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Interleukin-1 β Increases Gap Junctional Communication among Synovial Fibroblasts via the Extracellular Signal Regulated Kinase Pathway

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Abstract

Background information—The gap junction protein, connexin43, has been implicated in the etiology of osteoarthritis. Work from others has revealed that the size and number of gap junctions increases in synovial biopsies from patients with osteoarthritis. Further, pharmacologic inhibition of connexin43 function has been shown to reduce interleukin-1 β induced metalloproteinase production by synovial fibroblasts *in vitro*.

Results—In this study, we examine the link between interleukin-1 β and connexin43 function. We demonstrate that treatment of a rabbit synovial fibroblast cell line with interleukin-1 β markedly increases connexin43 protein in a dose- and time-dependent manner. The impact on connexin43 protein levels appears to occur post-transcriptionally as mRNA levels are unaffected by interleukin-1 β administration. Additionally, we show by fluorescence microscopy that interleukin-1 β alters the cellular distribution of connexin43 to cell-cell junctions and is concomitant to a striking increase in gap junction communication. Further, we demonstrate that the increase in connexin43 protein and the associated change in protein localization and gap junction communication following interleukin-1 β treatment are dependent upon activation of the extracellular signal regulated kinase signaling cascade.

Conclusions—These data show that interleukin-1 β acts through the extracellular signal regulated kinase signaling cascade to alter the expression and function of connexin43 in synovial fibroblasts.

Keywords

Connexin43; Arthritis; Inflammation; Extracellular Signal Regulated Kinase; Cell-Cell Interaction

Introduction

Osteoarthritis (OA) is a progressive disease of the joints characterized by degradation of articular cartilage, resulting in articular fibrillation, fissures, ulcerations, erosion of subchondral bone and osteophyte formation (Hamerman, 1989). Although disease initiation is multifactorial, the cartilage destruction appears to be a result of uncontrolled proteolytic extracellular matrix destruction with contributions from both articular chondrocytes and the synovial cells. Articular chondrocytes are the cells responsible for producing and maintaining the articular cartilage extracellular matrix, which provides the functional integrity of the articular cartilage. During OA these cells produce a non-functional extracellular matrix and

release catabolic enzyme that degrade the extracellular matrix (Kuettnner *et al.*, 1991). Synovial fibroblasts exist in a cellular network with synovial macrophages, forming a thin lining of synovial tissue surrounding the fibrous capsule of the joint. The physiologic role of synovial tissue is to produce a synovial fluid that lubricates the joints and supplies nutrients to the articular chondrocytes. During OA, the synovial cells mount a chronic, low-grade inflammatory response as a result of release of cartilage breakdown products into the synovial fluid (Benito *et al.*, 2005). The inflammatory cytokine, interleukin-1 β (IL-1 β), which is considered a key mediator of OA, drives cartilage destruction by inducing catabolic factors, such as matrix metalloproteinases and aggrecanases, from both synovial cells and articular chondrocytes (van den Berg, 1999; Chevalier, 1997).

Alterations in gap junctions have been linked to diseases of chronic inflammation (Green and Nicholson, 2008). Several studies suggest a role for the gap junction protein, connexin43 (Cx43), in the etiology of OA (Kolomytkin *et al.*, 2002; Marino *et al.*, 2004). Gap junctions are transcellular channels formed by juxtaposition of two connexon hemichannels present on adjacent cells. Gap junctions permit direct signal exchange and maintain metabolic continuity between cells via diffusion of ions, metabolites and small signaling molecules. These hemichannels are composed of six connexin monomers. Interestingly, hemichannels have also been shown, under some circumstances to be able to function independent of gap junction-formation by permitting direct communication between the cytoplasm and the extracellular milieu (Spray *et al.*, 2006; Saez *et al.*, 2005). Cx43 is a widely expressed gap junction protein that forms gap junctions with a relatively large pore, permitting the passage of molecules of up to ~1.2 kD (Elfgang *et al.*, 1995; Weber *et al.*, 2004; Nicholson *et al.*, 2000). Thus, gap junctional communication permits a rapid and efficient mechanism by which a population of cells may produce a synchronized and robust response to an extracellular cue. *In vitro* and *in vivo* studies have shown that synovial fibroblast express functional gap junctions (Kolomytkin *et al.*, 2000; D'Andrea *et al.*, 1998; Marino *et al.*, 2003; Iwanaga *et al.*, 2000). Work from Kolomytkin *et al.*, has suggested a role for the gap junctional communication in the production of collagenases by synovial fibroblasts. In these studies, a rabbit synovial fibroblast cell line (HIG-82) was stimulated with IL-1 β , and pharmacologic inhibitors of gap junctions were shown to attenuate the IL-1 β -dependent collagenase activity (Kolomytkin *et al.*, 2002). Subsequent studies by the same group, using synovial biopsies, show that there is a pathologic increase in both the size and number of gap junction plaques, and expression of the gap junction protein Cx43 in the synovial tissue of patients with OA, though the precise cell type in which the increase in coupling was not clearly defined (Marino *et al.*, 2004). The authors also show that pharmacologic inhibition of gap junctional communication with octanol or 18 α -glycyrrhetic acid could diminish the basal and IL-1 β -stimulated release of collagenases from explant cultures of synovial tissue from patients with OA, establishing that gap junction function can influence collagenase production.

Our own work and the work of others have demonstrated that Cx43 gap junctional communication modulates growth factor responses and subsequent signal transduction, leading to regulation of gene expression and cellular function (Stains *et al.*, 2003; Stains and Civitelli, 2005; Lecanda *et al.*, 1998; Iacobas *et al.*, 2004; Lima *et al.*, 2009). In this study, we wanted to ask whether IL-1 β can also influence Cx43 function and to examine the molecular mechanisms by which the pro-inflammatory cytokine IL-1 β and Cx43 intersect. By examining the molecular players involved in the synovial response to inflammatory cytokines, we hope to identify potential therapeutic targets to control the onset and/or progression of arthritic diseases.

Results and Discussion

Effects of IL-1 β on Cx43 gap junction protein expression and localization in synovial fibroblasts

Previous studies have linked gap junction function with both the basal and IL-1 β induced production of collagenases by synovial fibroblasts and/or multicellular synovial tissue biopsies from patients with OA (Kolomytkin *et al.*, 2002; Marino *et al.*, 2004). However, a direct effect of IL-1 β on Cx43 expression or function in synovial fibroblasts has not been shown.

Accordingly, we examined whether the pro-inflammatory cytokine IL-1 β may directly regulate the expression of Cx43 in HIG-82 cells, a rabbit synovial fibroblasts cell line. In figure 1A, we show by western blotting that HIG-82 cells increases the expression of the Cx43 protein when stimulated with IL-1 β for 16 hours. Indeed, densitometric analysis indicate increases in Cx43 expression (normalized to GAPDH) of 1.9-, 1.8- and 1.8-fold relative to vehicle treated controls after treatment with 0.1, 1.0, and 10ng/ml IL-1 β , respectively. Three bands for Cx43 are detected by western blot. These bands correspond to different phosphorylated forms of Cx43 (often referred as non-phosphorylated NP (~42 kD), P₁ (~44 kD), and P₂ (~46 kD) bands), arising from numerous phosphorylation events that can promote or inhibit migration, assembly and function of gap junctions (Solan and Lampe, 2009; Musil and Goodenough, 1991). In contrast to the protein data, real time PCR analysis of cDNA prepared from IL-1 β treated HIG-82 cells does not reveal an increase in Cx43 gene expression (Figure 1B). These data suggest that the upregulation of Cx43 protein by IL-1 β may be regulated post-transcriptionally.

By western blotting, we observed a sustained increase in Cx43 levels in HIG-82 cells treated for 16, 24 and 48 hours with 1 ng/ml IL-1 β (Figure 2A). A 1.7 fold increase in Cx43 protein abundance was observed after 16 hours of treatment with IL-1 β . Stimulation for 24 hours maximally increased the level of Cx43 protein, whereas prolonged stimulation 48h maintained but did not further induce further up-regulation (1.6- and 1.5-fold, respectively). As was observed in the IL-1 β dosing experiments, the effect on Cx43 protein abundance was not observed at the mRNA level, as real time PCR of cDNA from HIG-82 cells treated with 1 ng/ml IL-1 β did not detect a change in Cx43 levels (Figure 2B).

Next, we examined Cx43 cellular distribution in response to IL-1 β treatment by immunofluorescence microscopy. As illustrated in Figures 3A, under control conditions (vehicle added) HIG-82 cells exhibited two distinct patterns of staining. There is a considerable amount of cytoplasmic staining as well as modest punctate staining for Cx43 at the cell surface. This punctate pattern at the apposition of adjacent cells is characteristic of connexin present in gap junction plaques. Stimulation of the cells for 16 hours with IL-1 β (1 ng/ml) considerably increased the abundance of the punctuate membrane labeling as well as an increase in the cytoplasmic Cx43 staining.

Establishment of gap junctional intercellular communication (GJIC) among HIG-82 cells

In order to validate that IL-1 β is increasing the abundance of functional gap junctions between HIG-82 synovial fibroblasts, we examined gap junctional coupling by scrape loading (El-Fouly *et al.*, 1987). This assay utilizes the fluorescent tracer Lucifer yellow (LY), which is small enough (~0.5 kD) to transfer from cell-to-cell through Cx43 channels and a 10 kD rhodamine-dextran conjugate that cannot pass through the gap junction. A confluent monolayer of HIG-82 cells was mechanically “wounded” by producing a single scrape line with a scalpel. Gap junctional communication was quantitated by measuring the distance of LY transfer from the damaged cells at the scrape line to the most distal LY positive cell. Vehicle treated HIG-82 cells are moderately coupled with dye transfer routinely seen to spread ~ 2–4 cell layers from the wounded cell (Figure 4). Upon treatment with IL-1 β (16 hours) there is a striking, dose-dependent increase in the dye coupling of these cells. A maximal increase in GJIC is observed

in HIG-82 cells treated with 10ng/ml IL-1 β , which transmit dye ~5 times the distance of vehicle treated cells. To validate that the dye transfer among the cells is due to GJIC, we performed scrape loading experiments in the presence of the gap junction inhibitor, carbenoxolone (CBX). When HIG-82 cells are treated with 100 μ M CBX, there is a potent inhibition of IL-1 β -induced dye transfer synovial fibroblasts (Supplemental Figure 1). In total, these data show that treatment of a synovial fibroblast cell line with IL-1 β results in significant increase in the abundance of functional Cx43 protein and GJIC. Indeed, the marked increase in Cx43 observed in these cells is analogous to the increase in the size and number of gap junctions observed by transmission electron microscopy in the synovial biopsies from patients with severe OA relative to healthy controls (Marino *et al.*, 2004).

Role of Extracellular Signal Regulated Kinase (ERK) signaling in IL-1 β -mediated effects on GJIC in HIG-82 cells

IL-1 β is known to utilize several signaling pathways to mediate its effects, including NF- κ B and the p38, ERK and JNK mitogen activated protein kinase cascades, (Dunne and O'Neill, 2003). In fact, IL-1 β has been shown to induce the activation of ERK in primary synovial fibroblasts cultures isolated from the synovial tissue of patients undergoing total knee arthroplasty for osteo- or rheumatoid arthritis (Yun *et al.*, 2008; Yamamoto *et al.*, 2003). Increased phospho-ERK staining is also observed in the joints of mice with collagen induced arthritis and in synovial biopsies of patients with rheumatoid arthritis (Thiel *et al.*, 2007).

We were particularly interested in examining the ERK signaling cascade for several reasons. First, the ERK pathway has been shown to regulate Cx43 expression and function. ERK activation has been shown to upregulate Cx43 expression in several cell types (Boswell *et al.*, 2009; Jia *et al.*, 2008; Cushing *et al.*, 2005). Activation of ERK has been shown to phosphorylate Cx43 at multiple serine residues in the C-terminal tail and has been shown to regulate Cx43 function, typically inhibiting GJIC (Warn-Cramer *et al.*, 1998; Lampe and Lau, 2004). Second, we have implicated the ERK pathway in mediating the influence of alteration of Cx43 on osteoblast gene expression (Stains and Civitelli, 2005). In this study, we showed that disruption of Cx43 function could inhibit ERK signaling. Further, it has been also been shown that Cx43 hemichannels can regulate osteoblast apoptosis via activation of ERK (Plotkin *et al.*, 2002; Plotkin *et al.*, 2005). Thus, ERK activation can lie both upstream and downstream of Cx43 function. Finally, the ERK cascade has been implicated as a key mediator of arthritic diseases (Yamamoto *et al.*, 2003; Barchowsky *et al.*, 2000). Indeed, several inhibitors of the ERK cascade have recently been shown to reduce the severity of symptoms in a mouse models of collagen induced arthritis (Ohori *et al.*, 2007; Thiel *et al.*, 2007). Similarly, a dominant negative Ras construct, which suppresses ERK activation by IL-1, has been used to ameliorate inflammation and reduce joint destruction in a rat model of adjuvant induced arthritis (Yamamoto *et al.*, 2003). The impact of ERK cascade inhibition on arthritis seems to center on the fact that ERK is a critical mediator of the expression of catabolic factors involved in joint destruction. For example, the production of the protease stromelysin (MMP3) by cultured rabbit synovial fibroblasts has been shown to be dependent upon IL-1 α -induced activation of ERK (Thiel *et al.*, 2007); comparable results were obtained for the ERK-dependence of TNF α -induced MMP1 production in human synovial fibroblasts (Tagoe *et al.*, 2008) Given the role for the ERK cascade both upstream and downstream of Cx43 and its important role in the production of catabolic factors in arthritic disease, we examined whether the ERK cascade may be mediating the effects of IL-1 β on Cx43 protein expressions and function in synovial fibroblasts. Importantly, we have not ruled out the involvement of other pathways. We observe potent activation of the p38 and JNK MAPK pathways by IL-1 β in these cells as well (Supplemental Figure 2A).

Consistent with the literature, western blot analysis reveals that treatment with IL-1 β results in the phosphorylation of ERK (Figure 5A). ERK is phosphorylated within 5 minutes of treatment and is maintained for at least 15 minutes. Pre-incubation of HIG-82 cells with the ERK cascade inhibitor U0126 (10 μ M) prevents both basal and IL-1 β induced ERK phosphorylation (Figure 5B). Similar, but less effective, results are obtained with Mek1 inhibitor, PD98059 (Supplemental Figure 2B). Subsequently, we investigated the role of ERK activation on the IL-1 β mediated increase in Cx43 protein expression and communication. The induction of Cx43 protein expression by 1 ng/ml IL-1 β treatment is inhibited by treatment with 10 μ M U0126 (Figure 5C) as well as by PD98059 (Supplemental Figure 2C). This data indicates that the ERK pathway is required, at least in part, for the upregulation of Cx43 protein levels. Next we sought to examine whether the observed changes in Cx43 protein expression and function were likewise dependent on ERK signaling. By immunofluorescence microscopy, we observed that under basal conditions the detection of Cx43 is markedly reduced by the ERK cascade inhibitor (Figure 6A,C), suggesting a critical role for ERK in the accumulation of Cx43 at the plasma membrane of synovial fibroblasts. As shown earlier, IL-1 β induces a considerable accumulation of Cx43 at cell-cell contacts, in punctate patterns consistent with the formation of gap junction plaques (Figure 6A,B). However, treatment with 10 μ M U0126 results in a noticeable reduction of Cx43 in plaques at cell-to-cell contacts (Figure 6B,D). Indeed, it appears that Cx43 abundance at the membrane of IL-1 β -U0126 treated cells is comparable, if not less than, in vehicle treated synovial fibroblasts (Figure 6A,D). Indeed, nearly identical results were obtained with the Mek1 inhibitor, PD98059 (Supplemental Figure 3A-D). Given the apparent reduction in the amount of Cx43 accumulating in gap junction-like plaques at cell-to-cell contacts, we next sought to assess the impact of inhibition of ERK signaling on GJIC. Accordingly, we performed scrape loading dye coupling experiments on HIG-82 cells that been treated with IL-1 β (1 ng/ml) or vehicle in the presence or absence of 10 μ M U0126 (Figure 7). Treatment with U0126 alone did not dramatically affect the modest coupling among HIG-82 cells. As we have shown above, IL-1 β dramatically increases dye coupling among the cells. However, when treated with U0126, the impact of IL-1 β on dye coupling was fully abrogated. Similarly, PD98059 treatment reduced the IL-1 β induced GJIC (Supplemental Figure 3E). Thus, the activation of the ERK cascade by IL-1 β is, at least in part, required for the increase in GJIC. For the most part, ERK phosphorylation of Cx43 has been reported to inhibit GJIC (Warn-Cramer *et al.*, 1998; Ruch *et al.*, 2001; Solan and Lampe, 2009). However, there are exceptions where ERK activation can stimulate GJIC (Boswell *et al.*, 2009; Cushing *et al.*, 2005). Our data are consistent with an observed increase in Cx43 caused by IL-1 β in articular chondrocytes (Tonon and D'Andrea, 2000). The mechanistic difference between the inhibition or activation of GJIC by ERK are likely complex and involved cross talk among pathways. In future studies, we intend to systematically detect and dissect what changes are occurring in the complex phosphorylation status of Cx43 in these cells following IL-1 β treatment.

Collectively, these data demonstrate that the inflammatory cytokine, IL-1 β , markedly enhances the abundance of Cx43 in a synovial fibroblast cell line. It is unclear whether the synovial origin of these HIG-82 cells has an impact of the regulation of Cx43 or whether this is a generalized response. None the less, there is a profound impact of IL-1 β on Cx43 expression, accumulation of Cx43 in gap junction plaques, and an increase in GJIC. We suspect the action of IL-1 β on Cx43 expression and function may explain the observed increase in the size and number of gap junction plaques noted in synovial biopsies of humans with OA (Marino *et al.*, 2004). However, more rigorously defined *in vivo* approaches are required to validate the link between IL-1 β and Cx43 in arthritic tissue. In this study, we also establish that the ERK pathway is critical to the regulation of Cx43 by IL-1 β , as inhibition of the ERK cascade blocks the IL-1 β induced increase in Cx43 expression, the alteration in Cx43 cellular distribution and the increase in GJIC. We demonstrate an interrelationship between IL-1 β , ERK and Cx43, all of which have been implicated in the etiology of OA. While the functional consequences of increased Cx43 expression in synovial fibroblasts remain to be determined, work from others

has shown that there is a correlation between GJIC in synovial fibroblast and the production of matrix metalloproteinases (Marino *et al.*, 2004; Kolomytkin *et al.*, 2002). It is intriguing to hypothesize that alterations in Cx43 may result in an increased production of matrix metalloproteinases and other catabolic factors that have been implicated in joint destruction in arthritic disease. Indeed, we and others have shown in other cell types that alteration of Cx43 abundance or function can directly impact gene expression (Lecanda *et al.*, 1998; Stains *et al.*, 2003; Chung *et al.*, 2006; Lecanda *et al.*, 2000; Iacobas *et al.*, 2008; Li *et al.*, 2006; Lima *et al.*, 2009). Accordingly, Cx43 may prove to be a mediator of the inflammatory response that leads to cartilage destruction and may become a therapeutic target for slowing the onset or progression of arthritis. Given the role of the inflammation and IL-1 β specifically in rheumatoid arthritis as well as OA, we speculate that our hypothesis can also be extrapolated to rheumatoid arthritis. It is important to note that Cx43 in the joint is not limited to the synovial cells, as gap junctions have also been demonstrated *in vivo* in articular chondrocytes (Chi *et al.*, 2004), cells of the meniscus (Hellio Le Graverand *et al.*, 2001a; Hellio Le Graverand *et al.*, 2001b) and osteoblasts (Doty and Schofield, 1972; Stanka, 1975). Further, IL-1 β has been shown to alter Cx43 expression in articular chondrocytes *in vitro* (Tonon and D'Andrea, 2000; Tonon and D'Andrea, 2002). It has been postulated that gap junctions may play a central role in chronic inflammatory diseases and may sustain the inflammatory cycle (Green and Nicholson, 2008). Clearly, further *in vivo* experiments are needed to examine the effect of IL-1 β on GJIC in the complex multicellular joint compartment, so that we may more clearly define the consequence of altered gap junctional coupling in arthritis. Notably, *in vitro* and *in vivo* progress towards these ends continue to suggest that Cx43 should be considered a putative therapeutic target for slowing the onset or progression of arthritic disease.

Materials and methods

Chemicals and reagents

All chemicals were obtained from Sigma (St Louis, MO) unless otherwise stated. All reagents used for cell cultures were purchased from Cellgro (Herndon, VA). Human recombinant IL-1 β (Calbiochem; La Jolla, CA) was dissolved in Hank's Balanced Salt Solution (HBSS) supplemented with 0.1% bovine serum albumin to obtain a 10 μ g/ml stock solution. The antibody against the Cx43 was obtained from Sigma (St Louis, MO), anti-mouse-GAPDH from Chemicon (Temecula, CA) and the anti-rabbit IgG antibody conjugated to Alexa Fluor[®] 488 was from Invitrogen (Eugene, OR). The nuclear counterstain DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride) was from Roche (Indianapolis, IN). Anti-phospho-p44/42 ERK, anti-p44/42 ERK antibodies and the ERK selective inhibitors U0126 and PD98059 were purchased from Cell Signaling (Danvers, MA). U0126 and PD98059 were dissolved in dimethyl sulfoxide (DMSO).

Cells and cell culture

An established rabbit synovial fibroblast cell line (HIG-82) was used for all the experiments and was obtained from ATCC (American Type Culture Collection, Manassas, VA). Cells were cultured in modified Ham's F-12 1X medium supplemented with penicillin (50 IU/ml), streptomycin (50 μ g/ml) and 10% fetal bovine serum. The culture medium was renewed every 3 days. For all experiments, cells were serum starved (culture medium reduced to 0.1% FBS) 24 hours, prior to any treatments containing IL-1 β . Cultures were kept at 37°C in humidified atmosphere of 95% air and 5% CO₂. Cell viability was routinely monitored with a colorimetric CCK-8 assay (Alexis Biochemical, Farmingdale, NY). The number of viable cells under all conditions was consistently above 95% and did not statistically differ among groups.

RNA extraction and quantitative RT-PCR

At indicated time, total RNA was extracted from cells using RNeasy mini-kit (Qiagen, Valencia, CA), according to the manufacturer's recommendations. One μ gram of RNA was used to prepare first-strand cDNA with a mix of random hexamers and oligo(dT) primers using the SuperScript® III First-Strand Synthesis kit (Invitrogen; Eugene, OR). Newly synthesized cDNA strands were then used as templates to perform real time PCR on an Applied Biosystems 7300 Sequence Detection System using the SYBR green method (Applied Biosystems; Foster City, CA). Expression relative to 18S rRNA was calculated using the Δ CT method as described previously (Stains and Civitelli, 2005). The primer sequences for rabbit Cx43 were: 5'-GGG CAG GCA GGA AGT ACC AT-3' and 5'-TGG TTA TCA TCC GGG AAA TCA-3', respectively. The primer sequences for 18S rRNA were: 5'-CAT TAA ATC AGT TAT GGT TCC TTT GG-3' and 5'-TCG GCA TGT ATT AGC TCT AGA ATT ACC-3'. All real time PCR experiments were performed on triplicate cultures and repeated at least three times.

Protein extraction and western blot analysis

Whole cell extracts were harvested from adherent cells, grown on 6-well plates. Proteins were solubilized in 300 μ l/well modified RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS; completed with 10 mM sodium pyrophosphate, 10 mM β -glycerophosphate, 10 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate and 1X proteases inhibitor cocktail). The samples were sonicated, insoluble material removed by centrifugation and the supernatants stored at -20°C until use. Total protein concentration was determined using a BCA assay (Pierce; Rockford, IL), and 30 μ g total protein/sample were subjected to separation on 10% SDS-PAGE gels and then transferred to polyvinylidene difluoride membranes (Millipore; Bedford, MA). Subsequently, membranes were blocked in 5% nonfat dry milk, and then incubated overnight with the indicated primary antibody. Immunoreactive bands were detected with the horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence detection reagents (Pierce; Rockford, IL). Blots were acquired and analyzed using an UVP EpiChem gel documentation system (UVP; Upland, CA). Quantitation of western blots was performed with Lab Works 4.0 Image Acquisition and Analysis Software (UVP Bioimaging Systems, Upland, CA). A background corrected integrated optical density measurement of the observed bands intensity was calculated for each blot and normalized to its corresponding load control (e.g., GAPDH).

Immunofluorescence

Cells seeded on coverslips in 6-well plates were fixed for 15 min. in 4% paraformaldehyde and then blocked 30 min. at room temperature in HBSS containing 1% bovine serum albumin and 0.1% TritonX-100. Cells were incubated overnight at 4°C with a rabbit anti-Cx43 antibody (1:100) in blocking solution. After rinsing, cells were incubated at room temperature with Alexa Fluor® 488-conjugated chicken anti-rabbit IgG secondary antibody (1:400). DAPI (0.2 ng/ml) was added at the same time for nuclear labeling. Images were acquired on an inverted microscope that was equipped for epifluorescence illumination (Nikon Eclipse 50i, Nikon; Melville, NY), using a Photometrics Coolsnap camera system and interfaced to a computer using Roper Scientific Image (Tucson, AZ) software for analysis. All immunostaining was repeated at least three times.

Scrape loading/Dye transfer

Monolayers of cells grown to confluence in 6-well plates were prepared as previously described (Lima *et al.*, 2009). Cells were rinsed twice in Ca^{2+} -free HBSS 1X and incubated in 0.3% Lucifer yellow solution, containing 0.25% rhodamine dextran (10kD). A scrape with a surgical scalpel was made to allow the non-permeable dyes to penetrate the cells. After 5 min incubation

at room temperature, the dye medium was discarded and cells were washed three times with Ca^{2+} -containing phosphate-buffered saline and then fixed in 4% paraformaldehyde. Transfer of the dye from the scraped edge to the neighboring cells was observed under a fluorescence microscope with the proper filter (Nikon Eclipse 50i, Nikon; Melville, NY). The “injured” cells at the scrape line were discriminated from cells that received Lucifer yellow via GJIC by the presence of the 10kD molecular weight rhodamine dextran. Dye transfer was quantitated by measuring the distance from the Lucifer yellow positive, rhodamine dextran negative cells at the scrape line to the most distal Lucifer yellow positive cells perpendicular to the scrape line in the collected images. A minimum of 20 measurements were taken per field of view. Three fields of view were analyzed per experiment. The scrape loading was performed on triplicate cultures. All of the calculated measurements for dye diffusion were averaged for comparisons among groups. For most experiments, only the Lucifer yellow images are shown. Validation of that dye transfer is mediated by GJIC is shown in supplemental figure 1. In these experiments cells were preteated with 100mM CBX for 10 min prior to scrape loading. CBX was maintained throughout the loading procedure until fixation in 4% paraformaldehyde. All experiments were repeated at least three times.

Statistical analysis

All illustrated experiments were performed independently at least three times with similar results and one representative experiment is shown. All values are expressed as mean $\pm$ standard deviation (SD). Data was assessed for statistical significance using a two-tailed t-test (comparison of two data sets) or with analysis of variance (ANOVA, comparison of multiple data sets). A significant difference was determined as a p value being less than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

OA	osteoarthritis
IL-1 β	interleukin-1 β
Cx43	connexin43
RA	rheumatoid arthritis
GJIC	gap junctional intercellular communication
LY	Lucifer yellow
ERK	extracellular signal regulated kinase
DMSO	dimethyl sulfoxide
MAPK	mitogen activated protein kinases
HBSS	Hank's balanced salt solution
CBX	carbenoxolone

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Figure 1. IL-1 β increases Cx43 protein expression in a dose-dependent manner

(A) Serum-starved HIG-82 synovial fibroblasts were treated for 16 hours with vehicle or the indicated doses of IL-1 β . Western blots of whole cell extracts isolated from the cells were probed with anti-Cx43 antibodies, revealing a dose-dependent increase in Cx43 protein expression. GAPDH is used as a loading control. A representative blots is shown. Three bands are observed using the anti-Cx43 antibody, corresponding to various phosphorylated forms of Cx43. Densitometric quantitation of the western blots (n=5 blots) is shown to the right of the blot (mean $\pm$ s.d.). The densitometric value of the vehicle treated control sample is arbitrarily set to 1. An asterisk ‘*’, indicates a statistical significant difference from the vehicle control [p-value <0.05]. (B) Real time PCR analysis of Cx43 expression in serum-starved HIG-82 cells treated with the indicated amounts of IL-1 β for 16, 24 or 48 hours reveals no change in the relative abundance of Cx43 mRNA, despite the observed alteration in protein expression. Cx43 expression levels are expressed relative to the abundance of 18SrRNA in the sample. The data is shown as the mean ($\pm$ s.d.) from three independent experiments each performed on triplicate wells. No statistical difference is detected among groups.

Figure 2. IL-1 β increases Cx43 protein expression in a time-dependent manner

(A) Serum starved HIG-82 cells were treated with 1 ng/ml IL-1 β for 0, 16, 24 or 48 hours. Western blots of whole cell extracts isolated from the cells were probed with anti-Cx43 antibodies, revealing a time-dependent increase in Cx43 protein expression. GAPDH is used as a loading control. A representative blots is shown. Densitometric quantitation of the western blots (n=3 blots) is shown to the right of the blot (mean $\pm$ s.d.). The value of the '0 hour' control is arbitrarily set to 1. An asterisk '*', indicates a statistical significant difference from the control [p-value < 0.05]. (B) Real time PCR analysis of cDNA from serum starved HIG-82 cells treated as above with 1 ng/ml IL-1 β for 16, 24 or 48 hours reveals no change in the relative abundance of Cx43 mRNA, despite the observed alteration in protein expression. Cx43 expression levels are expressed relative to the abundance of 18S rRNA in the sample. The data is shown as the mean ($\pm$ s.d.) from three separate experiments each performed on triplicate wells. No statistical difference is detected among groups.

Figure 3. IL-1 β increases the amount and cellular distribution pattern of Cx43

(A) Confluent monolayers of HIG-82 cells cultured on glass coverslips were treated with vehicle for 16 hours, fixed, immunostained with anti-Cx43 antibodies and imaged using an epi-fluorescence microscope. Immunodetection of Cx43 (green) reveals significant cytoplasmic staining in vehicle treated cells with occasional punctate areas of staining near appositional cells, suggestive of gap junction plaques (arrows). Nuclei are labeled with DAPI (blue). (B) Treatment of the cells for 16 hours with 1ng/ml IL-1 β dramatically increases the total amount of Cx43 (green) observed by immunostaining both in the cytoplasmic compartment and in punctate plaques (arrows). The scale bars (red) indicate a distance of 10 μ m. Representative images are shown for each treatment.

Figure 4. IL-1 β increases GJIC in a dose dependent manner by scrape loading dye transfer
Prior to scrape loading serum starved HIG-82 cells were treated for 16 hours with vehicle, 0.1, 1, or 10 ng/ml IL-1 β , as indicated. Transfer of the low molecular weight fluorescent tracer, Lucifer yellow, from cells damaged by scraping to adjacent cells by gap junctions was imaged using an epi-fluorescence microscope. There is a striking increase in the distance of dye transfer that corresponds to the increasing dose of IL-1 β . Representative images of Lucifer yellow fluorescence are shown for each treatment. The graph shows the quantitation of the average distance of transmission of Lucifer yellow from the cells proximal to the scrape line to the most distally Lucifer yellow positive cell. Data (mean $\pm$ s.d.) is set relative to the vehicle control. An asterisk ‘*’, indicates a statistical significant difference from the vehicle control [p-value < 0.05].

Figure 5. The IL-1 β mediated effects on Cx43 expression are ERK dependent

(A) Whole cell extracts of serum starved HIG-82 cells treated for the indicated times with 1ng/ml IL-1 β were examined by western blotting with anti-phospho-ERK and total ERK antibodies, as indicated. Treatment of the cells resulted in an increase in phospho-ERK relative to total ERK by 5 minutes. ERK phosphorylation remained elevated for at least 15 minutes. (B) Cells were pre-treated with either DMSO or the ERK cascade inhibitor U0126 (10 μ M) for 10 minutes. Cells were then treated with vehicle or 1ng/ml IL-1 β for 10 minutes in the presence or absence of U0126. Pre-treatment of the cells with U0126 abrogates both the basal and IL-1 β stimulated phosphorylation of ERK. (C) Serum starved HIG-82 cells were treated with vehicle or 1 ng/ml IL-1 β for 16 hours in the presence or absence of DMSO or 10 μ M U0126.

Whole cell extracts were examined by western blotting with anti-Cx43 antibodies. IL-1 β increases the expression of Cx43 in the presence of DMSO. U0126 prevents the IL-1 β mediated increase in Cx43 protein expression. GAPDH is used as a load control. Representative blots are shown for each treatment group. For all panels, densitometric quantitation of at least three separate western blots is shown to the right of the blot (mean $\pm$ s.d.). The densitometric value of the control sample is arbitrarily set to 1. An asterisk '*', indicates a statistical significant difference from the control [p-value <0.05].

Figure 6. The IL-1 β mediated effects on Cx43 cellular distribution are ERK dependent

Confluent monolayers of HIG-82 cells cultured on glass coverslips were treated with (A) vehicle and DMSO (B) 1 ng/ml IL-1 β and DMSO (C) Vehicle and 10 μ M U0126, or (D) 1 ng/ml IL-1 β and 10 μ M U0126 for 16 hours, fixed, immunostained with anti-Cx43 antibodies and imaged using an epi-fluorescence microscope. (A) Immunodetection of Cx43 (green) reveals cytoplasmic staining in vehicle and DMSO treated cells with occasional punctate areas of staining near appositional cells, suggestive of gap junction plaques (arrows). (B) The pattern of staining is altered in IL-1 β and DMSO treated cells, with a marked increase in the cytoplasmic and punctate plaque-like staining. (C) Treatment with U0126 alone did not markedly alter the pattern of Cx43 staining with respect to the vehicle treated controls. (D) Treatment with U0126 blocked the alteration in Cx43 staining observed in IL-1 β treated cells, as the pattern of Cx43 staining appears nearly indistinguishable from the vehicle treated cells. For all images, nuclei are labeled with DAPI (blue). Representative images are shown for each treatment. The scale bars (red) indicate a distance of 10 μ m.

Figure 7. The IL-1 β mediated effects on GJIC are ERK dependent

Serum starved HIG-82 cells were treated as indicated for 16 hours. IL-1 β was used at a concentration of 1ng/ml and U0126 at 10 μ M. Transfer of the low molecular weight fluorescent tracer, Lucifer yellow, from cells damaged by scraping to adjacent cells by gap junctions was imaged using an epi-fluorescence microscope. Representative images of Lucifer yellow fluorescence are shown for each treatment. The graph shows the quantitation of the average distance of transmission of Lucifer yellow from the cells proximal to the scrape line to the most distally Lucifer yellow positive cell. Data (mean $\pm$ s.d.) is set relative to the vehicle control cultures. IL-1 β increases GJIC as measured by dye transfer. In the absence of IL-1 β , U0126 has no effect on basal dye transfer. In combination with IL-1 β , U0126 fully abolishes the

IL-1 β -mediated effects on dye transfer. An asterisk '*', indicates a statistical significant difference from the vehicle control [p-value <0.05]. Double asterisks '**', indicate a statistical difference from the IL-1 β treated samples, but no difference from the vehicle treated control cultures.