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Biochemical alterations in inflammatory reactive chondrocytes: evidence for intercellular network communication

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Abstract

Chondrocytes are effectively involved in the pathophysiological processes of inflammation in joints. They form cellular processes in the superficial layer of the articular cartilage and form gap junction coupled syncytium to facilitate cell-to-cell communication. However, very little is known about their physiological cellular identity and communication. The aim with the present work is to evaluate the physiological behavior after stimulation with the inflammatory inducers interleukin-1β and lipopolysaccharide. The cytoskeleton integrity and intracellular Ca^{2+} release were assessed as indicators of inflammatory state. Cytoskeleton integrity was analyzed through cartilage oligomeric matrix protein and actin labeling with an Alexa 488-conjugated phalloidin probe. Ca^{2+} responses were assessed through the Ca^{2+} sensitive fluorophore Fura-2/AM. Western blot analyses of several inflammatory markers were performed. The results show reorganization of the actin filaments. Glutamate, 5-hydoxytryptamine, and ATP evoked intracellular Ca^{2+} release changed from single peaks to oscillations after inflammatory induction in

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the chondrocytes. The expression of toll-like receptor 4, the glutamate transporters GLAST and GLT-1, and the matrix metalloproteinase-13 increased. This work demonstrates that chondrocytes are a key part in conditions that lead to inflammation in the cartilage. The inflammatory inducers modulate the cytoskeleton, the Ca^{2+} signaling, and several inflammatory parameters. In conclusion, our data show that the cellular responses to inflammatory insults from healthy and inflammatory chondrocytes resemble those previously observed in astrocyte and cardiac fibroblasts networks.

Keywords: Cell biology

1. Introduction

Osteoarthritis (OA) is a chronic progressive disease accompanied by low-grade inflammation that leads to pain and loss of joint mobility in horses and humans [1, 2, 3, 4, 5]. The OA joint includes pro-inflammatory mediators; interleukin-1 β (IL-1 β) and tumor necrosis factor (TNF)-α and Serum amyloid A (SAA) present in the early stages and antimicrobial peptides (AMPs) as defensines important for tissue repair [6, 7, 8]. Activity of IL-1 β has been detected in synovial fluid from joints [4] and has been shown to induce gene expression of matrix degrading MMP:s (matrix metallopeptidases) and ADAMTS (a distintegrin-like and metalloproteinase with thrombospondin type 1 motif) in chondrocytes. MMP-13 (collagenase 3) has been shown to be upregulated in articular cartilage explants after mechanically induced damage [9] and are localized in chondrocytes from patients with arthropathy of the temporomandibular joint discs and appears to play e key role in the degradation of the collagen type II network of the cartilage matrix [10].

In adult articular cartilage, chondrocytes exist as individual cells embedded in the extracellular matrix and gap junctions are mainly expressed by the flattened chondrocytes forming cellular processes facing the superficial layer [11]. Chondrocytes also exist as several cells such structures are referred to as chondrons [12]. Intercellular communication from paired chondrocytes is mediated through the gap junction channel protein connexin 43 (Cx43) [13, 14, 15, 16]. Cx43 enhances the expression of OA-associated genes such as matrix metallopeptidase (MMP)-1, MMP-13, and a disintegrin-like and metalloproteinase with thrombospondin type 1 motif (ADAMTS) in cultured rabbit synovial fibroblasts [14]. Although it is required for differentiation, whether Cx43 plays additional roles in chondrocytes remains unclear. Equine tenocytes are connected to each other in a three-dimensional network through cytoplasmic processes and linked gap junctions. Mechanical stimuli of both chondrocytes and tenocytes (such as strain) are sensed through gap junctions, which leads to the synthesis of collagen [15] [16]. Chondrocytes in the superficial layer unfold their cell membrane ruffles in a load-dependent manner as a protective measure against cell membrane rupture

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and necrosis [17]. Mitochondrial dysfunction and chondrocyte death of the cells in the superficial zone has been shown to be an acute response to mechanical injury [18]. Dynamic compression improves the synthesis of matrix molecules as aggrecan, collagen type II and lubricin by chondrocytes in the superficial layer [19] and physical activity improve the lubricative properties of articular cartilage by increasing lubricin synthesis [20].

Changes in intracellular Ca^{2+} levels have a profound influence on many cell functions, including matrix synthesis and degradation [21]. An increase in cytosolic Ca^{2+} levels can promote the release of signaling molecules via the process of regulated exocytosis. Such molecules can be transmitters, cytokines, prostaglandins, proteins, and peptides [22]. The gap junction channels are composed of two hemichannels, one provided by each of the joined cells [23]. Ca^{2+} signaling over long distances is analogous to, but much slower than, the propagation of action potentials [24]. Cytosolic Ca^{2+} plays a key role as a second messenger, and the control of Ca^{2+} signals is therefore critical. This involves coordination of Ca^{2+} entry across the plasma membrane, Ca^{2+} release from the endoplasmic reticulum, refilling of the endoplasmic reticulum stores, and extrusion across the plasma membrane [25]. There are two main types of Ca^{2+} communication: either intercellular communication through gap junctions or extracellular communication by diffusion of ATP, which binds to purinoceptors [26, 27]. In the presence of inflammation, an elevation of Ca^{2+} signaling in the cellular networks is observed; this is dependent on increased production and release of ATP through opening of hemichannels in the plasma membrane. ATP stimulates purinoceptors through autocrine or paracrine stimulation, resulting in increased release from internal stores in the form of Ca^{2+} oscillations, which may change the balance between Ca^{2+}-regulating processes [22]. This extracellular Ca^{2+} signaling attenuates the intercellular Ca^{2+} signaling, resulting in reduced communication via gap junctions [28].

The Na^{+}-Ca^{2+} exchanger, a Ca^{2+} transporter that controls the intracellular Ca^{2+} concentration, is driven by the Na^{+} electrochemical gradient across the plasma membrane. Referred to as an Na^{+}/K^{+}-ATPase, as it requires K^{+} to promote Na^{+}-Ca^{2+} exchange, levels of this pump indirectly affect Ca^{2+} signaling [29]. This is since inflammatory stimuli downregulate the Na^{+}/K^{+}-ATPase, thereby altering Ca^{2+} homeostasis in cellular networks [30, 31, 32, 33, 34]. The endogenous glutamate that is released by chondrocytes has important functions in both physiological and pathological conditions that are mediated via binding to glutamate receptors. Glutamate transport in chondrocytes is performed by GLAST and GLT-1[35] and is involved in chondrocyte proliferation [36]. Dynamic remodeling of the actin cytoskeleton, a critical event during inflammation, also plays an essential role in the migration and proliferation of cells [37].

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Exposure to lipopolysaccharide (LPS) and IL-1β can be used experimentally to generate inflammatory responses both *in vitro* and *in vivo*. LPS (a bacterial endotoxin) is a potent inflammatory activator [38] that stimulates Toll-like receptor 4 (TLR4) [34] [39]. TLR4 is upregulated in astrocytes under neuroinflammatory conditions [40], and its activation leads to release of the pro-inflammatory cytokines TNF-α and IL-1β [41].

A more faithful recapitulation of chondrocyte responses is ideally obtained by use of cells from non-osteoarthritic cartilage. However, obtaining chondrocytes from healthy joints of humans is prohibited for ethical reasons. To circumvent this issue we turned to equine articular cartilage as an alternative source of healthy chondrocytes.

The aim of this study was to explore how the chondrocytes coupled in cell networks behave over time with respect to Ca^{2+} signaling, and to determine if they exhibit similar properties to those of astrocytes and cardiac fibroblasts. We examined whether induction of an inflammatory response using IL-1β and LPS led to changes in glutamate, 5-HT, and ATP induced Ca^{2+} responses. Finally, we examined whether other inflammatory factors were altered in chondrocytes, and monitored actin filament remodeling during chondrocyte stimulation.

2. Material and methods

2.1. Experimental procedure

2.1.1. Materials

Macroscopically normal cartilage samples were obtained from middle carpal joints of three horses (1, 3 and 4-years old) without known clinical history of disease in the joints. Cartilage samples were obtained within 24 h after euthanasia. The horses were euthanized because of reasons unrelated to this study. The Ethical Committee on Animal Experiments, Stockholm, Sweden, approved the study protocol. (Dnr: N378/12). Following aseptic preparation, arthrotomies were performed, cartilage of the dorsal aspect of the radial facet of the third carpal bone was incised with a scalpel, and full-thickness cartilage samples were collected with forceps. The tissue was placed in a sterile saline (0.9% NaCl) solution with gentamicin sulfate (50 mg/l) and amphotericin B (250 μg/ml). Cartilage samples were transported chilled (approx. 5 °C) to the laboratory. Isolation and expansion of chondrocytes were performed as previously described [42].

2.1.2. Chondrocyte culture in monolayer

Chondrocytes was expanded in monolayer as earlier described [42]. Subsequently passage 3 cells were seeded at 20, 000 cells/cm^2 in Dulbecco's modified Eagles medium-high glucose (DMEM-HG) (Thermo Fisher Scientific; Waltham, MA,

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USA) supplemented with 14 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 10^{-7} M dexamethasone (Sigma-Aldrich), 1 mg/mL human serum albumin (Equitech Bio, Kerville, TX, USA), 1 × insulin–transferrin–selenium (Gibco, Life Technologies, Carlsbad, CA, USA), 5 μg/mL linoleic acid (Sigma-Aldrich), 1 × penicillin-streptomycin (PEST) (Sigma-Aldrich), and 10 ng/mL human transforming growth factor (TGF) β-1 (R&D Systems, Abingdon, UK) on glass coverslips (Nr 1, diameter 20 mm, BergmanLabora, Stockholm, Sweden) in 12 well culture plates for Ca^{2+} and immunohistochemistry analysis, or in 6 well culture plates for Western blot analysis.

2.1.3. Treatment with inflammatory inducers

The cells were incubated 3 to 7 days in monolayer culture and were then stimulated with recombinant IL-1β (5 ng/mL, R&D Systems) or LPS (10 ng/mL, *Escherichia coli* 055:B5, List Biological Laboratories, CA, USA) for 24 h. Unstimulated cells were used as controls.

2.1.4. Ca^{2+} imaging

Chondrocytes incubated with IL-1β, LPS or untreated controls were incubated at room temperature with the Ca^{2+} sensitive fluorophore probe Fura-2/AM (Invitrogen Molecular Probes, Eugene, USA) for 20 min (8 μL in 990 μL Hank 's HEPES-buffered saline solution (HHBSS), containing 137 mM NaCl, 5.4 mM KCl, 0.4 mM $MgSO_4$, 0.4 mM $MgCl_2$, 1.26 mM $CaCl_2$, 0.64 mM KH_2PO_4, 3.0 mM $NaHCO_3$, 5.5 mM glucose and 20 mM HEPES dissolved in distilled water, pH 7.4 (Sigma-Aldrich)). The fluorophore probe was dissolved in 40 μL dimethyl sulfoxide (DMSO) and 10 μL pluronic acid (MolecularProbes, Leiden, the Netherlands). The experiments were performed at room temperature using a Ca^{2+} imaging system and Simple PCI software (CompixInc., Imaging Systems, Hamamatsu Photonics Management Corporation, Cranberry Twp., PA, USA) connected to an inverted epifluorescence microscope (Nikon ECLIPSE TE2000-E) with a 20x (NA0.45) fluorescence dry lens and a Polychrome V, monochromator-based illumination system (TILL Photonics, CA, USA). The various substances glutamate (10^{-3} M), 5-HT (10^{-5} M), or ATP (10^{-4} M) (Sigma-Aldrich) were applied using a peristaltic pump (Instech Laboratories, Plymouth Meeting, PA, USA) at an approximate rate of 600 μl/min. One minute after the start of the experiment, the stimulating substance was pumped into the pump tubes for 30 s. The substance took approximately 60 s to reach the cells through the tubes. HHBSS continued to flow through the pump tubes and onto the cells throughout the experiment. The images were captured with an ORCA-12AG High Res Digital Cooled CCD Camera (C4742-80-12AG, Hamamatsu Photonics). The total area under the curve (AUC), which reflects the amount of Ca^{2+} released, was analyzed in order to measure the strength of the Ca^{2+} responses. The amplitude was

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expressed as the maximum increase of the 340/380 ratios, and the area under the Ca^{2+} peaks was calculated in Origin (Microcal Software Inc., Northampton, MA, USA).

2.1.5. Immunocytochemistry and actin visualization

The cells were fixed with 4% paraformaldehyde (Bie & Berntsen, Herlev, Denmark) for 10 min and washed twice with phosphate-buffered saline (PBS, Invitrogen) containing 1% bovine serum albumin (PBS–BSA, Sigma-Aldrich). The cells were permeabilized with PBS–BSA containing 0.05% saponine (PBS–BSA––Sap, Sigma-Aldrich) for 20 min. Subsequently, the cells were incubated for 1 h with a rabbit polyclonal COMP antibody (1:1000) provided by Professor Heinegård (Lund University, Sweden)(2) or rabbit IgG (X0903, Dako, Glostrup, Denmark) as isotype control using the same concentration as the primary antibody. The cells were washed with PBS–BSA–Sap for 3 × 5 min and incubated with a mixture of FITC conjugated F(ab′)2 fragment donkey anti-mouse IgG and a Dylight 594 conjugated F(ab′)2 fragment donkey anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch Europe Ltd, Suffolk, UK), both diluted 1:150. The cells was counterstained with an Alexa^{TM}488-conjugated phalloidin probe (Invitrogen) and washed with PBS–BSA–Sap for 3 × 5 min and finally rinsed with PBS. The cover slips were mounted on microscope slides with a fluorescent mounting medium (Dako) and viewed in a Nikon Optiphot-2 microscope. Digital pictures were taken with the NIS Elements D Ver.3.2 (Nikon, Tokyo, Japan).

2.1.6. Western blot analysis

Western blot analysis was carried out according to standard protocols. Briefly, protein extracts were prepared by cell lysis in RIPA buffer (Sigma-Aldrich) supplemented with a mammalian protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was determined using the Pierce BCA protein assay kit (Life Technologies), according to the manufacturer's instructions. Equal amounts of extracts (5 μg) were resolved on 4–12% Bis-Tris pre-cast gels (Life Technologies) and transferred onto nitrocellulose membrane (GE healthcare). Equal loading and transfer of the proteins was confirmed by Ponceau staining (0.1% in acetic acid, Sigma-Aldrich). The membranes were probed with the following primary antibodies; polyclonal rabbit anti-TLR-4 (M-300) (sc-30002, Santa Cruz), polyclonal rabbit anti-GLT-1 (pab0037, Covalab), polyclonal rabbit anti-GLAST (pab0036-P, Covalab), polyclonal rabbit anti-connexin 4371–0700, Life technologies), monoclonal mouse anti-Na+/K+-ATPase (A276, Sigma-Aldrich), and mouse monoclonal anti-β-actin (A5441, Sigma-Aldrich). The membranes were then probed with horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch). Protein bands were detected using Immobilon

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Western chemiluminescent HRP substrate (Millipore) in a ChemiDoc XRS+ instrument (Bio-Rad). The relative intensity of the protein bands in the linear exposure range were quantified using the ImageLab software (BioRad).

2.1.7. Assay for MMP-13

Concentration of MMP-13 were measured in the culture media using Fluorokine E Human Active MMP-13Fluorescent Assay (R&D Systems) after adding APMA (aminophenyl mercuric acetate), which activates latent MMP-13. The protein determination assay was performed according to the manufacturer's instructions using a DC Protein Assay (Bio-Rad, Hercules, CA, USA). Both the standards (0–4 mg/mL BSA) and the samples were mixed with the reagents, incubate for 15 min at 22 °C and subsequently read at 750 nm using a Versa-max microplate reader, and analyzed using SoftMax Pro 4.8 (Molecular Devices, CA, USA). Culture media (from stimulated and unstimulated cells) was diluted 1:2 and all assay analyses were performed on duplicate samples and were correlated to the protein concentration. The lowest detection limit was 8 pg/ml for active MMP-13. The concentration of activated MMP-13 in media was measured and correlated to total protein in cell lysate.

2.1.8. Glutamate release

The concentration of glutamate was measured in the culture media using ninhydrid reagent for photometric determination. Duplicate samples from culture media (from stimulated and unstimulated cells) were analyzed and correlated to the protein concentration. The lowest detection limit was 5 μmol/l of glutamate [43].

2.1.9. Statistical methods

The data were analyzed using GraphPad Prism software version 6.05 (La Jolla, CA). Statistical differences between sample groups were assessed using Mann-Whitney-test. The statistical analysis was performed with SPSS v19 (IBM Corp., Armonk, NY, USA software). Statistical significance was determined using Student's paired t-test or one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test. A significant difference was assumed at a p-value of ≤ 0.05. Unless otherwise stated, the data are expressed as the mean and standard error of the mean (SEM).

3. Results

3.1. Actin filaments and COMP immunocytochemistry

The chondrocytes were stained with Alexa^{TM}488-conjugated phalloidin probe. The unstimulated cells demonstrated F-actin organized in stress fibers (Fig. 1). The

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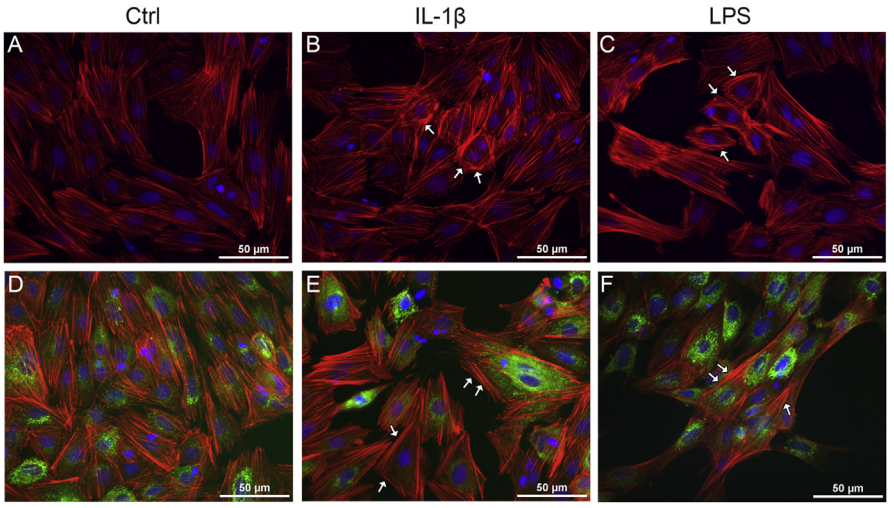

Fig. 1. Actin filament and immunostaining of COMP. Unstimulated chondrocytes and chondrocytes stimulated with IL-1β (5 ng/ml) or LPS (10 ng/ml) for 24 h were stained with Alexa^{TM}488-conjugated (red) phalloidin probe and for the chondrogenic matrix molecule COMP (green). The nuclei visualized with Hoescht33258 (blue). Untreated chondrocytes were dominated by F-actin organized in stress fibers and pronounced COMP-staining (**A, D**). Chondrocytes stimulated with IL-1β (**B, E**) or LPS (**C, F**) exhibited a more retracted organization and occasionally ring structures were demonstrated (white arrows). COMP-staining similar to unstimulated chondrocytes was observed (n = 3-4). Control sections with isotype staining were used (data not shown).

inflammatory induced cells, incubated with IL-1β or LPS for 24 h, showed signs of retracted actin structures and some actin ring structures. All cells, both unstimulated cells and cells stimulated with IL-1β or LPS for 24 h, demonstrated positive and distinct COMP-staining indicating a chondrogenic phenotype (Fig. 1).

3.2. Time-dependent Ca^{2+} responses

In order to evaluate an appropriate time point for the experimental conditions, the chondrocytes were stimulated with glutamate, 5-HT, or ATP and monitored using Ca^{2+} imaging between days 3 and 7. Chondrocytes stimulated with glutamate showed an increase in Ca^{2+}signaling at day 3, whereas no response was detectable on the other days (Fig. 2A). The 5-HT stimulus evoked Ca^{2+} signaling which peaked at day 4 (Fig. 2B), and both day 3 and day 4 were significantly higher compared to day 6. The ATP stimulation induced Ca^{2+} signaling peaked at day 5, where it was significantly higher compared to days 6 and 7 (Fig. 2C). Based on these data, we chose 4-day-old cultures for carrying out the following experiments.

3.3. Intracellular evoked Ca^{2+} release

Chondrocytes cultured for 4 days and then stimulated with IL-1β or LPS for further 24 h, were subjected to Ca^{2+} imaging experiments. The areas under the Ca^{2+}peak (AUC) were calculated. The glutamate evoked Ca^{2+} signaling was increased in

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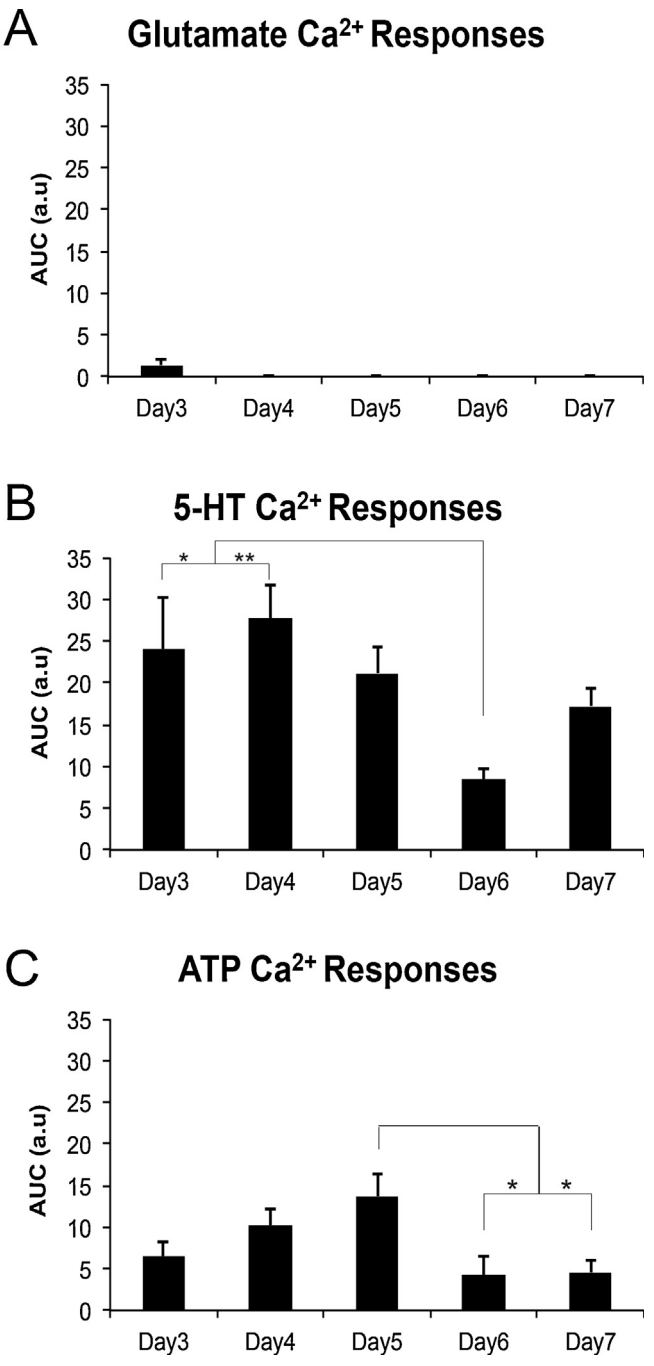

Fig. 2. Time-dependent evoked Ca^{2+} responses. All chondrocytes were stimulated in a Ca^{2+} imaging system over time with glutamate (10^{-3} M) (**A**), 5-HT (10^{-5} M) (**B**) or ATP (10^{-4} M) (**C**). The areas under the Ca^{2+} peaks (AUC) of Ca^{2+} transients were calculated. The cells were from 3–4 coverslips, and from 3 different seeding times. One-way ANOVA followed by Turkey's post hoc test was used for statistical analysis (n = 43) *$p < 0.05$, **$p < 0.01$.

inflammatory chondrocytes and significantly higher in cells stimulated with IL-1β compared to unstimulated controls (Fig. 3). Stimulation with 5-HT or ATP induced

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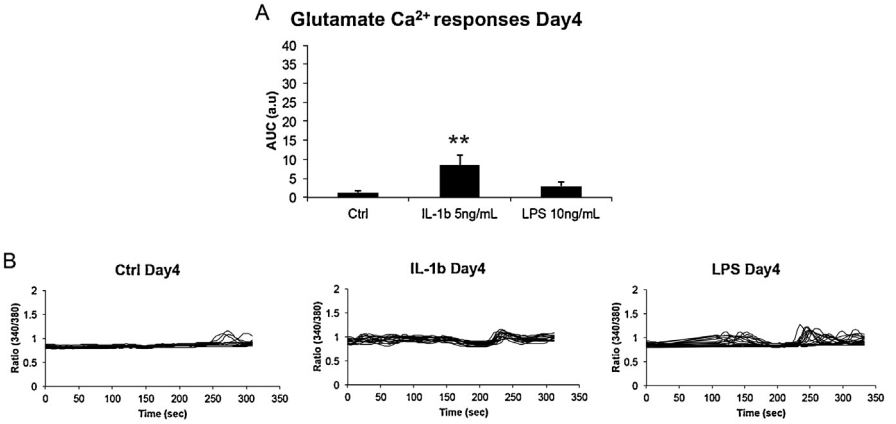

Fig. 3. Glutamate evoked Ca^{2+} responses in inflammatory chondrocytes. All cells were stimulated in a Ca^{2+} imaging system, and the areas under the Ca^{2+} peaks (AUC) of Ca^{2+} transients were calculated. Chondrocyte Ca^{2+} responses to glutamate (10^{-3} M) when stimulated with IL-1β (5 ng/ml) or LPS (10 ng/ml) for 24 h, unstimulated cells were used as control (**A**). The cells were from 3–4 coverslips, and from 3 different seeding times. The appearance of the Ca^{2+} transients is visualized; results are shown from a typical experiment (**B**). For statistical analysis, a paired student's t-test was used to compare unstimulated cells and IL-1β or LPS stimulated cells (n = 30), *$p < 0.05$, **$p < 0.01$.

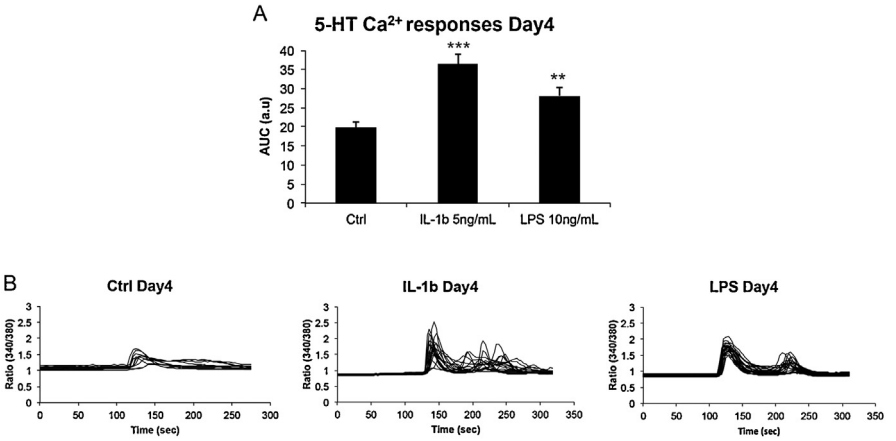

Fig. 4. 5-HT evoked Ca^{2+} responses in inflammatory chondrocytes. All cells were stimulated in a Ca^{2+} imaging system, and the areas under the Ca^{2+} peaks (AUC) of Ca^{2+} transients were calculated. Chondrocyte Ca^{2+} responses to 5-HT (10^{-5} M) when stimulated with IL-1β (5 ng/ml) or LPS (10 ng/ml) for 24 h, unstimulated cells were used as control (**A**). The cells were from 3–4 coverslips, and from 3 different seeding times. The appearance of the Ca^{2+} transients is visualized; results are shown from a typical experiment (**B**). For statistical analysis, a paired student's t-test was used to compare unstimulated cells and IL-1β or LPS stimulated cells (n = 30), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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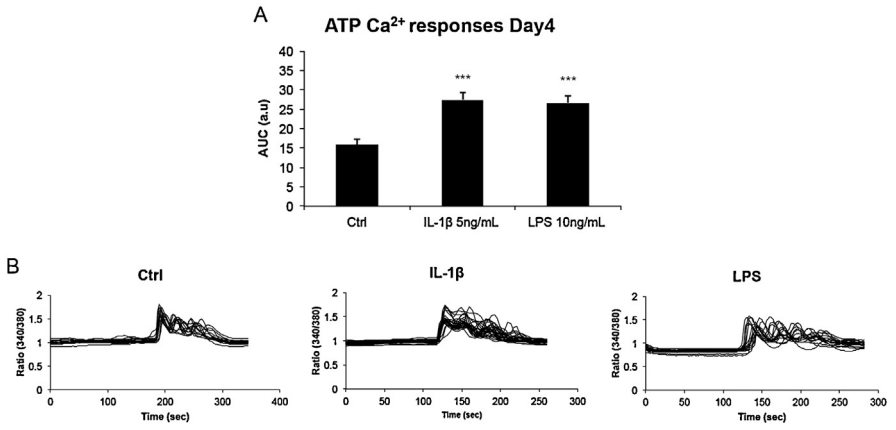

Fig. 5. ATP evoked Ca^{2+} responses in inflammatory chondrocytes. All cells were stimulated in a Ca^{2+} imaging system, and the areas under the Ca^{2+} peaks (AUC) of Ca^{2+} transients were calculated. Chondrocyte Ca^{2+} responses to ATP (10^{-4} M) when stimulated with IL-1β (5 ng/ml) or LPS (10 ng/ml) for 24 h, unstimulated cells were used as control (**A**). The cells were from 3–4 coverslips, and from 3 different seeding times. The appearance of the Ca^{2+} transients is visualized; results are shown from a typical experiment (**B**). For statistical analysis, a paired student's t-test was used to compare unstimulated cells and IL-1β or LPS stimulated cells (n = 30), *p < 0.05, **p < 0.01, ***p < 0.001.

significantly higher Ca^{2+} signaling in chondrocytes stimulated with IL-1β or LPS, compared to unstimulated controls (Figs. 4 and 5).

3.4. Activated MMP-13 release

The concentration of activated MMP-13 was increased after the chondrocytes had been incubated with IL-1β. No activated MMP-13 release was obtained after the chondrocytes had been incubated with LPS (Table 1).

Table 1. Concentrations of activated MMP-13. MMP-13 measured in culture, mean (pg/ml) ± SEM, supernatants of unstimulated, IL-1β or LPS-stimulated monolayer chondrocytes, compared to total amount of protein in cell lysate. A higher MMP-13 concentration in media from IL-1β stimulated cells compared to unstimulated control was found. One-way analysis of variance followed by the Bonferroni test was used for multiple group comparisons. $P < 0.05$ was considered statistically significant. NS = Non-significant. P-value; investigated group vs control.

	Control	IL-1β stimulated	LPS stimulated
Mean	0,02	3.94	0,022
±SDs	0,0005	0,8041	0,0008
p-Value	NS	P < 0,01	NS
N	4	4	4

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3.5. TLR4, Cx43, Na+/K+-ATPase, GLAST and GLT-1 expression in chondrocytes

The levels of several proteins involved in inflammatory and Ca^{2+} signaling was investigated in control cells and cells incubated with IL-1β or LPS. Chondrocytes incubated with LPS showed an increased expression of TLR4. Cx43 expression was increased both in IL-1β and LPS incubated chondrocytes compared to control cells. The glutamate transporters, GLAST and GLT-1 also increased in expression after LPS incubation. The Na+/K+-ATPase expression did not change markedly (Figs. 6 and 7).

3.6. Glutamate release

Glutamate release was increased in media from LPS stimulated chondrocytes (0,033 ± 0,016 μmol/l/mg protein) compared to unstimulated cells (0,013 ± 0,002

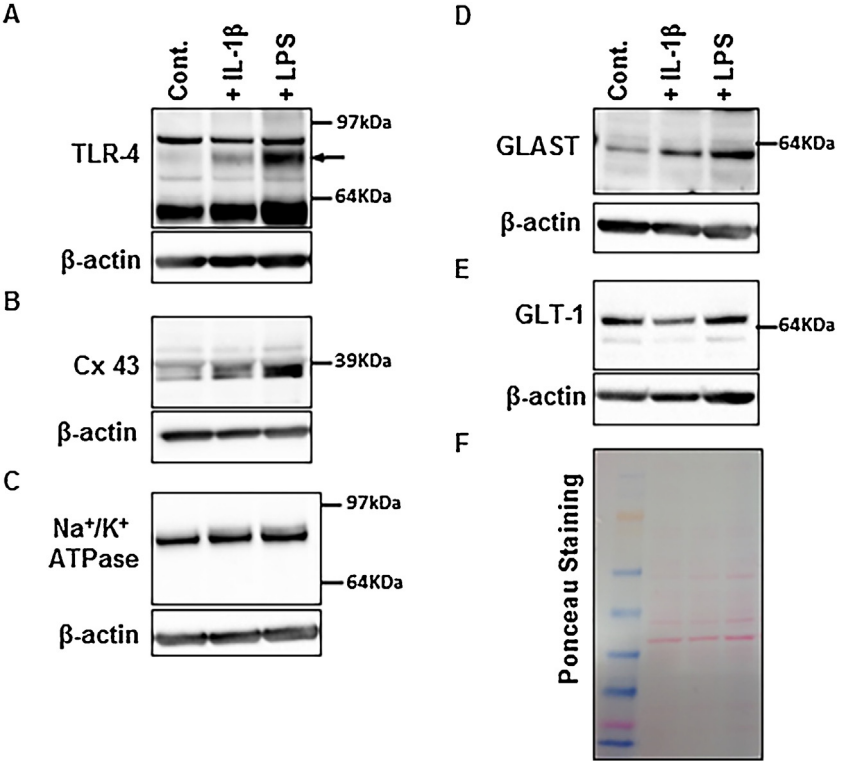

Fig. 6. **A-F**. The effect of IL-1β and LPS treatment on TLR4 and some membrane transporter levels in chondrocytes. Chondrocytes were treated with IL-1β (5 ng/ml) or LPS (10 ng/ml) for 24 h, and unstimulated cells were used as control. **A-E,** The cells were analyzed by western blotting with antibodies against TLR4, connexin 43 (Cx 43), Na^+/K^+ ATPase, GLT-1, GLAST-1, and β-actin as indicated on the figure. The blots are representative of three different horses. **F**, Representative Ponceau staining to confirm protein loading, which correlated to β-actin levels indicating it to be an appropriate internal control for normalization of protein levels.; Full size image is shown in supplementary documents (Fig. 6) of (Fig. 6A-F).

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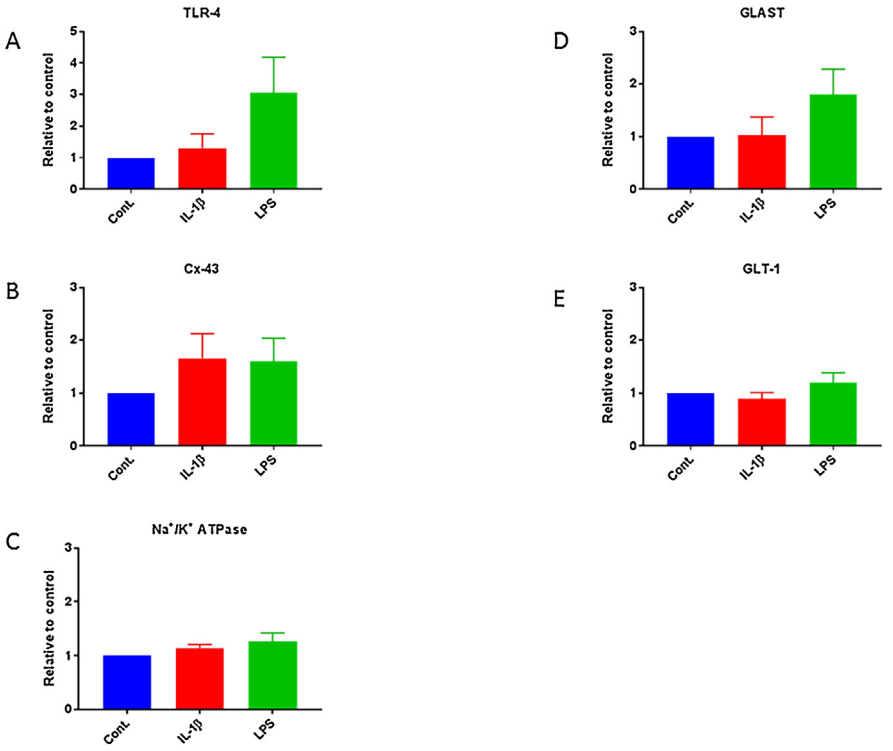

Fig. 7. A-E. The relative intensities of protein bands (mean ± SE) are shown in bar graphs for TLR4, connexin 43 (Cx 43), Na^+/K^+ ATPase, GLT-1, GLAST-1. Intensity were quantified using the ImageLab software in the linear exposure range and normalized to β-actin from the same blot. The intensity of the control protein band was set to 1.

μmol/l/mg protein), $p = 0.03$. Additionally, glutamate release was detected in media from IL-1β stimulated cells (0,020 ± 0,009 μmol/l/mg protein), however there was not a statistically significant difference when compared to the unstimulated cells.

4. Discussion

The aim of the current study was to explore whether chondrocytes exhibit similar biochemical properties as the network-coupled astrocytes and cardiac fibroblasts, and how inflammatory stimuli affect these parameters. Network-coupled cells are excitable, but do not express action potentials [24, 27, 44]. Rather, they are equipped with Ca^{2+} signaling systems that can be inter-and/or extracellular in nature. Our results show time-dependent Ca^{2+} responses of chondrocytes to glutamate, 5-HT, and ATP; this mimics the response of astrocytes in the central nervous system, which is one of the most well studied network-coupled cell systems and cardiac fibroblast [27, 45, 46]. Cells cultured in monolayer are prone to dedifferentiate, which could confound interpretation of results. Reassuringly, the COMP-positive staining we observed confirmed the phenotype of the articular

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cartilage chondrocytes [42, 47]. Intercellular communication gives tissues the ability to coordinate different cellular functions, such as the regulation of cell volume, intracellular ionic composition, and cell metabolism. A characteristic of network-coupled cells is their passive electrical properties, which allows them to provide the framework and metabolic support for different organs, but also to supply computational power and modify behavioral output. Intercellular communication takes place through gap junctions and extracellular communication through the diffusion of ATP, which then binds to purinoceptors [26, 27]. Chondrocytes are capable of sustaining propagation of intercellular Ca^{2+} waves in rabbits [13] as well as in equines [48, 49].

During inflammation and when cells are stimulated with inflammatory agents, Ca^{2+} signaling in network-coupled cells is disturbed. There is an increased release of IL-1β, which results in gap junction inhibition [50]. There is also an increased release of ATP, which provides a parallel system for intercellular Ca^{2+} communication [51] and triggers the release of glutamate [52]. Here we show that the inflammatory inducers IL-1β and LPS changed the glutamate-, 5-HT-, and ATP-evoked Ca^{2+} transients from single peaks to Ca^{2+} oscillations when the chondrocytes were incubated with IL-1β or LPS for 24 h.

Comparable results have been obtained with astrocytes, also coupled with gap junctions, in monoculture [41, 53, 54]. This suggests that the intracellular Ca^{2+} release from the endoplasmatic reticulum is increased after inflammatory induction, which leads to an overstimulation of the G_q protein coupled mGluR5-, $5\text{-}HT_{2A}$-, and P2Y-, respectively [26]. Intracellular Ca^{2+} is an important parameter due to its influences on many cell functions, including matrix synthesis and degradation [55]. An increase in cytosolic Ca^{2+} levels can induce release of signaling molecules, via exocytosis and such molecules can be cytokines, prostaglandins and transmitters [22].

OA is associated with a low grade inflammation and the presence of pro-inflammatory cytokines, IL-1β and TNF-α during the early stages of disease [56] [5]; this milieu leads to MMP activation and destruction of cartilage [57]. Our results showed after IL-1β stimulation of chondrocytes the concentration of secreted MMP-13 in its active form was higher compared to control and LPS-stimulated cells, indicating that the chondrocytes were responsive to inflammatory stimuli. Systemic (peripheral) inflammation with high serum levels of pro-inflammatory cytokines is associated with low-grade inflammatory diseases such as OA [2] and may be a physiological manifestation of metabolic syndrome [58]. When peripheral tissues are injured, pro-inflammatory mediators are released into the bloodstream. A systemic inflammatory response is then activated, resulting in alterations in several inflammatory parameters [59, 60].

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During inflammation the expression and affinities of several receptors is changed in many cell types. The expression of TLR4 increases in response to LPS [34, 39]. It was also prominent in chondrocytes stimulated with LPS or IL-1β, and has been confirmed in the biosynthetic activity in the cartilage [61].

A disruption of the actin filament with more pronounced ring-structures was found in inflammatory stimulated chondrocytes. F-actin reorganization engenders upregulation of inflammatory cytokines in many different cell types. Following LPS exposure, the production of pro-inflammatory cytokines, is high in pulmonary monocytes [62] as well as in astrocytes [41]. Increased IL-1β production is also observed in astrocytes after LPS stimulation, which seems to be initiated through TLR4 activation [63]. An upstream activator of IL-1β is a key component of immune response; the inflammasomes, which is an intracellular multiprotein complex involved in the response of inflammation [64].

Chondrocytes are connected to each other via cell-cell interactions and form functional gap junctions expressing Cx43 [13, 65]. In the adult articular cartilage, chondrocytes exists as individual cells embedded in the extracellular matrix, and gap junctions are mainly expressed by the flattened chondrocytes facing the superficial cartilage layer [11]. In diseased cartilage, chondrocytes divide and forms cluster of cells referred to as chondron. There is a possibility that the chondrocytes laying close to each other in the chondron are connected to each other via gap junctions. In our study confluent monolayer cells express the major gap junction protein Cx43 and there was an increased protein expression when the cells were stimulated with IL-1 β or LPS. This is in accordance to recent data [13] showing increased expression of Cx43 in IL-1β stimulated chondrocytes.

Chondrocytes release and accumulate glutamate in a Ca^{2+} dependent manner [66]. We show that chondrocytes express the glutamate transporters GLAST and GLT-1, which are upregulated after inflammatory induction. We also observed glutamate release in the media.

The present study show that chondrocytes are connected into gap junction coupled networks, responding to Ca^{2+}-evoked release from intracellular stores, which change in behavior after inflammatory induction. As a consequence of these cellular inflammatory changes in chondrocytes the actin filaments are reorganized and several biochemical parameters changed.

In conclusion, our data show that response of chondrocytes to pro-inflammatory stimuli is very similar to that of astrocytes and cardiac fibroblasts, and demonstrates that these cells are another example of a coupled-cell network.

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Declarations

Author contribution statement

Eva Skiöldebrand: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Anna Thorfve: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ulrika Björklund, Pegah Johansson, Ruth Wickelgren: Performed the experiments; Wrote the paper.

Anders Lindahl: Analyzed and interpreted the data; Wrote the paper.

Elisabeth Hansson: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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