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MECHANICAL INJURY SUPPRESSES AUTOPHAGY REGULATORS IN CARTILAGE SUPERFICIAL ZONE AND ITS PHARMACOLOGICAL ACTIVATION RESULTS IN CHONDROPROTECTIONB. Caramés^{1,2}, N. Taniguchi¹, D. Seino³, F. Blanco², D. D'Lima³, M. Lotz¹¹The Scripps Res. Inst., La Jolla, CA; ²Osteoarticular and Aging Res. Lab. INIBIC-Complejo Hosp.ario Univ.rio A Coruña, A Coruña, Spain; ³Shiley Ctr. For Orthopaedic Res. and Ed. At Scripps Clinic, La Jolla, CA

Purpose: Mechanical injury induces cell death in cartilage and triggers a remodeling process that ultimately can manifest as osteoarthritis (OA). Autophagy is a process for turnover of intracellular organelles and molecules that protects cells during stress responses. This study determined whether autophagy is activated and has a protective function in the cartilage response to mechanical injury.

Methods: Cartilage explants were obtained from mature (14-30 months old) bovine knee joints. Explants were subjected to single mechanical impact (40% strain, 500 ms). Cell viability was analyzed by Live/Dead cell assay, sulfated glycosaminoglycans (sGAG) released into supernatants were quantified using dimethylmethylene blue (DMB) method and cartilage explants were evaluated histologically by Safranin O staining. The autophagy regulators ULK1, Beclin1 and LC3 were evaluated by immunohistochemistry. To investigate the function of autophagy in response to mechanical injury we treated the cartilage explants with the autophagy inducer rapamycin (1 μ M).

Results: Mechanical injury induced cell death in a time-dependent manner. This increase was significant at 24, 48 and 96 hours compared to control explants without injury ($P < 0.01$). Expression of ULK1, Beclin1 and LC3 was decreased in the superficial zone at 48 hours. To address the role of autophagy after mechanical injury, we activated autophagy by treating cartilage explants with rapamycin. This treatment significantly reduced cell death induced by mechanical injury at 48 hours ($P < 0.05$). We studied the effect of mechanical stress on cartilage extracellular matrix by quantification of sGAG release into supernatants. These results showed an increase of sGAG release into supernatants after injury, which was significant at 48 and 96 hours compared to control explants without injury ($P < 0.001$). In the presence of rapamycin, the levels of sGAG in supernatants were significantly decreased at 48 and 96 hours after mechanical injury ($P < 0.001$).

Conclusions: Mechanical injury to cartilage does not activate autophagy and even suppresses Beclin1 and LC3 in the superficial zone. These results support the hypothesis that autophagy is compromised after mechanical injury, predominantly in the superficial zone where most of the cell death occurs. Pharmacological interventions that enhance autophagy may have chondroprotective activity after mechanical injury to articular cartilage.

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CALCIUM INPUT MODULATES TRANSFORMING GROWTH FACTOR- β 1-INDUCED EXPORT OF INORGANIC PYROPHOSPHATE BY CONTROLLING ANK EXPRESSION IN CHONDROCYTE: POSSIBLE INSIGHT TO THE PATHOPHYSIOLOGY OF HYPERCALCEMIA-RELATED CHONDROCALCINOSISF. Cailotto¹, S. Sebillaud², P. Reboul¹, P. Netter¹, J.-Y. Jouzeau¹, A. Bianchi¹¹LPPIA UMR 7561 Nancy Université CNRS, Vandoeuvre les Nancy, France; ²INRS, Vandoeuvre les Nancy, France

Purpose: Transforming Growth factor (TGF)- β 1 stimulates the extracellular inorganic pyrophosphate (ePpi) generation by chondrocytes, and promotes calcium pyrophosphate dihydrate deposition disease (CPPDD), which also occurs secondary to hyperparathyroidism-induced hypercalcemia. We previously demonstrated that ANK was the major player of TGF- β 1-induced increase in ePpi levels, and that ANK expression was regulated by ERK1/2 and Ca^{2+} -dependent PKC α/β . We investigated the mechanisms by which calcium fluxes may affect ePpi metabolism in articular chondrocytes, with special attention to the main ePpi regulating proteins ANK and PC-1.

Methods: Rat chondrocytes were stimulated with TGF- β 1 (10 ng/ml). The expression of ANK and PC1 (quantitative RT-PCR and western blotting) and ePpi levels (radiometric assay) were studied in the presence of ranging concentrations [1.25 to 5 mM] of extracellular Ca^{2+} (eCa²⁺). Experiments were also done during a disturbed mobilization of intracellular Ca^{2+} (iCa²⁺) induced either by Ca^{2+} ionophore Ionomycin (1 μ M), phospholipase C (PLC) inhibitor U73122 (10 μ M) or Ca^{2+} chelator BAPTA (1 μ M). The contribution

of Ca^{2+} voltage-operated channels (VOC) was assessed by measuring cellular Ca^{2+} input with the fluorescent probe Fluo-4 during blockade of VOC with a panel of selective inhibitors: Agatoxin (1 μ M) for P/Q-VOC, Conotoxin (1 μ M) for N-VOC, Lercanidipin (10 μ M) for L-VOC and NiCl₂ (500 μ M) for T-VOC. The involvement of the Ca^{2+} -sensing receptor (CaSR) was checked with the agonist GdCl₃ (100 μ M). The contribution of ERK1/2, PKC α/β and Sp1 signaling pathways to the TGF- β 1-induced Ppi export was investigated by western blotting. Any crosstalk between these pathways was evaluated using selective inhibitors [PD98059 (10 μ M) for ERK1/2, WP631 (1 μ M) for Sp1 and Gö6976 (5 μ M) for PKC α/β]. RNA silencing (10 nM) was assessed to evaluate the contribution of Sp1 to the ePpi generation. The activity of the Ank promoter was also evaluated by a gene reporter assay.

Results: eCa²⁺ stimulated TGF- β 1-induced ePpi levels in a dose-dependent manner. This effect was supported by the up-regulation of Ank and PC-1 mRNA and protein levels, as well as a major input of Ca^{2+} into chondrocytes. Blockade of the L- and T-VOC decreased significantly the stimulating effects of TGF- β 1 on ePpi levels (by 40 and 75% respectively), and the mRNA levels of Ank (by 60% and 95% respectively) and PC-1 (by 50% and 95% respectively). Lercanidipin and NiCl₂ reduced significantly the TGF- β 1-induced Ca^{2+} input, whereas the other blockers and the CaSR agonist were ineffective on the TGF- β 1 effects. An iCa²⁺ increase enhanced the stimulating effects of TGF- β 1, whereas its chelation reduced them. Blockade of PLC was ineffective, indicating that the regulating role of TGF- β 1 is on the input of eCa²⁺. The ability of TGF- β 1 to activate ERK1/2 and PKC α/β , and to increase Sp1 level, was strongly reduced by blockade of L- and T-VOC or Ca^{2+} chelation, while being enhanced by the Ca^{2+} ionophore. RNA silencing of Sp1 almost suppressed the stimulating effects of TGF- β 1 (ePpi level, ANK and PC-1 expression). Using the inhibitors of Sp1, ERK1/2 or PKC α/β , we demonstrated that these pathways were activated independently by TGF- β 1, and none of them modulated the Ca^{2+} input. In contrast, activation of the Ank promoter by TGF- β 1 was strongly reduced by blockade of L- and T-VOC, while being enhanced in a synergistic manner by overexpression of SP1 or ELK-1 (a transcription factor activated by ERK2).

Conclusions: Our data demonstrate that the input of eCa²⁺ is critical for the stimulating effects of TGF- β 1 on ePpi levels, due to its ability to enhance expression of ANK and PC-1. Entrance of eCa²⁺ is mediated by L- and T-VOCs, and precedes activation of Sp1, ERK1/2 and PKC α/β signaling pathways to regulate the Ank promoter. These data provide, for the first time, a possible mechanism to explain the pathophysiological role of a high eCa²⁺ level in the occurrence of secondary CPPDD.

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THE TRANSCRIPTION FACTOR ISOFORM EPITHELIAL SPECIFIC ETS (ESE)-1B IS AN INHIBITOR OF CARTILAGE CATABOLISM AND DEGRADATION

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Purpose: It has been postulated that the transcription factor epithelial specific Ets (ESE)-1 plays a role as pro-inflammatory molecule in arthritis. Here we investigated the expression and function of ESE-1 in articular cartilage.

Methods: The presence of ESE-1 was assessed on the protein and mRNA level by immunohistochemistry and PCR in human articular cartilage. Expression vectors containing either the full length or a truncated, dominant negative form of ESE-1b were generated in order to transfect primary human articular chondrocytes as well as the chondrocyte cell line C-28/I2. Additionally, stimulation experiments using IL-1 β or native type II collagen were performed in primary human articular chondrocytes or C-28/I2 cells. Quantitative real-time PCR (qPCR) and Western immunoblotting was used to determine differences in mRNA and protein expression of matrix proteinases metalloproteinase (MMP)-13. Additionally, mRNA expression of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and -5 as well as interleukin (IL)-1 β was assessed.

Results: The splice variant ESE-1b was dominantly expressed in articular cartilage and was up-regulated with increasing tissue damage. Functional analysis revealed that ESE-1b represses the mRNA/protein expression of MMP-13, as well as the mRNA expression of ADAMTS-4/-5 and IL-1 β in primary human articular chondrocytes and C-28/I2 cells. Under the influence of catabolic signals, ESE-1b expression is initially decreased but up-regulated in a situation of chronic tissue degeneration due to arthritis, where it aims to suppress proteinase expression.