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Mechanical exposure and diacerein treatment modulates integrin-FAK-MAPKs mechanotransduction in human osteoarthritis chondrocytes

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ABSTRACT

Background: Progression of osteoarthritis (OA) is characterized by an excessive production of matrix degrading enzymes and insufficient matrix repair. Despite of active research in this area, it is still unclear how the combination of mechanical exposure and drug therapy works. This study was done to explore the impact of the disease modifying OA drug (DMOAD) diacerein and moderate tensile strain on the anabolic metabolism and the integrin-FAK-MAPKs signal transduction cascade of OA and non-OA chondrocytes.
Methods: Cyclic tensile strain was applied in terms of three different intensities by the Flexcell tension system. Influence on catabolic parameters such as MMPs, ADAMTS, and IL-6 were assessed by qPCR. Changes in phosphorylation of FAK, STAT3 as well as MAP kinases were verified by western blot analysis. Intracellular calcium was measured fluorimetrically using fura-2.
Results: Tensile strain at moderate intensity (SM/SA profile) proved to be most efficient in terms of reducing production of matrix degrading enzyme and IL-6 expression. Treatment with diacerein by itself and diacerein in combination with SM/SA stimulation reduced phosphorylation of FAK and STAT3, which is more pronounced in OA cells. Pretreatment with diacerein for 7 days resulted in an increase in the sensitivity to Yoda1, the agonist for the mechanically activated ion channel Piezo1. However, in OA chondrocytes a significant reduction in Piezo1 expression was observed following treatment with diacerein.
Conclusion: Our results demonstrated for the first time that diacerein intensively intervenes in the regulation of FAK and STAT3 and influences components considered relevant for the progression of OA, even in the presence of mechanical stimulation.

1. Introduction

Progression of osteoarthritis (OA) is characterized by destruction of articular cartilage, thickening of subchondral bone, and formation of osteophytes. The destruction, remodeling, and inflammation of joint tissues leads to joint failure, which manifests as joint pain and loss of function [1], creating a serious socio-economic burden [2]. Current pharmacological treatment paradigms such as analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) only provide symptomatic relief, but fail to stop disease progression or achieve remission. Therefore, new therapeutic strategies that combine effectiveness and safety are urgently needed. The disease modifying OA drug (DMOAD) diacerein functions as a slow acting drug through anti-inflammatory, anti-catabolic, and pro-anabolic effects on cartilage and the synovial membrane [3,4]. As reported 2016 the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO) considers diacerein beneficial for the treatment of OA [5,6].

Articular damage due to OA results from an imbalance in degradation (catabolic) and promotion (anabolic) activity. A crucial aspect of matrix damage is the overexpression of matrix degrading enzymes, such as matrix metalloproteinases (MMPs) produced by chondrocytes. Direct inhibition of either MMP activity, or the signaling pathways that regulate MMPs are being explored as therapeutic targets. Mechanical loading of joint tissue directly affects the homeostasis of matrix degrading enzyme production and matrix repair [7–9]. On the other hand disuse or immobilization can lead to cartilage atrophy [10].

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Many different mechanisms have been shown to be involved in chondrocyte mechanotransduction [11,12]. Piezo channels, a family of cation-permeable, mechanically directly activated ion channels were recently identified [13,14]. Piezo channels are expressed in chondrocytes and it has been demonstrated that blocking the channel can prevent mechanical stress induced cell death, possibly by reducing Ca^{2+} influx [15].

In addition, the biomechanical stability of cartilage tissue is determined by the composition and integrity of the extracellular matrix (ECM). Integrins, a family of cell adhesion receptors, are involved in the regulation of cell proliferation, survival, differentiation and matrix remodeling by binding cartilage matrix proteins [16,17]. In many cell types, focal adhesion kinase (FAK) is a major hub for integrating signals generated by integrins and growth factors [18]. Many of the signaling intermediates activated by the integrin pathway converge on the mitogen activated protein kinase (MAPK) family which includes the extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK1–3), and p38 resulting in downstream regulation of gene transcription [18,19]. Hence, integrin signals work in concert with signals generated by growth factors, cytokines and mechanical forces.

Due to the fact that MAPKs also function as central regulators of additional cell signaling pathways that control cell proliferation, survival, matrix synthesis, and production of pain mediators, they are considered potential therapeutic targets for several diseases, including OA [20]. The aim of this study was to analyze the effects of diacerein and cyclic tensile strain on the integrin-FAK-MAPK mechanotransduction cascade in OA and non-OA chondrocytes.

2. Methods

2.1. Cell culture

Cells from two immortalized human chondrocyte cell lines, C-28/I2 and T/C-28a2, originating from cells isolated from rib cartilage, were used throughout the study [21]. Cells were cultured using Dulbecco's modified Eagle's medium (DMEM high glucose; GIBCO, Invitrogen, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (all GIBCO, Invitrogen). Cells were kept at 37 °C in a humidified atmosphere of 5% CO_2 and passaged by trypsinization upon reaching confluence. C-28/I2 cells were always stimulated with IL-1β (10 ng/ml; Sigma-Aldrich, St. Louis, MI) as a model for inflammatory OA.

2.2. Mechanical stimulation of chondrocytes

The Flexcell FX5K Tension System (Flexcell International Corp, Burlington, NC) was used to apply mechanical cyclic tensile stain on chondrocytes. The FX5K is a computer-based system that uses a vacuum to strain cells adhered to flexible silicon membranes (BioFlex plates) in a six well plate format. The deformation of the flexible bottom of the plates causes the attached cells to deform (Fig. 1A). Chondrocytes were seeded (5×10^4 cells/well) onto the pronectin-coated BioFlex plates. After the cells were approximately 70% to 80% confluent, chondrocytes were subjected to three different strain profiles: SM (consisting of 8 h resting and 16 h slow-moving activity with 0.2 Hz and 2% elongation), SM/SA (consisting of 8 h resting and four repetitions of alternate 2 h slow-moving activity (0.2 Hz, 2% elongation) and 2 h high intensity (0.5 Hz, 15% elongation)), and SA (consisting of 8 h resting and 16 h strong activity with 0.5 Hz and 15% elongation) (Fig. 1B). The mechanical stimulation was applied for 48 h. Control cultures were grown under the same conditions but without the strain protocol.

2.2.1. Real-time RT-PCR

Total RNA was isolated from treated and untreated cells using the RNeasy Mini Kit and DNase-I treatment according to the manufacturer's manual (Qiagen, Hilden, Germany). Three µg RNA were reverse transcribed with the iScriptcDNA Synthesis Kit, (BioRad Laboratories Inc., Hercules, CA) using a blend of oligo(dT) and random hexamer primers for 30 min at 37 °C. Amplification was performed with the Sso Advanced Universal SYBR Green Supermix (Bio-Rad) and measured by the CFX96 Touch (BioRad). Each qPCR run consisted of a standard 3-step PCR temperature protocol (annealing temperature of 60 °C) followed by a melting curve protocol to confirm a single gene-specific peak and to detect primer dimerization. Relative quantification of expression levels was obtained by the ΔΔCt method based on the geometric mean of the internal controls glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), and hypoxanthine phosphoribosyl-transferase (HPRT-1), respectively. The expression level (C_T) of the target gene was normalized to the reference genes (ΔC_t), and the difference between the ΔC_t value of the test sample and the ΔC_t of the control sample gave the $\Delta\Delta C_t$ value. Finally, the expression ratio was expressed as $2^{-\Delta\Delta Ct}$. The following QuantiTect primer assays (Qiagen) were used for real time RT-PCR: IL-6, ADAMTS5, MMP1, MMP3, and MMP13. Primer sequences for the other genes were derived from the Primerbank database (http://pga.mgh.harvard.edu/primerbank): *FAK*: fwd 5' GGTGCAATGGAGCGAGTATT 3′, rev 5' GCCAGTGAACCTCCTCTGA 3′; Piezo: fwd 5´CATCTTGGTGGTCTCCTC TGTCT 3′, rev 5´CTGGCATCCACATCCCTCTCATC 3′.

2.2.2. MAPK protein array

The Proteome Profiler Human Phospho-MAPK Array Kit (ARY002B; R&D Systems, Minneapolis, MN) for determination of the phosphorylation status of the major families of MAPKs, ERK1/2, JNK1–3, and different p38 isoforms (α/β/δ/γ) was used according to the manufacturer's instructions. Cells were treated with 30 µM diacerein and mechanically stimulated as described above. Untreated and unstimulated cells were used as controls.

2.2.3. Western blot analysis

For immunoblotting, whole cell protein extracts were prepared with lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM NaF, 1 mM EDTA, 1% NP-40, 1 mM Na3 VO4, and protease inhibitor cocktail; Sigma Aldrich), subjected to SDS-PAGE and blotted onto Amersham Protran Premium 0.45 µM nitrocellulose membranes (GE healthcare Life science, Little Chalfont, UK). All steps were performed on ice. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. Primary antibodies against Integrin β1, Akt, phospho-Akt, p44/42 (Erk1/2), phospho-p44/42 (Erk1/2) (Thr202/Tyr204), STAT3, phospho-STAT3 (Ser727), phospho-STAT3 (Tyr705) and β-actin were purchased from Cell Signaling Technology (Leiden, Netherlands). Blots were developed using a horseradish peroxidase-conjugated secondary antibody (Dako, Jena, Germany) at room temperature for 1 h and the Amersham ECL prime western blotting detection reagent (GE Healthcare), in accordance with the manufacturer's protocol. Chemiluminescence signals were detected with the ChemiDocTouch Imaging System (BioRad) and images were processed with the ImageLab 5.2 Software (BioRad). For a better understanding within the figures the blots were further divided in non-OA and OA subgroups.

2.2.4. STAT3 activity assay

STAT3 activity was measured with the ELISA-based TransAM STAT3 transcription factor assay (Active Motif North America, Carlsbad, CA) according to the manufacturer's protocol. After diacerein treatment (50 µM), nuclear and whole cell protein extracts were isolated. Cells were collected in ice-cold PBS containing phosphatase inhibitors and following cell lysis, cytoplasmic and nucleic extracts were prepared in the presence of a protease inhibitor cocktail.

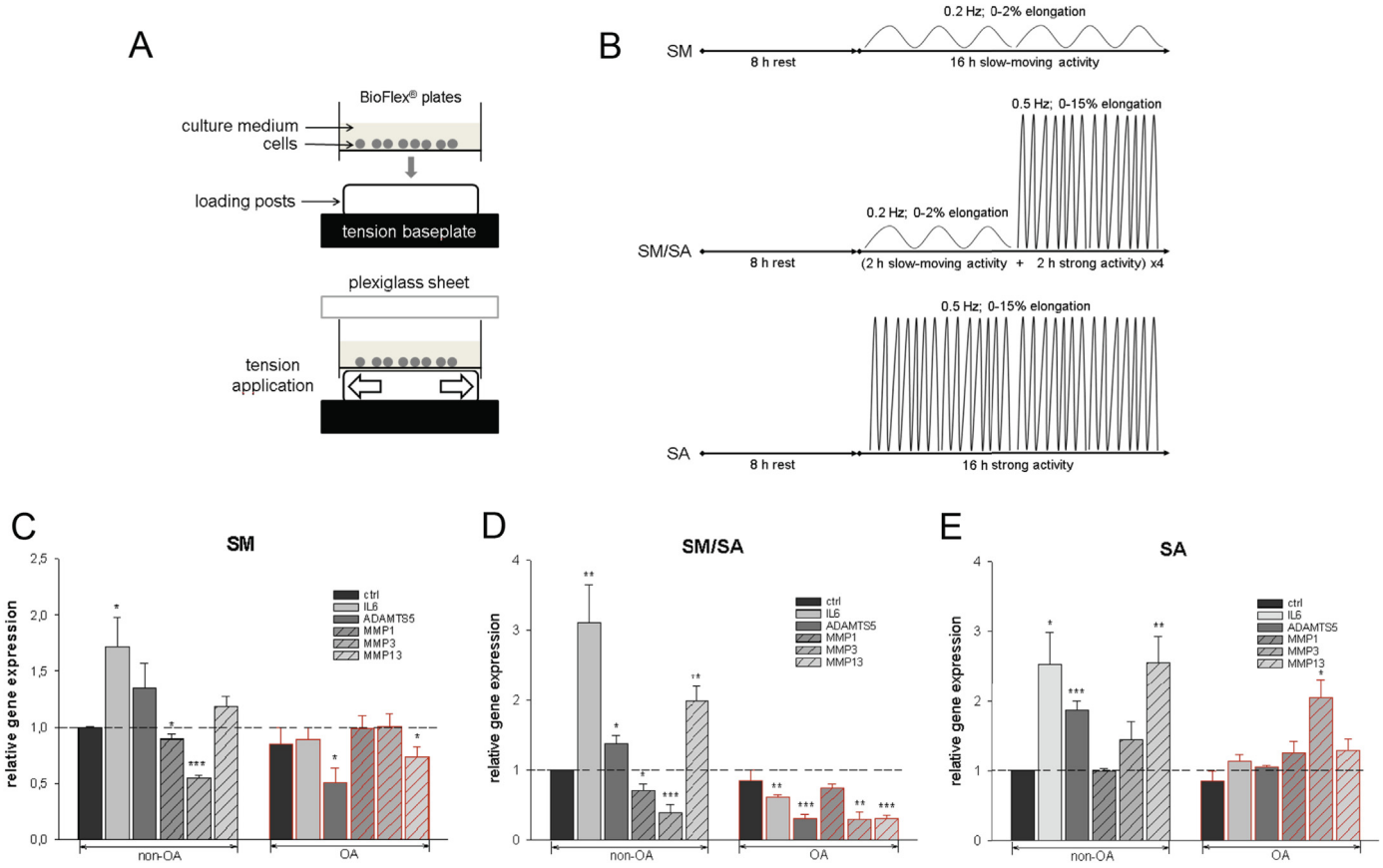

Fig. 1. Experimental set-up for the mechanical stimulation and the influence of cyclic tensile strain on inflammation markers and matrix degrading enzymes. (A) Chondrocytes were mechanically stimulated via vacuum applied to the flexible-bottom of the BioFlex culture plates. (B) Non-OA and OA chondrocytes were subjected to three different strain protocols: SM (consisting of 8 h resting and 16 h slow-moving activity with 0.2 Hz and 2% elongation), SM/SA (consisting of 8 h resting and four repetitions of alternate 2 h slow-moving activity (0.2 Hz, 2% elongation) and 2 h high intensity (0.5 Hz, 15% elongation)), and SA (consisting of 8 h resting and 16 h strong activity with 0.5 Hz and 15% elongation. Control cultures were grown under the same conditions but without the strain protocol. After 48 h stimulation with the (C) SM, (D) SM/SA, and (E) SA profile relative gene expression of IL6, ADAMTS5, MMP1, MMP3, and MMP13 was determined by qRT-PCR. Cyclic alternation between rest periods, slow-moving activity, and strong activity (SM/SA profile) caused a significant decrease in matrix degrading enzymes, especially in OA chondrocytes. Unstimulated cells were measured as controls (mean ± SD; non-OA: n = 8; OA: n = 6).

2.3. Calcium imaging

The intracellular calcium concentration (Ca_i^{2+}) was assessed with the ratiometric fluorescent dye fura2-AM (Life Technologies, Carlsbad, CA). After treating the cells with 10 µM Fura2-AM (Sigma Aldrich) for 1 h at 37 °C, coverslips were washed to remove unloaded dye, kept in Tyrode (NT) solution (concentrations in mM: NaCl 137, KCl 5.4, $NaHCO_3$ 2.2, $MgCl_2$ 1.1, NaH_2PO_4 0.4, HEPES/Na 10, $CaCl_2$ 1.8, glucose 5.6, pH 7.4) and placed in a Nikon fluorescence microscope. Acquisition of fluorescence images with excitation wavelengths of 340 nm and 380 nm and an emission wavelength of 510 nm was performed with the VisiView Imaging system (Visitron Systems, Puchheim, Germany). The data sampling rate was 1 Hz. Background subtraction, rationing of images and calculation of Ca_i^{2+} were performed offline using the Sigma Plot software (Systat Software Inc., San Jose, CA). Yoda1 (2-[5-[[(2,6-Dichlorophenyl)methyl]thio]-1,3,4-thiadiazol-2-yl]-pyrazine (Sigma-Aldrich), was applied at concentrations of 0.1, 0.3, 1 and 3 µM in NT solution (as indicated) by a superfusion system with a 7-channel perfusion pipette. The system was driven by a valvebank (TSE, Bad Homburg, Germany) with a solution exchange time of < 500 ms. Before starting calcium imaging, cells were treated with diacerein for 7 days at a concentration of 30 µM. Calcium influx was verified by omitting Ca^{2+} from the extracellular solution and through depletion of intracellular calcium stores by cyclopiazonic acid (20 µM, Sigma-Aldrich) before perfusing cells with different concentrations of Yoda1.

2.4. Statistical analysis

Data from RT-qPCR are presented as the mean (± standard error) of at least four single experiments, whereby RT-qPCR as well as ELISA experiments were performed in triplicates. Statistical significance was determined by the two-sample Student *t*-test (parametric data), if normality test failed, the Mann-Whitney Rank Sum Test (non-parametric) was used and specifically cited in the figure legends. Data were presented as mean values and 95% confidence interval, p-values are given. Graphical data and data analysis were performed with the SigmaPlot software (Systat Software Inc., San Jose, CA).

3. Results

3.1. Cyclic mechanical strain

In order to select the optimal loading condition we implemented three different protocols for mechanical stimulation, each protocol with a different magnitude of mechanical stimulation. Non-OA and OA chondrocytes were stimulated for 48 h with the SM, SM/SA, and SA profile (Fig. 1B). In non-OA chondrocytes all three protocols caused a significant increase in expression of the inflammatory marker IL-6, whereas under OA conditions the moderate SM/SA profile achieved a

Table 1
Expression of the inflammation-specific markers and the degrading matrix metalloproteinases (MMP) affected by three different mechanical loading protocols. mRNA levels of IL-6, ADAMTS5, MMP1, MMP3, and MMP13 were normalized to the mechanically unstimulated control group (ratio = 1). The values are mean values ± SD of independent experiments performed in triplicate (non-OA: n = 8; OA: n = 6); *P < .05; **P < .01; ***P < .001.

Chondrocytes	Genes	Mechanical stimulation profile SM	SM/SA	SA
Non-OA	IL-6	1.67 ± 0.6	3.10 ± 1.5 (p = .006)	2.53 ± 1.2
	ADAMTS5	1.29 ± 0.6	1.37 ± 0.3	1.86 ± 0.4 (p = .0002)
	MMP1	0.89 ± 0.1 (p = .04)	0.70 ± 0.2 (p = .03)	0.99 ± 0.1
	MMP3	0.54 ± 0.1 (p = 2.7E-11)	0.39 ± 0.3 (p = .0006)	1.44 ± 0.7
	MMP13	1.18 ± 0.2	2.09 ± 0.53 (p = .002)	2.56 ± 0.9 (p = .005)
OA	IL-6	0.88 ± 0.2	0.61 ± 0.09	1.13 ± 0.2
	ADAMTS5	0.51 ± 0.3 (p = .01)	0.31 ± 0.13 (p = 7.8E-6)	1.02 ± 0.1
	MMP1	0.89 ± 0.3	0.75 ± 0.1 (p = .005)	1.25 ± 0.4
	MMP3	1 ± 0.27	0.30 ± 0.2 (p = .001)	2.04 ± 0.7 (p = .01)
	MMP13	0.73 ± 0.2 (p = .03)	0.31 ± 0.4 (p = 1.5E-6)	1.28 ± 0.4

reduction of IL-6 (Fig. 1C-E). An analog effect was observed with the ADAMTS5 expression. This reduction in the expression of inflammatory markers is desirable in the treatment of OA. The cyclic alternation between resting periods, slow-moving activity, and strong activity (SM/SA profile) caused a significant decrease of the MMP1, MMP3, and MMP13 expression especially in OA chondrocytes (Fig. 1D). All data were listed in Table 1 (mean values ± 95% confidence intervals; non-OA: n = 8; OA: n = 6). According to these data, we decided to use the SM/SA profile for further experiments.

3.2. Influence of the DMOAD diacerein on phosphorylation of FAK and MAPKs

A first insight into the modulation of MAPK activities was gained by testing a MAPK array with proteins isolated from untreated control cells compared to OA chondrocytes stimulated with diacerein under the influence of mechanical stimulation (SM/SA) (Fig. 2A). The results showed a decrease in the phosphorylation of the protein kinase B isoform (Akt1) in the presence of diacerein by 28%. Other isoforms displayed only minor effects, except AKTpan which showed a reduction in phosphorylation by 39% when mechanical stimulation was combined with diacerein. Decreased phosphorylation of CREB from 37% to 69% was detected in all three treatment scenarios. Phosphorylation dropped under combined treatment for ERK1 by 39% and GSK-3β by 41%, in contrast to ERK2 where the phosphorylation increased for all three treatment options with an average of 68%. For JNKpan, p38δ and MKK3 a decrease in phosphorylation by 24%, 18%, and 20% was detected for DIA-SM/SA treatment when compared to control values (Fig. 2B).

As a next step, the influence of diacerein treatment on different phosphorylation sites of the focal adhesion kinase (FAK), and the phosphorylation of the MAPKs STAT3, Akt, and Erk 1/2 were investigated. After treatment with 50 µM diacerein (DIA) whole cell lysates were extracted from the cells and prepared for western blot analysis. Untreated cells were used as control (ctrl). In the case of FAK, diacerein treatment decreased the phosphorylation at tyrosines Y397 and Y925 both in non-OA and OA chondrocytes, but increased Y576/Y577 phosphorylation only in OA chondrocytes (Fig. 3A). Additional western blot analysis indicated a clear decrease in phosphorylation of STAT3 (in non-OA and OA cells) and Erk1/2 (in non-OA cells), whereas Akt phosphorylation increased under OA conditions (Fig. 3B). The TransAM STAT3 transcription factor assay confirmed the observed significant downregulation. Consistently, STAT3 activity decreased under diacerein treatment both in the nuclear protein extracts (non-OA: 0.161 ± 0.01 vs 0.116 ± 0.03; p = .005. OA: 0.113 ± 0.01 vs 0.089 ± 0.005; p = .0009) and the whole cell lysate (non-OA: 0.199 ± 0.003 vs 0.152 ± 0.007; p = .0001. OA: 0.158 ± 0.004 vs 0.12 ± 0.009; p = .002) of non-OA and OA chondrocytes (Fig. 3C). Only a small reduction in protein expression of the integrin receptor was observed in non-OA and OA chondrocytes (Fig. 3D). Fig. 3E shows a schematic depiction of the described effects.

3.3. Influence of mechanical stimulation in combination with diacerein on phosphorylation of FAK and MAPKs

To investigate the combined effect of cyclic tensile strain and diacerein, non-OA and OA chondrocytes were treated with 50 µM diacerein and stimulated with the SM/SA profile (DIA+). The effect of cyclic tensile strain alone is also shown (ctrl+). Cyclic tensile strain alone showed only small effects on the phosphorylation of FAK. Only the combination of mechanical strain and diacerein treatment decreased phosphorylation at tyrosine Y397 (both in non-OA and OA chondrocytes) and phosphorylation at tyrosine Y925 (in non-OA chondrocytes). Interestingly, the combined treatment increased Y576/Y577 phosphorylation under non-OA and OA conditions (Fig. 4A). Phosphorylation analysis of the downstream pathways revealed a markedly increased phosphorylation of STAT3 after combined treatment with cyclic tensile strain and diacerein under non-OA and OA

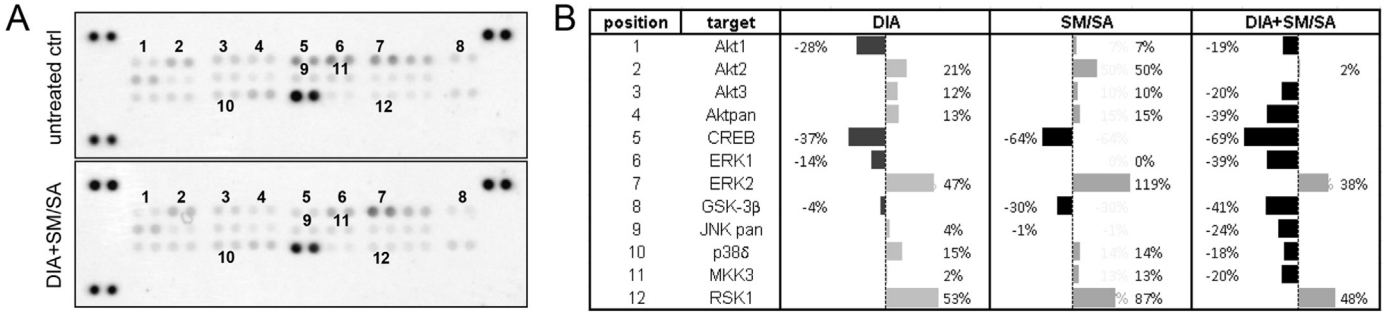

Fig. 2. MAPK activity assay. The Human *Phospho-Mitogen-Activated Protein Kinase (MAPK)* Antibody Array was probed with protein extracts from untreated control cells and diacerein plus SM/SA stimulates OA chondrocytes. (A) Relevant kinases are assigned on the dot blot with specific numbers and are listed under B (left column). (B) Percent changes within the status of phosphorylation are given as horizontal bars.

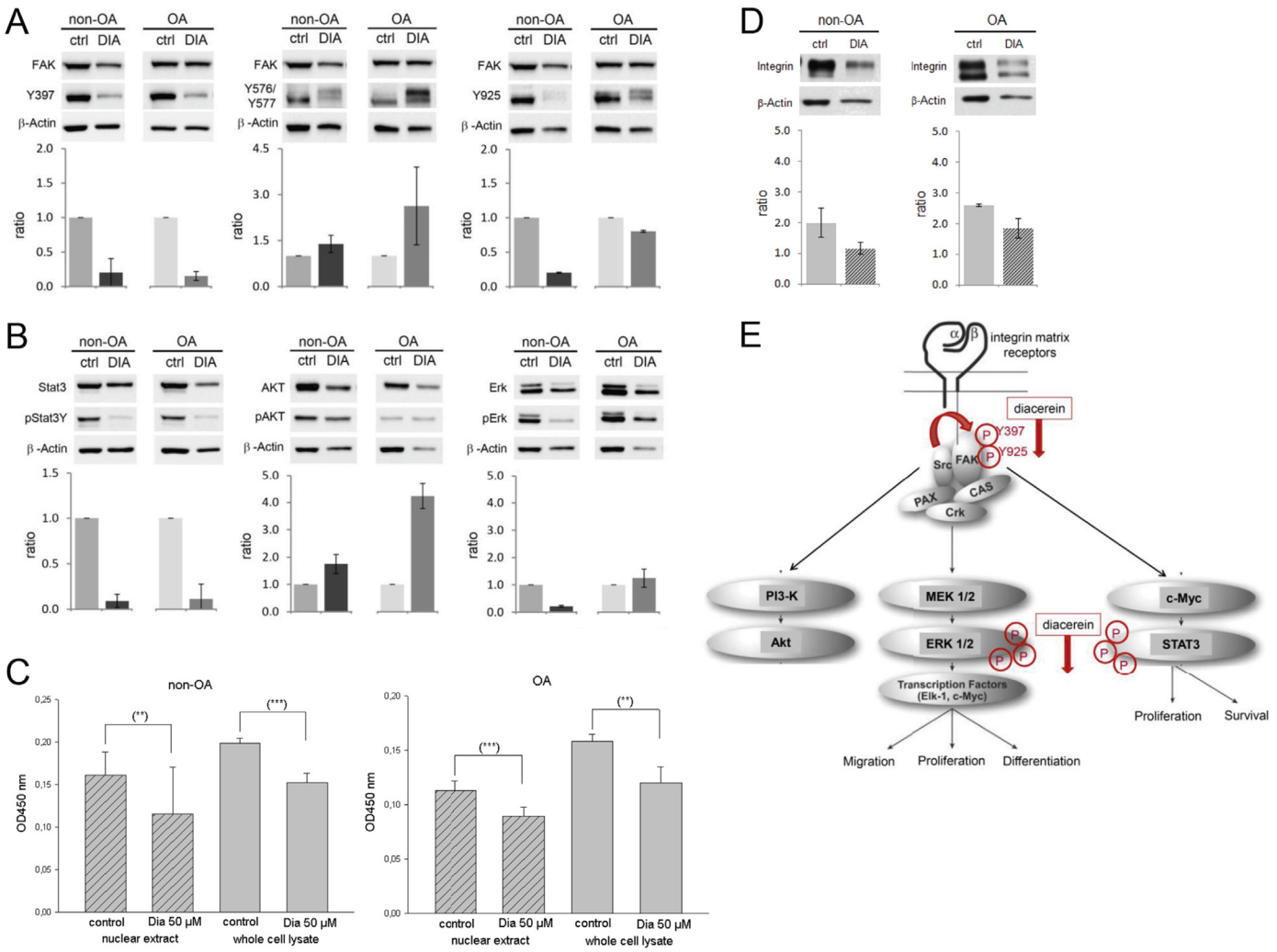

Fig. 3. Effect of diacerein on the FAK-MAPK phosphorylation. Phosphorylation of (A) different phosphorylation sites of the focal adhesion kinase (FAK) and (B) the MAPKs STAT3, Akt, and Erk1/2 is shown by immunoblotting after 48 h treatment with 50 µM diacerein. One representative blot out of three repeats is depicted. β-actin was used as loading control. The bar charts are the mean value ± SD (n = 3). For a better understanding the original full-length blots were further divided in non-OA and OA subgroups. (C) STAT3 activity from nuclear extracts and whole cell lysates of non-OA and OA chondrocytes. (D) Protein expression of the integrin β1 receptor under the influence of 50 µM diacerein. (E) Schematic representation of the diacerein effect on the integrin-FAK-MAPKs signal transduction pathway.

conditions. However, phosphorylation of Akt seemed to increase to a lesser degree. Under non-OA conditions the combination of cyclic tensile strain and diacerein treatment caused significantly decreased phosphorylation of Erk 1/2 (Fig. 4B).

3.4. Modulation of Piezo1 by diacerein

Based on the fact that Piezo1 is known to modulate the activity of integrin we tested for Piezo1 being modulated by diacerein. Fura-2 loaded cells, treated beforehand for 7 days with diacerein were exposed to different concentrations of the Piezo1 activator Yoda1 and the resulting changes in Ca_i^{2+} were measured fluorometrically. Removal of extracellular Ca^{2+} greatly diminished the Ca^{2+} response whereas depletion of Ca_i^{2+} stores with cyclopiazonic acid had no effect on the Yoda1 response, showing that Yoda1 causes an influx of Ca^{2+}. In non-OA cells, pretreated with diacerein, Yoda1 at a concentration of 0.3 µM evoked a 2.2 fold higher Ca^{2+} response compared to control values (Ca_i^{2+}: DIA: 495.7 ± 173.9 nM; ctrl: 223 ± 56.0 nM (p = .005) (Fig. 5A). This increased sensitivity against Yoda1 by diacerein was also detected in OA cells, whereby the Ca^{2+} response to 0.3 µM Yoda1 increased from 280.0 ± 169.9 nM Ca_i^{2+} to 772.4 ± 254.9 nM Ca_i^{2+} (p = .002) and with 1 µM Yoda1 from 738.5 ± 298.4 nM Ca_i^{2+} to 1054.4 ± 216.0 nM Ca_i^{2+} (p = .03) (Fig. 5B). By probing the gene expression level for Piezo1, we identified a reduction by 44% (p = .03) reduction in Piezo1 mRNA availability within OA-cells treated with diacerein (Fig. 5C). Likewise the expression of FAK seemed to be reduced in OA-cells treated with diacerein, although this observation was not statistically significant.

4. Discussion

The widely used human chondrocyte cell lines C28/I2 and T/C-28a2 (both already being distributed by the Merck group) represent model cell lines used for studying normal and pathological cartilage repair mechanisms related to chondrocyte biology and physiology [21,22]. The T/C-28a2 cell line was established by transfecting primary cultures of costal cartilage with a retroviral vector expressing simian virus SV40 large T antigen, whereas the C-28/I2 cell line was derived by immortalization of the non-clonal T/C-28a4 cells. Due to the fact that C-28/I2 cells display the highest levels of matrix-anabolic and matrix-catabolic genes and thus are presumably preferable for use in investigating chondrocyte anabolic and catabolic activity and its regulation we decided to treat this clonal cell line with IL-1β to transfer cells to a more inflammatory like situation [23]. The T/C-28a2 cells play the

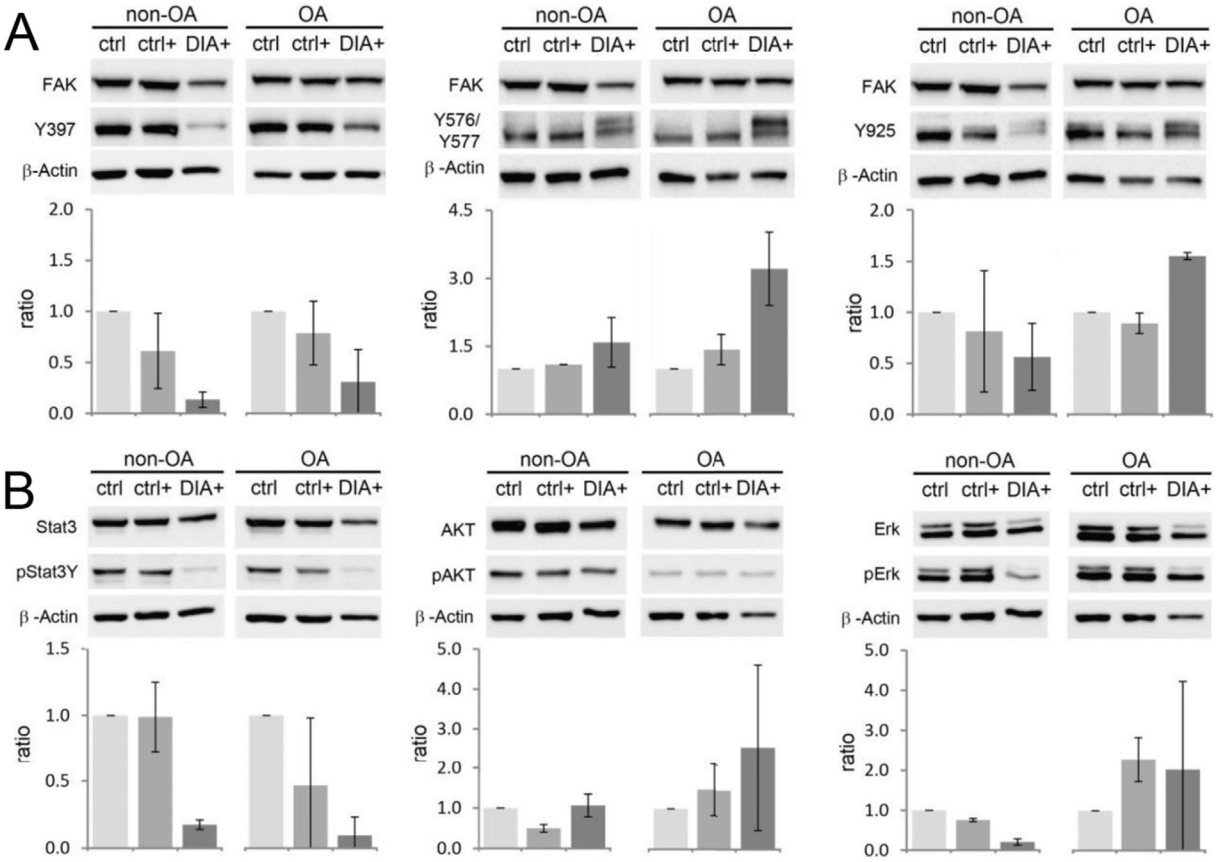

Fig. 4. Effect of diacerein treatment in combination with cyclic tensile strain on the FAK phosphorylation. Analysis of the different phosphorylation sites of the focal adhesion kinase (FAK) (A) FAK Y397, FAK Y576/577, FAK Y925 and (B) STAT3, Akt, and Erk1/2 was performed by immunoblotting protein extracted from cells treated for 48 h with 50 μM diacerein and application of SM/SA (DIA+), consisting of 8 h resting and four repetitions of alternate 2 h slow-moving activity (0.2 Hz, 2% elongation) and 2 h of high intensity profile (0.5 Hz, 15% elongation). Untreated cells (ctrl) or cells undergoing tensile strain alone (ctrl+) were used as control. One representative blot and the mean values ± SD (n = 3) are shown. For a better understanding the original full-length blots were further divided in non-OA and OA subgroups. β-actin was used as loading control.

part of a low developed to non-developed OA status.

From the literature and our own experiences we learned that the application of IL-1β increases the expression of pro-inflammatory metabolites as well as matrix destructing enzymes in chondrocytes.

Apart from its anti-inflammatory properties, diacerein has been shown to have anti-catabolic and pro-anabolic [22] effects on cartilage and on the synovial membrane, as well as protective effects against subchondral bone remodeling [23]. In addition, diacerein reduces IL-6 expression by blocking the IL-1β pathway via MEK/ERK and NFκB DNA binding [24]. In a previous study, we demonstrated that diacerein treatment in combination with tensile strain influenced the inflammatory environment and growth factor expression of human chondrocytes [25]. The present study demonstrates that different loading conditions had different effects on the expression of inflammatory markers and degenerative enzymes in chondrocytes. Derived from general knowledge that physical and movement therapy improves cartilage function, the moderate SM/SA profile was designed to closely resemble the physiological movement pattern including

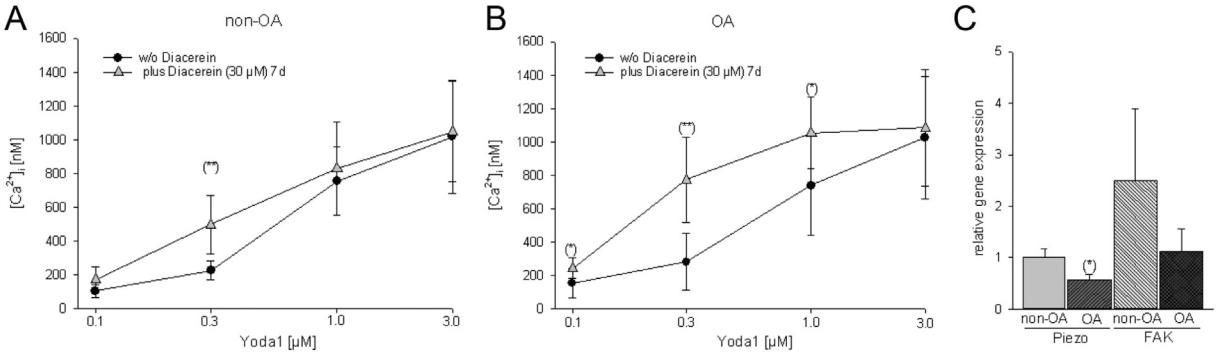

Fig. 5. Effect of diacerein on intracellular Ca^{2+} concentration. (A) Non-OA and (B) OA chondrocytes, pretreated with diacerein, were exposed to different concentrations of the Piezo1 activator Yoda1 and the resulting changes in intracellular Ca^{2+} were measured fluorometrically. Both non-OA and especially OA cells showed an increased sensitivity against Yoda1 following diacerein treatment. (C) Gene expression analysis revealed a reduction by 44% (p = .03) in Piezo1 mRNA availability within OA-cells treated with diacerein and a reduction of FAK mRNA expression level.

strong activity sections alternating with phases of slow motion. SM/SA led to a significantly decreased expression of the inflammatory marker IL-6 and the degenerative enzymes ADAMTS3, MMP1, MMP3, and MMP13. Since this was the desired effect, we used the SM/SA profile for all further experiments.

Despite the critical importance of mechanotransduction its mechanism in chondrocytes is not yet fully understood. In many cell types from different species including mammalians, Piezo channels have been identified as a family of directly mechanically activated ion channels [14,26]. These channels are Ca^{2+} permeable and can rapidly inactivate subsequent mechanical gating. Mammalian chondrocytes express these Piezo channels and high strain leads to Ca^{2+} influx into the chondrocytes [15].

If cartilage tissue is not exposed to adequate mechanical load in vivo, it tends to undergo atrophy due to a decrease in metabolic activity of chondrocytes and a decrease in production of components of the ECM. Through daily exercise, applying mechanical force to cartilage can increase the thickness and glycosaminoglycan content of joint cartilage [27]. Integrin receptors physically connect chondrocytes to the ECM [28] and act as mechanotransducers that activate intracellular signaling pathways and mediate ECM component production [29]. Following activation of integrins, the focal adhesion kinase (FAK) autophosphorylates at Y397, generating a high-affinity binding site for the Src homology 2 (SH2) domain of Src family kinases [30,31]. Recruitment of Src family kinases results in the phosphorylation of FAK tyrosine residues Y576 and Y577 in the catalytic domain, and tyrosine residues Y871 and Y925 in the carboxy-terminal region [32,33].

In our experiments only a small reduction of the protein expression of the integrin receptor β1 was observed, although we saw a differential regulation of downstream FAK phosphorylation sites due to diacerein. Generally, phosphorylation of FAK was decreased by diacerein in non-OA and OA chondrocytes, and only Y576/Y577 phosphorylation increased under OA conditions. Functionally, this increase seems to correlate with activation of Akt whereas the phosphorylation of STAT3 and Erk1/2 is diminished. This inhibition could be the caused by inhibition of the other FAK phosphorylation sites. A similar correlation was found when cyclic tensile strain was applied. The downregulation of FAK phosphorylation on tyrosine residues Y397 and especially Y925, was enhanced in non-OA chondrocytes whereas FAK Y576/Y577 phosphorylation was significantly increased. The phosphorylation of STAT3 was particularly inhibited by the combination of diacerein and tensile strain.

Mechanical stimulation influences integrin activity via R-Ras and activates Piezo1, a mechanically activated ion channel. Changes in the Ca_i^{2+} levels lead to calpain induced regulation of focal adhesion turnover. In our experiments pretreatment of cells with diacerein reduced the expression of Piezo 1 and rendered the channels more sensitive to Yoda, an activator of the Piezo channel. Although an increase in Ca^{2+} by the application of Yoda was measured one has to consider that elements of uncertainty remain due to the fact that Yoda might not act highly specific and/or activate kinase like Akt and ERK although the data represented by Dela Paz and Frangos (2018) showing western blots are not convincing [34]. The more pronounced effect in OA cells is consistent with the observation that Piezo1/2 could potentially serve as a therapeutic target for posttraumatic OA, based on the finding that channel blocking protected chondrocytes from cell death following injury [15,35]. The diacerein induced reduction in STAT3 phosphorylation could explain the mechanism of diacerein treatment in OA, since IL-6 induces chondrocyte catabolism mainly via STAT3 signaling. It has been demonstrated that blocking STAT3 activity can alleviate OA in mice [36].

Our results demonstrated for the first time that diacerein intensively intervenes in the regulation of FAK and STAT3 and influences components considered relevant for the progression of OA, even in the presence of mechanical stimulation. The reduction in FAK phosphorylation by diacerein may imply a modulation of focal adhesion.

Declarations of interest

None.

Ethics statement

No institutional review board approval was required for this project.

All methods were carried out in accordance with relevant guidelines and regulations.

Competing interests

The authors declare no competing interests.

Authors' contributions

Concept and design: BL, BSF; Analysis and interpretation of the data: BL, LW, BSF; WK, AL, drafting of the article: BL, BSF; critical revision and final approval of the article: BL, LW, WK, AL, BSF, HK, NS, AM; statistical expertise: LW; acquisition of data: HK, NS, AM.

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