBα IgG and rabbit anti-IκBβ IgG (Santa Cruz, CA). Subsequently, cells were observed under an epifluorescence microscope.

Western blot analysis. For semiquantitative measurements of proteins synthesis we used Western blot analysis. Cells lysates were loaded on the SDS-10% acrylamide gels. The proteins were electrophoretically transferred to nitrocellulose membranes and identified by mouse anti-phosphoNF-κB(Ser 536) or Ser 276), rabbit anti-p65 IgG, rabbit anti-IκBα IgG and rabbit anti-IκBβ IgG. Bands were semiquantitatively assessed by densitometric analysis

IKK assay. Kinetics of IKK activation was investigated using a kinase assay. IKK proteins was immunoprecipitated from cells lysates using anti IKKα/IκBα and then incubated with 2ug of GST-IκBα and 0.5umol of cold ATP at 30°C for 2h. The GST-IκBα was size separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with either anti-phospho-IκBα (Ser32/36) or anti-IκBα.

Results:
(1) DTF blocks IL-1β-induced TNFα induction at both mRNA and protein level in a sustained manner, as evidenced by Real Time PCR and ELISA. (2) DTF inhibits phosphorylation, nuclear translocation, and synthesis of NF-κB. Examination of NF-κB mRNA expression by RT-PCR revealed that DTF suppresses IL-1β-induced NF-κB mRNA synthesis by 70% within 12h followed by 12h of rest. Densitometry analysis of Western blots revealed that DTF also down regulates IL-1β-induced NF-κB p65 phosphorylation at Ser 536 by 70%, within 4 h which is sustained over the next 20 h (Figure1). However, sustained exposure to DTF for 20 or 24 h inhibited NF-κB p65 phosphorylation at Ser 536 by less than 20%. These observations are consistent with the immunofluorescence analysis shown in Figure 2, where DTF inhibited IL-1β-induced NF-κB nuclear translaction following exposure to IL-1 and DTF for 4/20, 8/16, 12/12, 16/8 h of DTF/REST while 20/4 or 24/0h of DTF/REST did not block NF-κB nuclear translocation completely. (3) Anti-inflammatory effects of DTF are mediated by increased expression and synthesis of NF-κB inhibitors, IκBα and IκBβ, that sequesters NF-κB in the cytoplasm. Real-Time PCR data showed increase in IκBα and IκBβ mRNA expression in IL-1β-activated chondrocytes, while DTF/REST suppressed IκBα and IκBβ induction, as revealed by the Western blot analysis and immunofluorescence staining of both proteins. (4) DTF downregulates IL-1β-induced IκB kinase (IKK) activation as shown by lower level of GST-IκBα substrate phosphorylation in cells treated with DTF/REST for 4/20, 8/16, 12/12, and 16/8.

Fig 2. DTF inhibits IL-1β-induced NF-κB nuclear translaction and synthesis

Conclusions: 1. Continuous exposure of chondrocytes to DTF induces a marked suppression of IL-1β-dependent proinflammatory genes induction. 2. The effects of DTF are persistent and continue to attenuate IL-1β-induced proinflammatory proteins for several hours after the removal of DTF even in an inflammatory environment. 3. The extent of persistence of anti-inflammatory signals generated by DTF is dependent upon the duration of exposure of DTF to the chondrocytes. 4. The sustained effects of DTF are mediated by the downregulation of nuclear translocation of NF-κB. 5. Signals generated by mechanical strain inhibit IKK activation and thus block NF-κB-dependent proinflammatory gene transcription. 6. Mechanical signals act in two ways to suppress inflammation, by inhibiting (i) mRNA expression and synthesis of NF-κB, IκBα and IκBβ, and (ii) activation of IKK and thus downregulating IκB degradation and thus inhibition of NF-κB nuclear translocation. 7. Our results demonstrate a necessity of choosing adequate time intervals of physical therapies for its sustained effects in the optimal management of joint inflammation.

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