The therapeutic nuclear magnetic resonance changes the balance in intracellular calcium and reduces the interleukin-1β induced increase of NF-κB activity in chondrocytes

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Abstract

Objective

Osteoarthritis as the main chronic joint disease is characterised by the destruction of articular cartilage. Developing new, more effective and in particular non-invasive methods to achieve pain reduction of OA patients are of exceptional interest. Clinical observations demonstrated positive effects of therapeutically applied low nuclear magnetic resonance (NMRT) for the treatment of painful disorders of the musculoskeletal system. In this study the cellular mechanism of action of NMRT was examined on chondrocytes.

Methods

Cal-78 human chondrosarcoma cells were kept under inflammatory conditions by application of IL-1β. NMRT treated cells were tested for changes in histamine induced Ca^{2+} release by fura-2 calcium imaging. The effects of IL-1β and of NMRT treatment were further tested by determining intracellular ATP concentrations and the activity of MAP-kinases and NF-κB.

Results

NMRT influenced the intracellular calcium signalling by elevating the basal $[Ca^{2+}]_i$. The peak calcium concentration evoked by 10 μM histamine was increased by IL-1β and this increase was reversed under NMRT treatment. Screening of different kinase-activities revealed an apparent increase in activity of MAPK/ERK and MAPK/JNK in NMRT stimulated cells, p38 was downregulated. The IL-1β-induced decline in intracellular ATP and the elevated NF-κB activity was reversed under NMRT stimulation.

Conclusion

Under inflammatory conditions, NMRT influenced cellular functions by modulating cellular calcium influx and/or calcium release. Further, NMRT induced changes in MAPK activities such as down-regulation of NF-κB and increasing intracellular ATP might help to stabilise chondrocytes and delay cartilage damage due to OA.

Key words

calcium signalling, chondrocytes, nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB), nuclear magnetic resonance therapy (NMRT), osteoarthritis

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Introduction

Osteoarthritis (OA) represents the main chronic joint disease by causing destructions of the articular cartilage leading to pain and disability (1, 2) and constitutes a multifactorial disorder where low grade inflammation plays a central role (3). Patients with increased levels of the inflammatory mediator interleukin-1β (IL-1β) were at higher risk of progression of OA (4). Although, different reasons for the occurrence of OA are described, medical means to treat main clinical symptoms are limited and comprise physiotherapy, drug treatment, usually directed only to pain such as non-steroidal anti-inflammatory drugs (NSAID) or symptomatic slow acting drugs in osteoarthritis (SYSADOA), plus finally joint replacement (5-7). With the exception of joint replacement none of these treatments are to date sufficiently developed and able to achieve a definite clinical remission. More effective, in particular non-invasive therapeutics are needed to inhibit the process that drive OA pathology.

Studying the therapy with nuclear magnetic resonance (NMRT), clinical observations demonstrated positive effects when NMRT was applied to treat painful disorders of the musculoskeletal system (8-11). Following NMR-therapy clinical studies showed significant pain relief of patients with low back pain and increased function in finger joint mobility in patients with OA (Heberden's/Bouchard's nodes) (12-14). By NMRT positive adaptations of the cartilaginous structures and pain improvements from patients with knee OA were outlined. Despite the observation of clinical relevant improvements, molecular details concerning the mode of action of NMRT are unclear. Various studies postulated a stimulation of chondrocytes by NMRT leading to an augmented synthesis of collagens, elevation of the cell proliferation rate and distinct changes both in cellular and extracellular components (15, 16). In human chondrocytes changes in the modulation of signal transduction pathways involved in cartilage degeneration by NMRT have been proposed (17).

Cell signalling involving calcium ions (Ca^{2+}) is widely recognised to play a fundamental role in the regulation of many biological processes. Transient changes in cytoplasmic Ca^{2+} concentration represent a key step for the modulation of cell membrane excitability. Evidence has been accumulated to define the involvement of Ca^{2+} in nociception and antinociception, including analgesic effects (18). Likewise, chondrogenesis appears to be controlled by complex mechanisms that involve Ca^{2+} sensitive signalling components (19). Connected to oxidative stress, redox sensitive transcription factors (*e.g.* nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB) are upregulated, which may result in an uncontrolled inflammatory response (20), thus unlike other signalling pathways the NF-κB kinases function as potential therapeutic OA targets (21). Generally, an increased Ca^{2+} influx through L-type-calcium-channels and reduced endoplasmic reticulum (ER) calcium storage has been described for low-grade systemic inflammation and increased cytokine levels (22).

In this study the cellular mechanism of action of NMRT was explored. Chondrocytes under inflammatory conditions were tested for NMRT induced changes in the concentrations of intracellular calcium as a signal transmitter and activity of mitogen-activated protein kinases (MAPK). Cellular consequences induced by IL-1β and the influence of NMRT were further tested for adenosine triphosphate (ATP) and NF-κB activity.

Material and methods

Cell culture

Chondrocytes (Cal-78 chondrosarcoma cells, DSMZ, Braunschweig, Germany) were cultured in Dulbecco's-modified Eagle's medium (GIBCO, Invitrogen, Darmstadt, Germany), containing 10% fetal bovine serum (FBS), 1% L-glutamine, 10.000 units/ml Penicillin, 100 µg/ml Streptomycin, 0.5 µg Amphotericin B (all GIBCO, Invitrogen) and 50µg/ml ascorbic acid. Cells were kept at 37°C in a humidified atmosphere of 5% CO_2 and were passaged by trypsinisation after reaching 80–90% confluence. The amount of seeded cells, if not separately indi-

cated, was 10.000 cells/cm^2. Three to four days after cell seeding, medium was replaced by a HEPES containing culture medium ± IL-1β (10 ng/ml, Sigma-Aldrich, MO, USA).

Nuclear magnetic resonance therapy (NMRT)

NMRT treatment was performed by a specific magnetic nuclear resonance therapy device adapted for cell cultures (MedTec Company, Wetzlar, Germany) producing a magnetic field of 0.23 mT and an electromagnetic field of about 100 kHz. The NMRT treatments lasting for 1 h performed at room temperature outside the incubator, were generally started one hour after the addition of IL-1β. Untreated control cells were placed on a laboratory table to keep the temperature and humidity conditions comparable for all groups. HEPES containing culture medium was used to keep the pH value within the neutral range.

Fluorokine MAP human MMP kit

Cal-78 cells were plated into six well plates with a cell density of 7500 cells/cm^2. IL-1β stimulation in this experimental set up was for three days. Duplicate supernatants were pooled and diluted 2.5 fold in dilution buffer and concentrations of the metalloproteinases MMP-1, -3, and -13 were determined according to the Fluorokine MAP Human MMP Kits manufacturer's instruction (R&D Systems, Minneapolis, USA). Samples were measured via a luminex analyser (Luminex 100/200, Austin, USA)

Enzyme Immuno Assay ELISA

Ready-to-use Sandwich ELISAs (human interleukin 6 (IL6) and interleukin 8 (IL-8) Platinum ELISA, eBioscience, San Diego USA) were used to quantify IL-6 and IL-8. Supernatants were used undiluted or diluted as required from 1:5 to 1:100 and proceeded according to the manufacturer's instruction. All measurements were performed in duplicates at 450 nm with micro plate reader SpectraMax Plus 384 (Molecular Devices, Sunnyvale, US) or Anthos 2010 (Anthos Labtec Instruments GesmbH., Wals, Austria).

Calcium imaging

The intracellular calcium concentration was assessed with the ratiometric fluorescent dye Fura2-AM (Life Technologies, CA, USA). Cells were treated with NMRT for 1h hour at room temperature (RT) in culture medium completed with 10 μM Fura2-AM (Sigma Aldrich, MO, US) (23). 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, $NaH_2PO_{0.4}$, HEPES/Na 10, $CaCl_2$ 1.8, glucose 5.6, pH7.4) and placed in a Nikon fluorescence microscope. Acquisition of fluorescence images with excitation wavelengths of 340 nm and 380 nm and emission wavelength of 510 nm was performed with the QC2000 Imaging system from VisiTech (Sunderland, UK). The data sampling rate was 1 Hz. Background subtraction, rationing of images and calculation of the intracellular calcium concentration ($[Ca^{2+}]_i$) were performed offline using the Sigma Plot software (Systat Software GmbH, Erkrath, Germany).

Histamine (Sigma-Aldrich, MO, USA) at concentrations of 1, 3, 10, 30 or 100 μM in NT solution (as indicated) was applied by a superfusion system with a 7-channel perfusion pipette (List-electronic, Darmstadt, Germany). The system was driven by a valvebank (TSE, Bad Homburg, Germany) with a solution exchange time of less than 500 ms. Before starting calcium imaging, Cal-78 cells underwent a 1 h pretreatment with IL-1β followed by NMRT stimulation for an additional hour. For the inhibitor studies cells were preincubated with SB 203580 (Sigma-Aldrich, MO, USA) for 30 min at a concentration of 10 μM from a stock solution of 10 mM in DMSO ((24)).

Protein array

The proteome profiler human phospho-MAPK array kit (ARY002B; R&D, MN, USA) is suitable for the multiplex phosphor protein detection of 24 human mitogen-activated protein kinases. Cells were treated with NMRT and control conditions for 1 h and whole cell protein extracts were prepared with lysis buffer. The detection procedure was performed as described in the manufacturer's instructions on nitrocellulose membranes on which capture and control antibodies of the major MAP kinases have been spotted in duplicates.

Western blot analysis

For immunoblotting, whole cell protein extracts were prepared with frakelton buffer (10 mM Tris-HCl, 50 mM NaCl, 50 mM NaF, 50 mM $Na_4P_2O_7$, adjusted to pH 7.1 and immediately before use 1 mM PMSF, 500 μM Na_3VO_4, 0.2 mM DTT, 1% Triton-X100, and 1 tablet cOmplete, MiniProtease Inhibitor Cocktail (Roche)), subjected to SDS-PAGE (10%) and blotted onto PVDF membranes (Merck Millipore, Darmstadt, Germany). Membranes were probed with primary antibodies at 4°C over night in 5% BSA/TBS buffer primary antibodies against P-p38 MAPK and p38 MAPK, P-ERK1/2 and ERK1/2, were purchased from Cell Signaling Technology (Danvers, MA, US) and used diluted 1:1000. Blots were developed using horseradish peroxidase-conjugated secondary antibodies (Dako, Jena, Germany) at room temperature for 1 h and the Thermo Scientific Pierce ECL Western Blotting Substrate (Waltham, USA), in accordance with the manufacturer's protocol. Image processing and signal intensity measurements were performed with ImageJ. Measured data from phosphoprotein and non-phosphoprotein detection before the treatments as well as after 1 h room temperature (control) and after 1 h NMRT were obtained. Signal intensities of the two treatments, RT and NMRT, were normalised to signal intensities measured before starting both of the treatments. The ratio between signals from antibodies directed against the phosphorylated and the correlating non-phosphorylated kinase was calculated.

Cellular ATP detection

Total levels of cellular ATP were measured via the Luminescent ATP Detection Assay Kit (Luminescent ATP Detection Assay Kit, Abcam, Cambridge, UK), an adenosine 5'-triphosphate (ATP) monitoring system based on firefly luciferase with the emitted light being proportional to the ATP concentra-

tion inside the cell. Cells were plated in 96 well plates, 10.000 cells per well and treated with IL-1β followed by NMRT treatment for 1 h. Control cells were kept at room temperature for the same time. After trypsinisation, luminescence was determined with an Enspire plate reader (Perkin Elmer, Waltham, MA, USA)

NF-κB transcription factor activity

The used NF-κB p50/p65 Transcription Factor Assay Kit (Abcam, Cambridge, UK) allows quantification of NF-κB activation by detecting specific transcription factor DNA binding activity in nuclear extracts. 10.000 cells per cm^2 were plated on 25 cm^2 flasks and the assay was started after cells reached 80% confluence. A specific double stranded DNA (dsDNA) containing the NF-κB response element is immobilised onto the wells of a 96-well plate. NF-κB contained in a nuclear extract binds specifically to the NF-κB response element. NF-κB p50 and p65 are detected by addition of a specific primary antibody directed against NF-κB p50 or p65. A secondary antibody conjugated to HRP is added to provide a sensitive colorimetric readout at 450 nm. After treating the cells with NMRT or mock treatment at room temperature, nuclear extracts were isolated from cells with the Nuclear Extraction Kit (EpiSeeker Nuclear Extraction Kit, Abcam, Cambridge, UK). 100 µl nuclear extract per sample was used to determine NF-κB activity.

Statistical analysis

Statistical significance was determined by the Student *t*-test and by the Wilcoxon-Mann-Whitney-Test; *p*-values <0.05 were considered to be significant (***p<0.001; **p<0.01; *p<0.05), all p-values are two-sided. Data analysis was performed with the SigmaPlot 11.0 software. Median values (25^{th}/75^{th} percentile) or mean values ± standard errors are given throughout the manuscript.

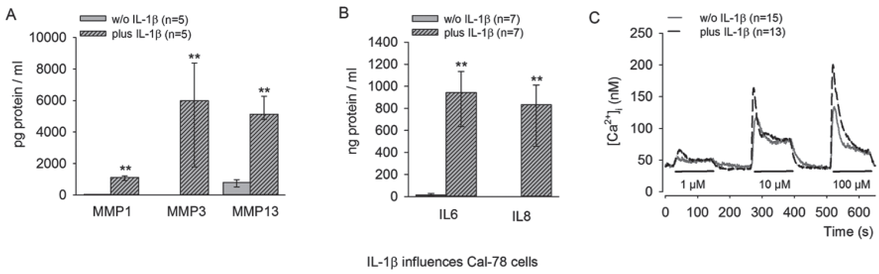

Fig. 1. Impact of interleukin-1β (IL-1) on Cal-78 cells.
Changes in the expression of metalloproteinases (**A**) and interleukins (**B**) in Cal-78 cells by IL-1β are given. Bars represent median values with the 25^{th} and 75^{th} percentile. Significance of differences between IL-1β unstimulated and IL-1β stimulated cells are marked (**p<0,01). Number of experiments is given in brackets. (**C**) Time courses of averaged intracellular calcium concentration $[Ca^{2+}]_i$ under different concentrations of histamine applied. Control conditions (dark grey solid line) and conditions under IL-1β treatment (black short dashed line) are given.

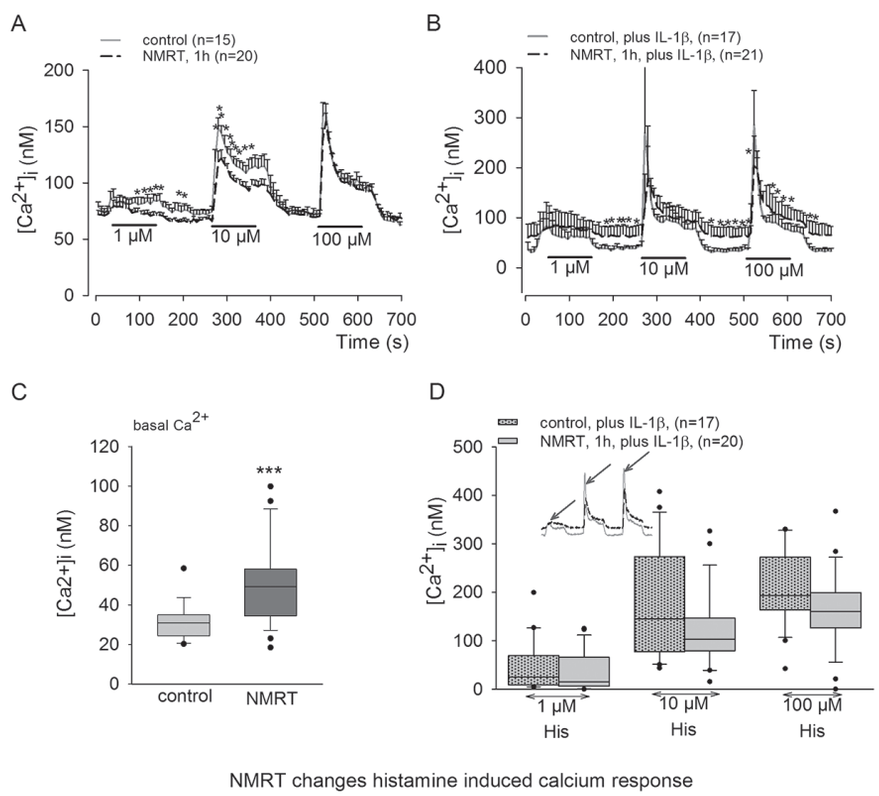

Fig. 2. NMRT influences histamine induced calcium release.
Averaged (±S.E.M.) intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ for untreated (**A**) and IL-1β treated cells (**B**) under control conditions (dark grey solid line) and NMRT application (black short dashed line). Different concentrations of histamine are indicated by black bars, significant changes are marked by asterisks, with significance levels of p<0.05 to p<0.01. (**C**) A comparison of basal calcium values from control and NMRT treated cells under IL-1β, with ***p<0.001. Changes on histamine induced calcium increase, calculated from measured peak calcium minus basal calcium levels, are shown in (**D**).
The inserts represent the Ca^{2+} time course given under (**B**) where arrows mark the time points on which peak calcium values have been measured.

Results

The influence of IL-1β on chondrocytes

Chondrocytes demonstrated a significant change in expression of matrix metalloproteinases by IL-1β treatment. Over a period of three days the concentration of MMP-13 increased 7-fold and 34-fold for MMP-1 whereas MMP-3 reached the 845 fold level compared to control cells (Fig. 1A). Likewise, comparing median values, a significant IL-1β-induced increase in the expression of the two pro-inflammatory cytokines IL-6 (944.4 ng/ml (634.6/1135.2)) and IL-8 (833 ng/ml (454.8/1012.1)) compared to control values (IL-6: 15.5 ng/ml (10.3/28.7); IL-8: 0.9 ng/ml (0.7/1.4)) was observed (Fig. 1B). When stimulating Cal-78 cells with histamine, changes

in the intracellular concentration of calcium due to an inositol-triphosphate (IP_3) induced calcium release was detected. The increase was dose dependent as shown by applying histamine in a concentration of 1, 10 and 100 μM, respectively (Fig. 1C). Pretreatment of cells with IL-1β enhanced the observed response, in particular at the higher histamine concentrations.

NMRT changed histamine-induced calcium response

NMRT treatment for one hour lowered the histamine-induced response in intracellular calcium with highest efficiency at a histamine concentration of 10 μM (Fig. 2A). A 15% reduction of the calcium increase under perfusion of the cells with 10 μM histamine was observed. Under inflammatory conditions induced by the application of IL-1β, NMRT stimulation increased the basal and decreased the peak calcium levels (Fig. 2B). The basal intracellular calcium concentration $[Ca^{2+}]_i$ was measured before starting the histamine perfusion and was significantly increased (NMRT: 49.6 (34.5/58.0) *vs*. control: 30.9 (24.2/34.9), $p<0.001$, Fig. 2C). In contrast to the observed augmentation in basal calcium, reductions in peak calcium induced by histamine application were measured ($[Ca^{2+}]_i$, nM). Even though clear differences were observed at 1 μM histamine (NMRT: 15.1 (6.2/65.8) *vs*. control: 25.8 (8.8/69.5)), 10 μM histamine (NMRT: 103.2 (78.9/146.4) *vs*. control: 144.6 (77.2/273.4) and 100 μM (NMRT: 160.3 (126.4/199.3) *vs*. control: 193.9 (163.4/272.4)), respectively, values did not depict statistical significant changes (Fig, 2D).

Activity of MAP kinases was modulated by NMRT

First insight into the modulation of MAPK activities was given by testing a MAPK array with proteins isolated from Cal-78 cells stimulated by NMRT for 1 h and under control conditions in the presence of IL-1β (Fig. 3A). The results showed a decrease in the phosphorylation of protein kinases B (Akt1, Akt2 and Akt pan) by an averaged value of 13.3%. The phosphorylation

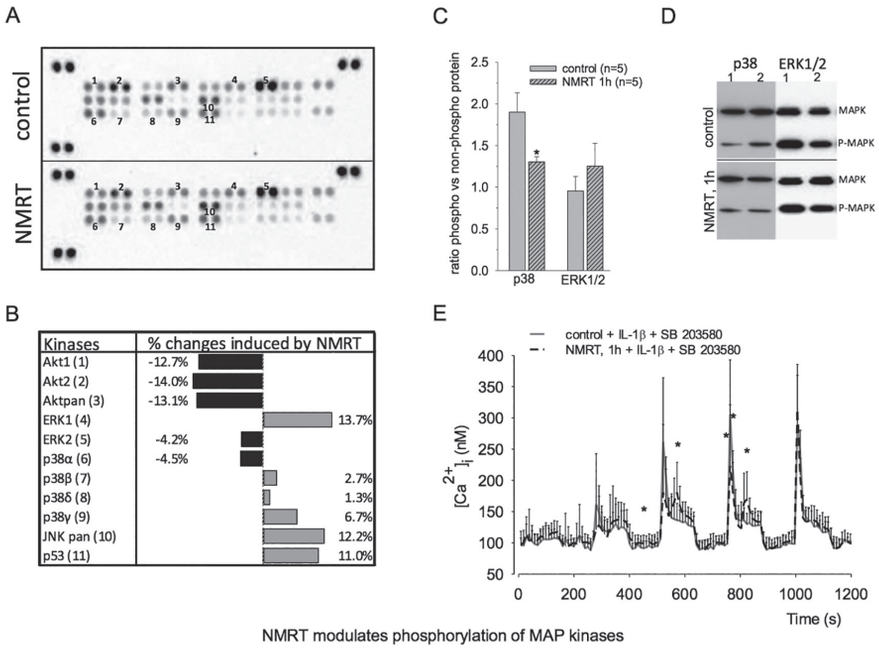

Fig. 3. NMRT influences MAP kinase activity in Cal-78 cells treated with IL-1β.
The Human *Phospho-Mitogen-Activated Protein Kinase (MAPK)* Antibody Array was probed with protein extracts from Cal78 + IL-1β cells under control and NMRT conditions (**A**). Relevant kinases are assigned with specific numbers and are listed under (**B**), (left column). Percent changes within the status of phosphorylation are given as horizontal bars (B, right column). Western blot analysis under (**C**) represents the ratios of normalised signals obtained using phospho-MAPK or MAPK antibody; examples for immunoblot signals are given in (**D**); samples before (lane 1) and after (lane 2) treatment at room temperature and NMRT, respectively. Time courses of intracellular Ca^{2+} concentration induced by different concentrations of histamine with or without the inhibitor of p38 MAPK SB 203580 (**E**).

in extracellular signal-regulated kinase (ERK) 2 and mitogen-activated protein kinase 14 (p38α) dropped by 4.2% and 4.5%. Quite the opposite was detected for ERK1, p38β, p38δ, p38γ, c-Jun N-terminal kinase (JNK) pan and the tumor suppressor p53 with increases in phosphorylation when compared to control values of 13.7%, 2.7%, 1.3%, 6.7%, 12.2% and 11%, respectively (Fig. 3B). A significant decline in the phosphorylation of p38 MAPK was detected under the influence of NMRT treatment by western blot analysis with a ratio of phosphorylated to non phospho-protein for NMRT treatment of 1.3±0.06 *vs*. control of 1.9±0.23 ($p<0.05$). Furthermore, the comparison between the phosphorylated and the non-phosphorylated form of ERK1/2 revealed a small increase by NMRT stimulation of the cells (NMRT: 1.3±0.3 *vs*. control: 0.9±0.2) (Fig. 3C). Measurements of histamine induced calcium release in Cal-78 cells pre-treated with the inhibitor for p38, SB 203580, abolished the NMRT induced increase in basal calcium whereas the reduction in peak calcium seen by NMRT treatment was enhanced (Fig. 3E).

Cellular production and IL-1β induced reduction in ATP varied under NMRT stimulation

In Cal-78 cells under control conditions an overnight treatment with IL-1β led to a reduction in cellular adenosine-triphosphate (ATP) by almost 20% (given as luminescent counts: control: 64610.9±5895.4 and under IL-1β: 55508.9±13214.6) (Fig. 4). In the absence of IL-1β NMRT treatment increased significantly the luminescence by 37% (NMRT: 87985.9±8868.5; $p=0.03$). Although under IL-1β it was still possible to detect an increase in ATP by NMRT of 17%, the detected effect was not significant (NMRT+IL-1β: 64477.7±12025.2; $p=0.62$). Thus, the decline of the ATP concentration due to IL-1β was more pronounced when cells were treated with NMRT.

IL-1β and NMRT changed NF-κB p50 and p65 DNA binding activity

By using a specific ELISA, the binding

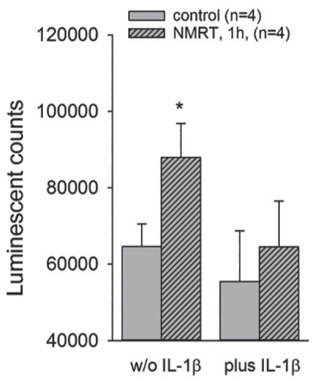

Fig. 4. Detection of changes in cellular ATP by NMRT.
ATP was measured from whole cell extracts and is represented as luminescent counts. The bar chart represents mean and SEM. values of cells treated with IL-1β (n=4) and without IL-1β (n=3) measured in quadruplicates. Significant difference is given: *p<0.05.

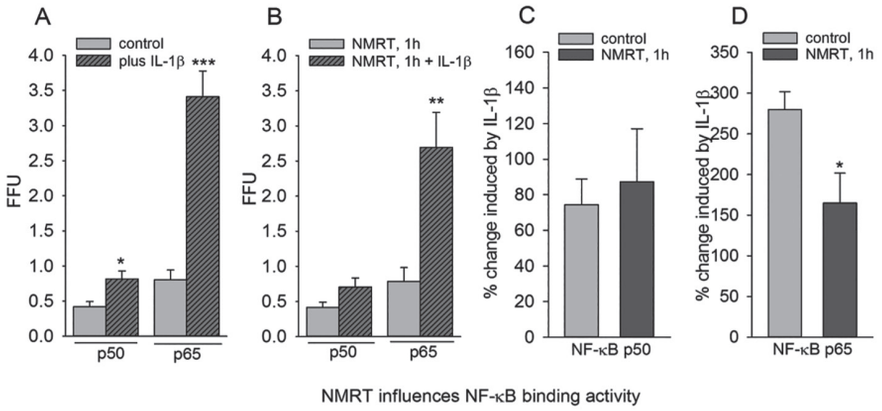

Fig. 5. NMRT attenuates the IL-1β-induced increase in NF-κB p65.
The influence of IL-1β (**A**) and NMRT (**B**) is shown for the binding activity of NF-κB protein p50 and p65, respectively. Measured fluorescence forming units were normalised to amount of protein. IL-1β-induced percent change in DNA binding activity given under control and NMRT treatment are pictured (**C**, **D**). Number of experiments n=4 / in duplicates for experiments without IL-1β and n=5 / in duplicates for experiments with IL-1β; significant differences are given: *p<0.05, **p<0.01, ***p<0.001.

activity of NF-κB isolated from nuclear extracts was measured.

In the presence of IL-1β the binding activity of both classes of NF-κB proteins, p50 and p65, significantly increased by 93% for p50 (p50/control: 0.42±0.08 *vs*. p50/IL-1β: 0.81±0.12, p=0.011; p65/control: 0.80±0.14 *vs*. p65/IL-1β: 3.41±0.34, p<0.001) (Fig. 5A). In the absence of IL-1β, NMRT did not influence NF-κB activity (p50/NMRT: 0.42±0.07; p65/NMRT: 0.70±0.13) (Fig. 5B). While, for NF-κB/p50 the response to IL-1β was not affected by NMRT (0.79±0.20), for NF-κB/p65 the IL-1β-induced increase was weakened (2.69±0.50). The percental change by IL-1β for p50 was 74.3%±14.6 under control and 87.4%±29.7 under NMRT stimulation (Fig. 5C). To the contrary, on addition of the inflammatory cytokine IL-1β, NMRT was able to halve the amount of bound NF-κB/p65. The increased binding activity of 280.0%±21.7 due to IL-1β alone was down regulated to 164.8%±37.0 by additional stimulation with NMRT (Fig. 5D). The observed change in p65 binding activity was significant at the p<0.05 level (p=0.018).

Discussion

Osteoarthritis is the rheumatic disease with the highest prevalence and increasing socioeconomic impact. Its degenerative characteristics is driven by, amongst other things, signalling mechanisms affected by stress- and inflammation induced factors (25). While pharmacological treatments are essentially related to relief of symptoms, disease-modifying OA treatments to reduce symptoms and to slow down or stop disease progression are very rare and neither fully explored nor developed. For these reasons, alternative new and effective treatment strategies in OA are indispensable.

Findings from clinical studies point to NMRT as a possible therapeutical option to treat OA pain and to induce regeneration of affected cartilage (11, 14). Despite these findings from clinical studies, little is known about the mechanism of action at the cellular level. Studying the influence of NMRT on chondrosarcoma Cal-78 cells, our investigations evinced an influence of NMRT on cell signalling.

In the experimental setup the influence of NMRT on IL-1β and non-IL1β treated cells was compared. The inflammatory state of cells treated with IL-1β was demonstrated by the increased expression in MMP1, 3, 13, IL-6 and IL-8 (26-28). IL-1 proteins are potent, pro-inflammatory cytokines capable of inducing multiple signalling cascades that can serve in host defense or contribute to inflammatory tissue injury (29). Analysing chondrosarcoma cells under the influence of IL-1β, a massive increase in the OA characterising proteins MMP-1,-3,-13, IL-6 and IL8 could be demonstrated and proofed the inflammatory status of the investigated cells (30, 31). It has further been demonstrated that IL-1 signalling depends on focal adhesions (32). SHP2 (Src homology phosphatase 2), involved in focal adhesion dynamics, is crucial for IL-1-induced phosphorylation of PLCγ enhancing Ca^{2+} release from the endoplasmic reticulum (33). Our experiments indicated that the histamine induced Ca^{2+} release in Cal-78 cells was increased when treated with IL-1β. The supplementary NMRT treatment reversed the IL-1β-induced increase in peak calcium and in addition raised the basal $[Ca^{2+}]_i$. Changing the IL-1β-induced Ca^{2+} release by NMRT could suppresses downstream pathways known to mediate matrix destruction (34). Modulation of intracellular signalling pathways, regulated by mitogen-activated protein (MAP) kinases, influences the production and activity of multiple mediators of joint tissue destruction (35). In addition to cytokine regulation of MMP expression (36), matrix fragments generated during the development of arthritis including fibronectin fragments are able to activate MAP kinases and r induce expression of MMPs (37). NMRT treatment resulted in changes in the phosphorylation of different kinases. While members of the stress inducible p38 mito-

gen-activated protein kinases (p38α, β, γ, δ) seemed divergently regulated, NMRT enhanced the phosphorylated isoform of extracellular signal-regulated kinase 1 (ERK1) and reduced ERK2 and proteinkinase B (PKB/Akt) activity, respectively. The down-regulation of p38 was confirmed by western blot, indicating once more that NMRT could counteract the cartilage destroying processes. It has been shown that inhibition of p38 also inhibited harmful MMP-13 expression and that chondroprotective effects were associated with reduced activation of ERK1/2 and p38 (38). Interestingly, in our studies, blocking p38α and p38β with SB203580 abolished the NMRT induced increase in basal calcium, whereas reduction in peak calcium was still detectable (39). Both observations point to a selective and/or isoform specific regulation of chondrocytes by NMRT. Alongside the modulation of MAPKs by NMRT, the treatment induced increase in ATP might regulate AMP-activated protein kinase (AMPK) which plays a key role as a master regulator of cellular energy homeostasis (40). Bioenergy-sensing by AMPK and SIRT1 provides 'stop signals' for oxidative stress, inflammatory, and matrix catabolic processes in chondrocytes (41). NFκB proteins constitute a family of transcription factors that are stimulated by pro-inflammatory cytokines, chemokines, stress-related factors and extracellular matrix (ECM) degradation products. Upon stimulation, the activated NF-κB molecules trigger the expression of an array of genes which induce destruction of the articular joint, leading to onset and progression of osteoarthritis (OA) (42). In addition NF-κB regulates the expression of many cytokines, chemokines, adhesion molecules, inflammatory mediators and matrix degrading enzymes such as MMPs. Under these aspects it is of interest that the NMRT treatment reduced the IL-1β-induced NF-κB activity in chondrosarcoma cells.

In summary, we found that NMRT exhibit a modulatory effect on intracellular Ca^{2+} release, changes in MAPK activities with the particular reduction in p38 activity and a down-regulation of NF-κB, an important target to control OA. These results point out that NMRT exerts positive effects on the sub-threshold inflammatory mechanisms of cultured chondrocytes. Counteracting the IL-1β-induced reduction in ATP, NMRT opens new strategies to stabilise the metabolism of chondrocytes.

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