



Celecoxib alleviates nociceptor sensitization mediated by interleukin-1beta-primed annulus fibrosus cells

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Abstract

Purpose This study aims to analyze the effect of pro-inflammatory cytokine-stimulated human annulus fibrosus cells (hAFCs) on the sensitization of dorsal root ganglion (DRG) cells. We further hypothesized that celecoxib (cxb) could inhibit hAFCs-induced DRG sensitization.

Methods hAFCs from spinal trauma patients were stimulated with TNF- α or IL-1 β . Cxb was added on day 2. On day 4, the expression of pro-inflammatory and neurotrophic genes was evaluated using RT-qPCR. Levels of prostaglandin E2 (PGE-2), IL-8, and IL-6 were measured in the conditioned medium (CM) using ELISA. hAFCs CM was then applied to stimulate the DRG cell line (ND7/23) for 6 days. Then, calcium imaging (Fluo4) was performed to evaluate DRG cell sensitization. Both spontaneous and bradykinin-stimulated (0.5 μ M) calcium responses were analyzed. The effects on primary bovine DRG cell culture were performed in parallel to the DRG cell line model.

Results IL-1 β stimulation significantly enhanced the release of PGE-2 in hAFCs CM, while this increase was completely suppressed by 10 μ M cxb. hAFCs revealed elevated IL-6 and IL-8 release following TNF- α and IL-1 β treatment, though cxb did not alter this. The effect of hAFCs CM on DRG cell sensitization was influenced by adding cxb to hAFCs; both the DRG cell line and primary bovine DRG nociceptors showed a lower sensitivity to bradykinin stimulation.

Conclusion Cxb can inhibit PGE-2 production in hAFCs in an IL-1 β -induced pro-inflammatory in vitro environment. The cxb applied to the hAFCs also reduces the sensitization of DRG nociceptors that are stimulated by the hAFCs CM.

Keywords Intervertebral disk · In vitro model · 3R principle · Celecoxib · Dorsal root ganglion · DRG · Annulus fibrosus · Cyclooxygenase 2 inhibitor · COX-2 · Inflammatory cytokine

Introduction

Chronic low back pain (LBP) is one of the world's leading causes of disability. In 26–42% of all cases, the degenerative intervertebral disk (IVD) can be identified as a cause of pain (discogenic pain) [1]. This may indicate a maladaptive communication between the degenerated IVD tissue and the sensory nervous system. Indeed, in healthy IVD, sensory nerve fiber only penetrates the outer third of the annulus fibrosus (AF), while the painful, degenerative IVD was found to be associated with deeper innervation [2, 3]. The nerve fibers penetrating the degenerative IVD express

the neuropeptide transmitters substance P (SP) [3]. SP and calcitonin gene-related peptide (CGRP) are also markers of peptidergic nociceptors which are neurons that generally respond to damaging stimuli. Pain sensitizations are thought to be at least partly induced by hyperexcitability and spontaneous discharges of the nociceptors [2]. The neuronal cell body of these sensory nerve fibers is located in the dorsal root ganglion (DRG), an oval structure within the foramina at lumbar levels [4]. The inflamed degenerative IVD tissue contains increased levels of pro-inflammatory cytokines such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α). Together they trigger higher expression of nerve growth factor (NGF), interleukin 8 (IL-8), interleukin 6 (IL-6), and prostaglandin E2 (PGE-2) synthesizing enzyme cyclooxygenase 2 (COX-2) in IVD cells [5]. PGE-2 is known to be involved in nerve sensitization including a

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subthreshold response and enhanced excitation in DRG neurons [6].

Modulation of the COX-2 pathway by non-steroidal anti-inflammatory drugs (NSAID) represents first-line therapy for patients with low back pain [7]. Celecoxib (cxb) hinders the conversion of free arachidonic acid into PGE-2 by selective inhibition of COX-2. A disadvantage of this treatment is that highly effective doses can lead to side effects when administered systemically, such as gastrointestinal and cardiovascular risks [8]. In contrast, local cxb administration is expected to reach effective local concentrations while minimizing distal off-target effects. Indeed, promising results were reported in experimental dogs with early spontaneous IVDD, where intradiscally injected cxb led to pain relief [9, 10]. Moreover, it has been shown that intradiscal cxb delivery can also prevent IVD degeneration [11]. Nevertheless, the role of cxb in treating IVD degeneration and local IVD-nerve communication remains largely unexplored.

The present study first aimed to assess the anti-inflammatory effect of cxb on human AF cells (hAFCs) stimulated with pro-inflammatory cytokines in vitro. Further, the conditioned medium (CM) of the cytokine-treated hAFCs with/without cxb treatment was applied to DRG neurons to analyze the effect of cxb in alleviating the AF cell-mediated DRG sensitization.

Methods

Isolation and culture of human AF cells

IVD tissue was obtained from three trauma patients (Table 1) undergoing discectomy surgery. The AF tissue was isolated from the nucleus pulposus (NP) tissue as previously described [12]. In brief, the AF tissue was washed with PBS containing 1% penicillin–streptomycin (PS, Gibco, UK), cut into small pieces, and treated with 50 mL 0.2% pronase (Roche, DE) on a shaker at 37 °C for 1 h. The tissue was then washed twice with PBS, treated with 50 mL 130 U/mL collagenase type II solution (Worthington, NJ, US), and incubated on a shaker at 37 °C for 12 h. Undigested tissue was removed using a 100 µm cell strainer, and the isolated hAFCs were seeded for expansion at 2×10^5 cells/cm² and cultured in α MEM (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (FCS, Gibco, Paisley, UK) (growth medium) at 37 °C, 2% oxygen, and 5% CO₂. Passage 5 hAFCs were used for the experiments.

Cell Titer-Blue assay

To determine safe concentrations for subsequent experiments, we assessed the effects of cxb and DMSO on hAFCs

Table 1 Human annulus fibrosus cell donor information

Donor ID	Sex	Age	Pfirmann grade
# 1	Male	55	II–III
# 2	Male	50	II
# 3	Female	45	III

using the CellTiter-Blue assay, which measures cell viability by monitoring the reduction of resazurin to resorufin. The resulting fluorescence, detected at 579Ex/584Em, correlates with the number of viable cells and is a well-established indicator of cell viability [13]. Additionally, this assay can serve as an indicator of cellular energy metabolism, since resazurin is reduced by oxidoreductases in living cells.

Cxb (Sigma-Aldrich, St. Louis, USA) was dissolved in DMSO at 10 mM. hAFCs were seeded in 96-well plates (2×10^3 cells/well) and incubated at 2% O₂. After 24 h, different concentrations of cxb (0.1 µM, 1 µM, 10 µM, and 50 µM) or DMSO (0.001%, 0.01%, 0.1%, and 0.5%) were added. After incubation for 24, 48, and 72 h, the cells were washed with PBS and then exposed to the Cell Titer-Blue® reagent (Promega Corporation, Madison, WI, USA) diluted 1:5 in DMEM. Fluorescence intensity was determined with the Viktor 3 plate reader (PerkinElmer, Waltham, MA, USA) after 4 h of incubation (ex/em 560/590 nm).

Cytokine stimulation and cxb treatment of hAFCs

hAFCs were seeded in 12-well plates at a density of 2×10^5 cells / well and incubated in 1 mL basal medium containing high glucose Dulbecco's Modified Eagle Medium (DMEM-HG) (Gibco, Paisley, UK) with 1% insulin–transferrin–selenium (ITS) (Gibco, Paisley, UK), 50 µg/mL ascorbic acid-2-phosphate (As-2P) (Sigma-Aldrich, St. Louis, USA), and 1% non-essential amino acids (NEAA) (Sigma-Aldrich, St. Louis, USA). On day 1, hAFCs were stimulated with 10 ng/mL of either IL-1 β or TNF- α (Thermo Fisher Scientific, Reinach, CH). On day 2, 1 µM or 10 µM cxb was added. The same volume of DMSO was added as vehicle control. On day 4, cell lysates were collected from half of the samples. For the other half, the medium was changed to basal medium, and CM was collected on day 6 (Fig. 1).

Real-time quantitative polymerase chain reaction (RT-qPCR)

On day 4, hAFCs were lysed in TRI reagent (Molecular Research Center, Ohio, USA). Total RNA was extracted, and cDNA was synthesized using SuperScript™ VILO™ (Invitrogen, CA, USA) according to the manufacturer's instructions. RT-qPCR was performed using TaqMan™

Gene Expression Master Mix and Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) or custom primers and probes (Online resource supplementary table 1). The mRNA expression was quantified using the $2^{-\Delta\Delta C_t}$ method. *RPLP0* was used as a reference gene. $C_{q_{Cutoff}}$ was set to 35, and missing data were assigned to $C_{q_{Cutoff}} + 1$ [14].

Enzyme-linked immunosorbent assay (ELISA)

On day 4, cells were washed (to remove cytokines and cxb), and the basal medium was added. After incubation for 48 h, the CM was collected on day 6. Concentrations of IL-6, IL-8, brain-derived nerve factor (BDNF), NGF, and PGE-2 were measured using ELISA kits (R&D Systems) according to the manufacturer's instructions.

Culturing and experimental design for neural sensitization measurement

ND7/23 cells were derived from cell fusion of N18Tg2 mouse neuroblastoma and primary neonatal rat DRG neurons (Sigma-Aldrich, MI, USA). These DRG-like neurons were cultured in DMEM-HG containing 10% FCS, 1% penicillin/streptomycin, and 0.11 g/L sodium pyruvate. The cells were maintained at 37 °C and 5% CO₂ and passaged every 3 days. Passage 8 cells were seeded in 48-well plates

at a seeding density of 5×10^3 cells/cm². The medium was replaced by respective hAFCs CM 1 h after cell seeding (Fig. 2). Neural differentiation of the cells was induced by the addition of 10 mM cAMP (Sigma-Aldrich, MO, USA) and 10 ng/mL NGF (R&D Systems Inc., MN, USA). Cells were incubated for 6 days, and sensitization of the neurons was evaluated using calcium imaging (Fig. 2).

Calcium imaging

Calcium imaging was performed as formerly reported [15]. Briefly, an intracellular calcium signal (5 μM Fluo-4 AM, Thermo Fisher Scientific, Reinach, Switzerland) was recorded in 150 μL Krebs-Ringer's solution (NaCl 119 mM, KCl 2.5 mM, NaH₂PO₄ 1.0 mM, CaCl₂ 2.5 mM, MgCl₂ 1.3 mM, HEPES 20 mM, and D-glucose 11.0 mM) using a confocal laser scanning microscope (LSM 800, Zeiss) at 10× magnification, 85 μm pinhole, excitation of 488 nm, and emission at 509 nm. Overall, 200 s was recorded with one image per second. In the first 100 s, cells were recorded without any stimulation to evaluate the spontaneous calcium oscillation intracellularly. At 100 s, 150 μL bradykinin (1 μM, Sigma-Aldrich, Buchs, CH) was added for a final concentration of 0.5 μM to evaluate the calcium response to bradykinin. Using ImageJ Fiji (version 1.52p), the region of interest (ROI) for cell bodies was defined using an automatic

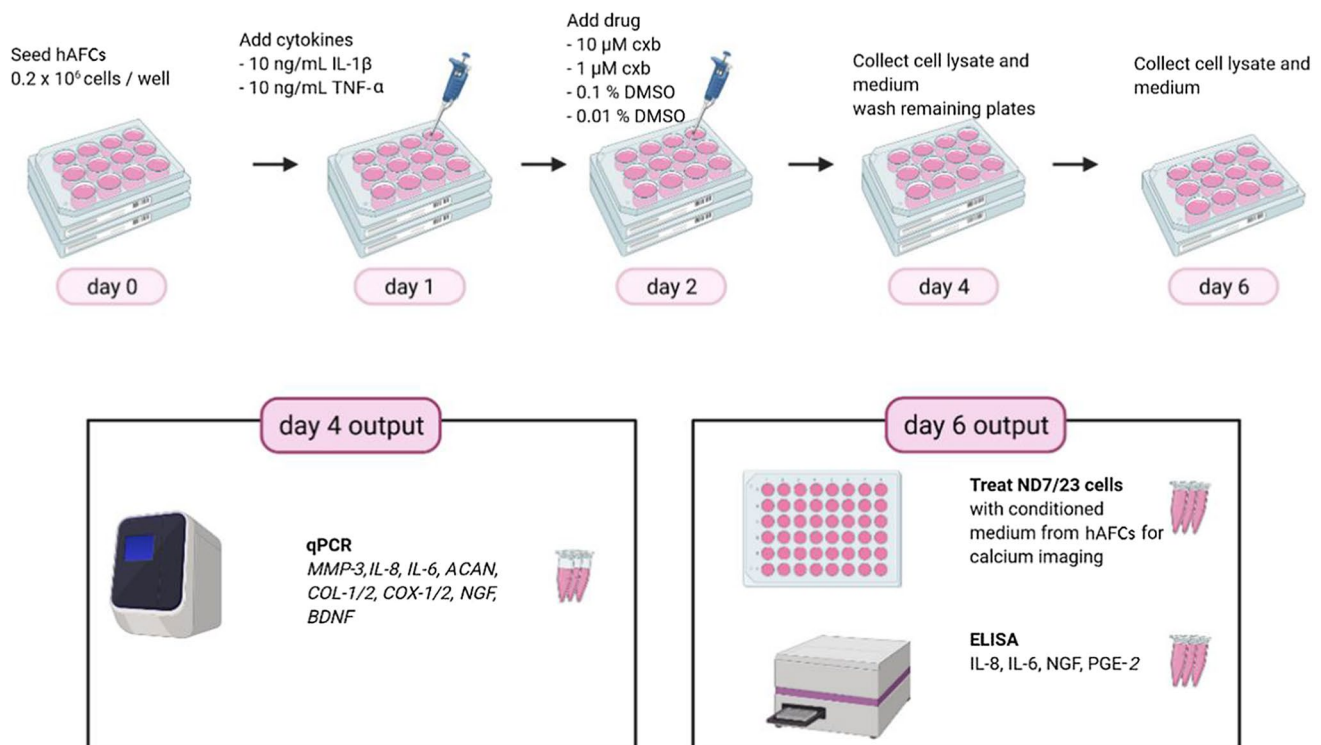


Fig. 1 Overview of the experimental design and timeline. hAFCs: human annulus fibrosus cells; cxb: celecoxib. ND7/23: rodent dorsal root ganglion cells. Figure created with Biorender

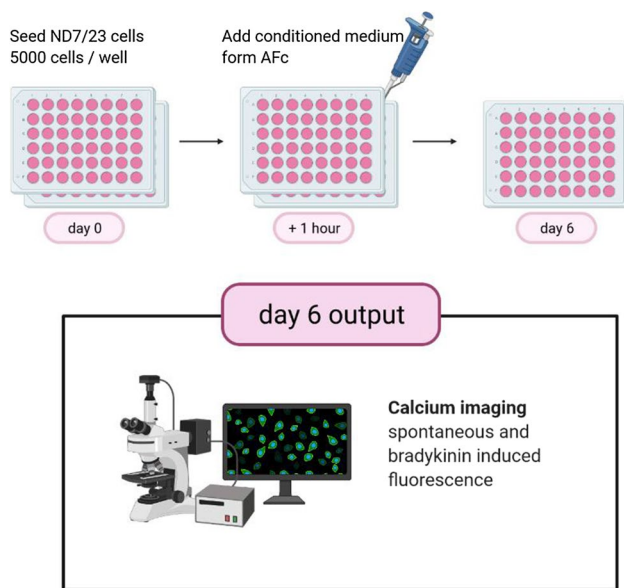


Fig. 2 Experimental setup for rodent dorsal root ganglion cells (ND7/23). Cells were seeded in 48-well plates. After one hour they were treated with conditioned medium and cultured for 6 days. Subsequently, calcium imaging was performed. Figure created with Biorender. AFCs: human annulus fibrosus cells

thresholding method. The fluorescence over time within each ROI was imported to R (R Studio version 1.3.959 and R version 3.6.2) and normalized by the baseline level. Peak heights of both 1–100 s and 100–200 s were compared among groups representing spontaneous and bradykinin-stimulated responses.

Bovine DRG calcium imaging

DRGs were obtained from two cattle (one male and one female, 10–12-month-old) at a local abattoir. The bovine DRG cell culture has been described formerly [16]. In brief, cells were dissociated using 3 h of digestion in 4 mg/mL collagenase P (Roche, Mannheim, DE) and mechanical trituration using a P1000 pipette tip. Undigested tissues were removed by passing through a 100 μ m cell strainer (Falcon, NY, USA) and density gradient centrifugation over 5 mL of 15% BSA in a 50 mL Falcon tube. Cells were resuspended in DMEM/F12 medium (50% v/v, DMEM from Gibco, UK, and F-12 Ham from Sigma, UK) supplemented with 1% penicillin/streptomycin, 20 mM HEPES (Thermo Fisher, Bleiswijk, NL), 1% ITS, and 10% FCS (Corning, CA, USA). Cells were seeded at the density of 5000 neurons/cm² in ' μ -Slide 18 Well ibiTreat' (Ibidi, DE). The DRG culture medium was changed to the respective CM after 48 h (disk CM from donors #1 and #2). The CM stimulation lasted for 3 days.

Calcium imaging was combined with immunofluorescence to evaluate the sensitization of nociceptors which were labeled by CGRP. Calcium imaging was performed as described for the ND7/23 cell line except for a potassium chloride (KCl 50 mM) stimulation at the end to identify living neurons that were immediately depolarized by KCl. Then, the neurons were fixed using 4% buffered formalin. The cells were double stained using rabbit anti-CGRP (1:1000, Immunostar, WI, USA) and mouse anti-neurofilament 200 (NF200, 1:100, Thermo scientific, NL) primary antibodies at 4 °C overnight. Afterward, the cells were incubated with the goat anti-mouse and donkey anti-rabbit secondary antibodies conjugated with AlexaFluor 488 and AlexaFluor 680, respectively (both 1:500, Thermo Fisher, OR USA), at room temperature for 1 h. The CGRP-labeled nociceptors were detected in the same field of view as the calcium images. This was achieved by moving the confocal stage with the same numbers of fields from the up and left corners of wells to the same location.

Statistical analysis

Quantification of the ELISA and calcium imaging data was performed using R (R Studio Version 1.3.959 and R version 3.6.2). Plots were created using GraphPad Prism (version 9.9.1) and the 'ggplot2' package in R. The technical replicates of respective donors were averaged to perform statistics. Two-way ANOVA was performed using the 'dplyr' package, and the Tukey Honest Significant Differences (Tukey HSD) method was used to perform multiple pairwise comparisons. Data in different groups are paired by donors. The normality of difference values was tested using the Shapiro–Wilk method.

Results

Celecoxib did not show cytotoxic effects on human annulus fibrosus cells

None of the tested cxb and DMSO concentrations showed significant toxicity to the hAFCs. Therefore, our experiments were performed with 1 μ M, 10 μ M cxb, 0.01%, and 0.1% DMSO (Fig. 3).

Celecoxib did not affect gene expression of annulus fibrosus cells in an inflammatory environment

Stimulation of hAFCs with TNF- α and IL-1 β led to a strong upregulation of IL-6 and IL-8, COX-2, and Matrix metalloproteinase 3 (MMP-3). This effect was more pronounced with IL-1 β compared to TNF- α . A slight upregulation of BDNF and NGF was observed but was not significant. By

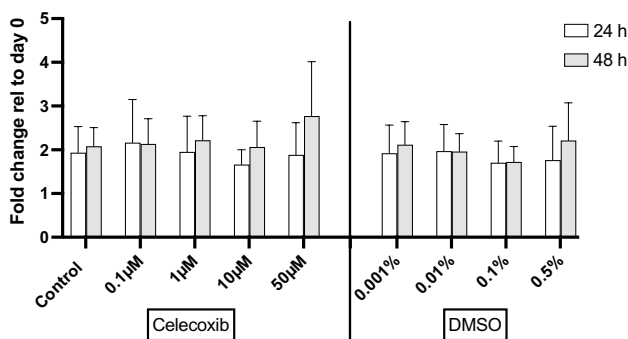


Fig. 3 Relative metabolic activity of human annulus fibrosus cells after 24 h and 48 h of celecoxib or dimethylsulfoxide (DMSO) exposure. Means + standard deviations are shown. Data are from two donors assessed in triplicates. The y-axis is the ratio of the recorded fluorescent of differently treated cells relative to the fluorescent recorded at day 0 of cell culture

treating cells with cxb, these effects could not be reversed (Online resource supplementary Fig. 1).

Celecoxib reversed the IL-1β-induced increase of prostaglandin E2

IL-1β significantly elevated IL-6 release, whereas the effect of TNF-α on IL-6 release was less significant. (Fig. 4A)

Both IL-1β and TNF-α increased IL-8 concentration in the hAFCs CM. (Fig. 4B) 10 µM cxb was not found to significantly influence the IL-6 and IL-8 levels (Fig. 4A-B).

PGE-2 level in the hAFCs CM was significantly increased by IL-1β stimulation. This upregulation of PEG-2 release was completely reversed by subsequent treatment of 10 µM cxb. NGF was not detected in the CM (lower than the detection range of the ELISA kit: 31–2000 pg/mL).

Calcium imaging

The effect of cxb on hAFCs-mediated nerve sensitization was tested in a model in that hAFCs were stimulated by IL-1β. TNF-α was not able to upregulate PGE-2 release and was thus not suitable to study the effect of cxb which targets the PEG-2 synthase. The nerve sensitization was evaluated using calcium imaging, because the transient increase of intracellular calcium in DRG nociceptors is associated with neuronal discharge and neurotransmitter release. Calcium signals in DRG nociceptors at least partly contribute to chronic pain mechanism, although pain is far more complex involving regulations from the central nervous system [17].

We found that spontaneous calcium peak frequency in DRG cells was elevated by the IL-1β stimulation of hAFCs in donors #1 and #3 but remained unchanged in donor #2 (Fig. 5A). The spontaneous calcium peak frequency was not

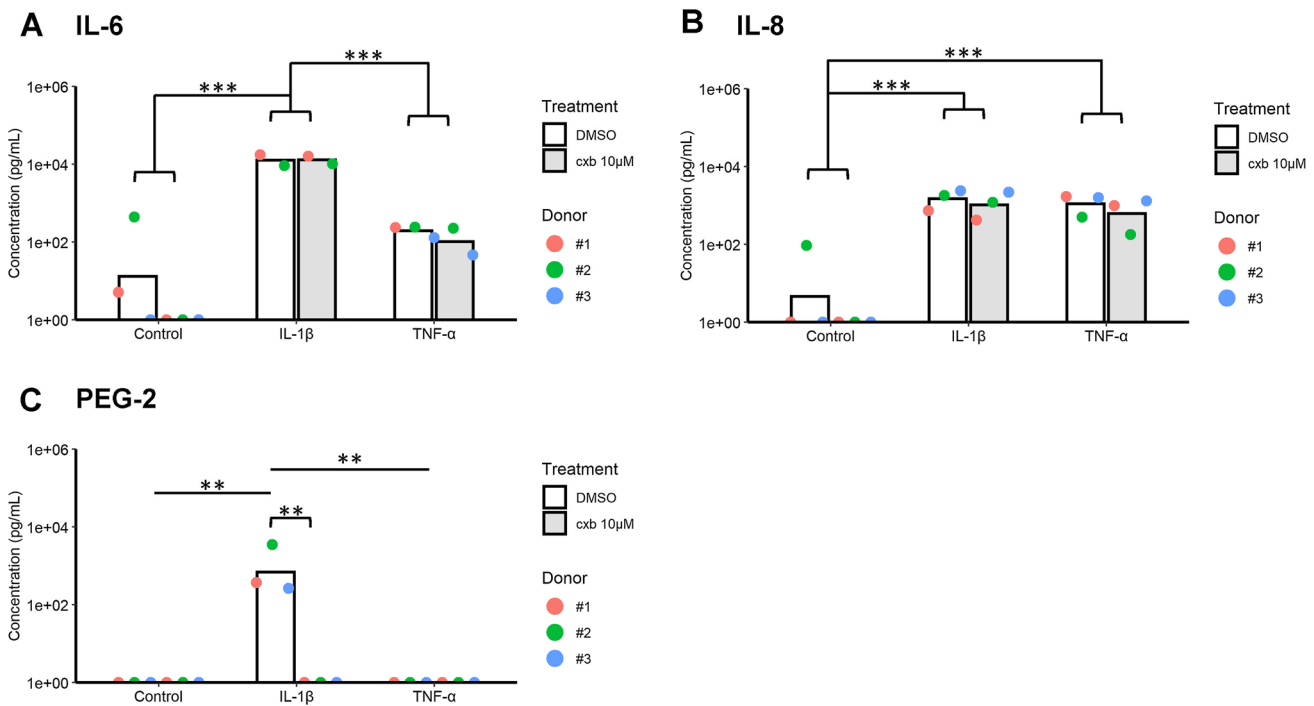


Fig. 4 Influence of celecoxib (cxb) on interleukin-6 (IL-6) (A), interleukin-8 (IL-8) (B), prostaglandine-E2 (PGE-2), and (C) levels in the conditioned media of human annulus fibrosus cells after 6 days

of culture. Data points represent the mean level of each donor. Bars show the mean values of the data points. n=3 donors and each donor have 2 technical replicates, **p*<0.05, ***p*<0.01, ***<0.001

found to be influenced by cxb treatment (Fig. 5B), and cxb alone did not change DRG cells' calcium signal without previous stimulation with IL-1 β (Fig. 5C).

Calcium response to bradykinin indicates a neuronal nociceptive response to inflammatory cytokines. Strikingly, the bradykinin-stimulated calcium response of DRG cells appeared slightly elevated by the CM of IL-1 β stimulated hAFCs (Fig. 6A–D and I). Following the IL-1 β treatment, cxb at a concentration of 10 μ M significantly reversed this effect in all the 3 donors as compared with carrier DMSO control (Fig. 6E–H and J). Cxb at a lower concentration (1 μ M) did not show a significant effect (Fig. 6J). Additionally, we investigated whether celecoxib alone could induce intracellular calcium flux changes in DRG cells. DRG cells treated with cxb were compared against a DMSO control group and a non-DMSO control group (consisting only of basal medium), without prior stimulation with IL-1 β . Our findings suggest that cxb alone did not elicit any significant changes in calcium signaling in DRG cells (Fig. 6K).

The effect of cxb was then tested in primary DRG cell culture from a large animal species (bovine) (Fig. 7A–D). Next, nociceptors were labeled by CGRP (Fig. 7D), and the sensitization of these nociceptors was evaluated by calcium imaging (Fig. 7A–C). The CGRP(+) nociceptors displayed lower spontaneous calcium response frequency (Fig. 7E and F), bradykinin-stimulated calcium peak duration (Fig. 7I and J) and peak height (Fig. 7K and L) in the cxb-treated cells compared with the DMSO controls. Spontaneous peak height in CGRP(+) nociceptors was only marginally decreased by cxb. For all the results, the 2 AF cell donors (#1 and #2) were showing the same trend. There was not enough CM from donor #3 to be tested in the primary bovine DRG model.

Discussion

Our study shows that cxb significantly decreased the secretion of PGE-2 in CM of IL-1 β -stimulated hAFCs. Cxb also alleviated the nociceptor sensitization induced by the CM of IL-1 β -stimulated hAFCs. Cxb is thus potential in targeting the IVD-nerve communication which contributes to discogenic pain.

Celecoxib's effect on IL-1 β -treated hAFCs

The findings of this study suggest that localized delivery of cxb could be effective. Local injection of cxb has been studied for osteoarthritis [17], and more recently, research has focused on controlled release methods, such as cxb-loaded in situ forming gel [18] and polyesteramide microspheres [11]. To apply these approaches in the IVD-nerve environment, it is important to understand the action of cxb. Our study used cell samples from patients with Pfirrmann grades II–III, as we aimed to focus on mild degeneration, which could benefit from cxb therapy. While this study is the first to investigate the effect of cxb-related signaling on human AF cells, previous research has explored the impact of cxb on human NP cells and in other species. In a canine model, cxb-loaded microspheres reduced PGE2 production by up to 76% without affecting GAG/DNA levels in the AF. Another recent study focused on the use of cxb in rat NP cells, which showed promising results in restoring extracellular matrix formation and minimizing oxidative stress by activating the mammalian target of rapamycin and downstream proteins to accelerate autophagy machinery. However, this effect has not been investigated in AF cells [11, 18, 19].

In terms of PEG-2's effect on nerve sensitization, former studies showed that PEG-2 induced a bradykinin-evoked

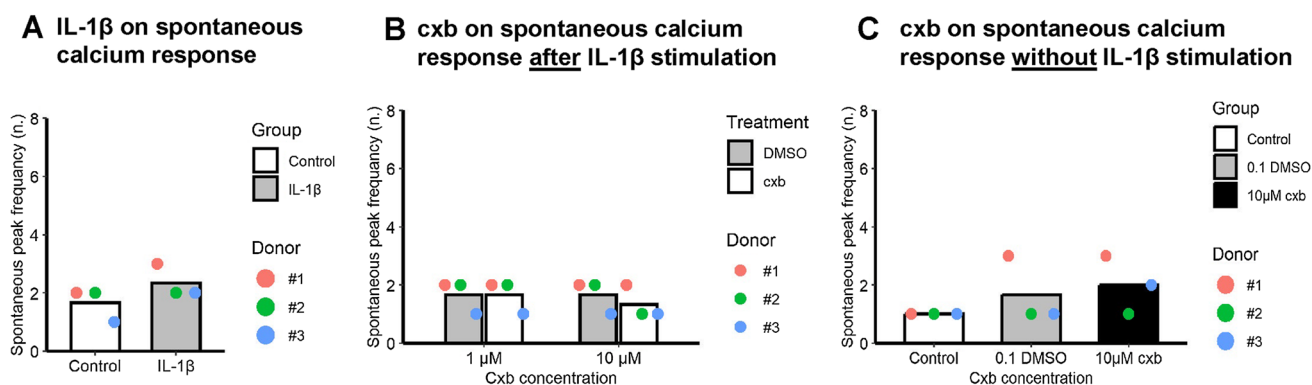


Fig. 5 Calcium imaging evaluating the influence of human annulus fibrosus cells conditioned medium on spontaneous calcium oscillation in rodent dorsal root ganglion cells (ND7/23 cells). **(A)** The effect of 10 ng/mL interleukin-1 β (IL-1 β) only. **(B)** The effect of

celecoxib treatment [1 and 10 μ M] after IL-1 β stimulation. **(C)** The effect of celecoxib treatment [1 and 10 μ M] without IL-1 β stimulation. Data points represent the respective median value of each annulus fibrosus cell donor. n = 3 donors with 2 technical replicates

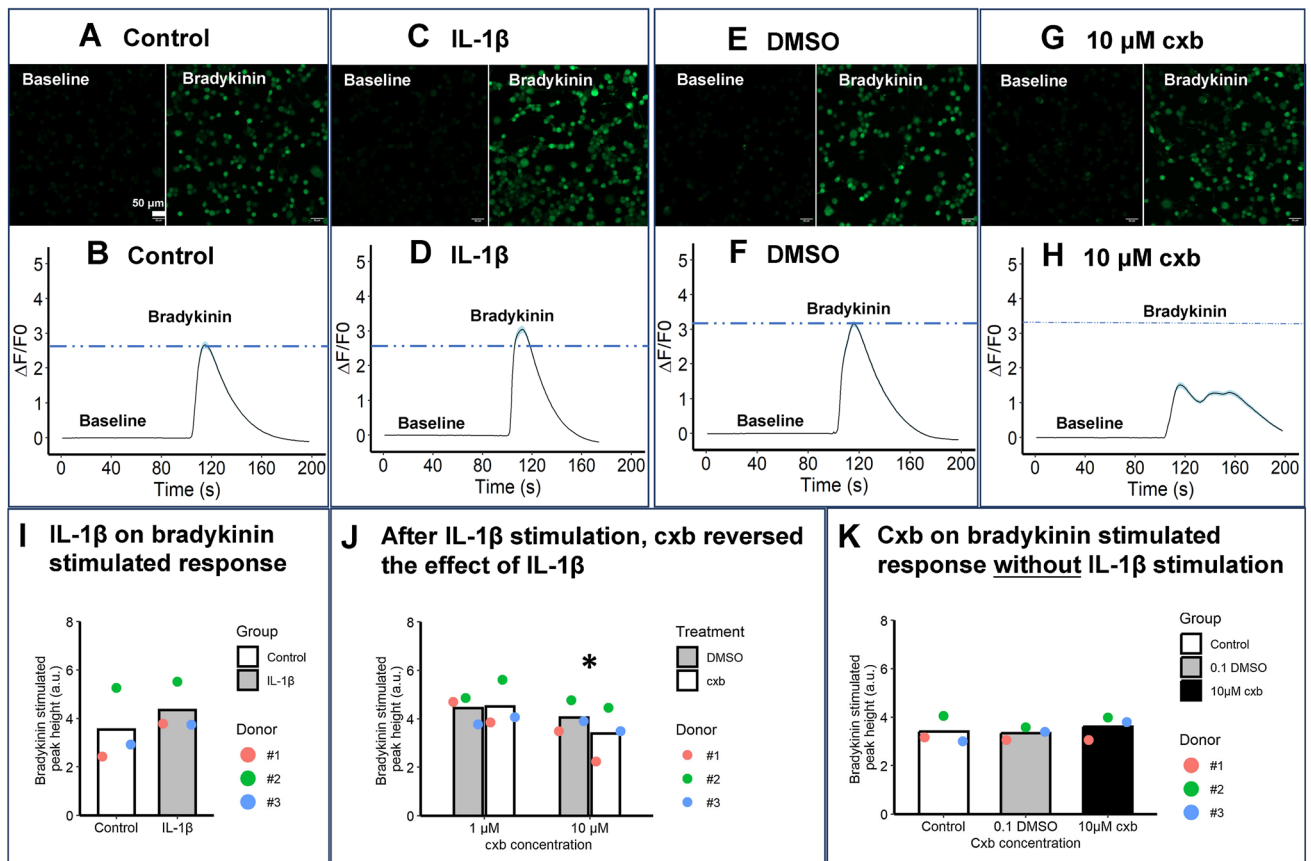


Fig. 6 Calcium imaging evaluating the influence of human annulus fibrosus cell conditioned media on dorsal root ganglion cell line's (ND7/23) response to bradykinin. **(A–H)** Representative images of the bradykinin-stimulated response. **(A), (C), (E), and (G)** show the fluorescent change of cells following bradykinin stimulation, and **(B), (D), (F), and (H)** are the averaged curves of normalized fluorescence over time. **(I)** hAFCs treated with 10 ng/mL interleukin-1 β (IL-1 β)

enhanced the bradykinin-stimulated response in dorsal root ganglion cells. **(J)** After IL-1 β treatment, 10 μ M celecoxib significantly decreased the bradykinin-stimulated response in dorsal root ganglion cells. **(K)** Effect of celecoxib only without prior IL-1 β stimulation. For I, J, and K, data points represent the median value of each annulus fibrosus cell donor. $n = 3$ donors with 2 technical replicates each, $*p < 0.05$

sensitization in neonatal rat DRG neurons through a PKA-phosphorylation mechanism [6]. A selective COX-2 inhibitor was formerly shown to inhibit pain-associated behavior in rats acting upstream to down-regulate IL-6 in DRG [20]. In our study, however, cxb did not inhibit IL-6 release in hAFCs. A recent study on COX-2 inhibition in NP cells also showed that IL-6 could not be downregulated by inhibiting COX-2 after induction with IL-1 β [21]. Similarly, our results showed that 10 μ M cxb did not significantly reverse the effects on IL-6 and IL-8 genes as well as protein expression following IL-1 β and TNF- α stimulation. However, PGE-2, which is the main target of cxb, was significantly reduced at the protein level.

Regarding the neurotrophins NGF and BDNF, studies showed that in vitro BDNF gene expression in cultured hAFCs has a significant positive correlation with IVD degeneration [3]. Also, it is known that NGF induces BDNF expression [2]. However, we could not detect BDNF and

NGF protein in CM of hAFCs under either unstimulated or pro-inflammatory cytokine-treated conditions. Agreeing with our results, former IVD histology studies localized these neurotrophins in regions of vessel ingrowth, that they are more likely to be produced by vessel cells, but not AF cells [3].

To control for donor variation, we performed statistical comparisons within different groups using the same donor. By using data from the same donor for all groups, we attempted to minimize the impact of donor variation on group differences. Nevertheless, we recognize that a larger sample size of human donors should be employed in future studies to enhance the generalizability of our findings.

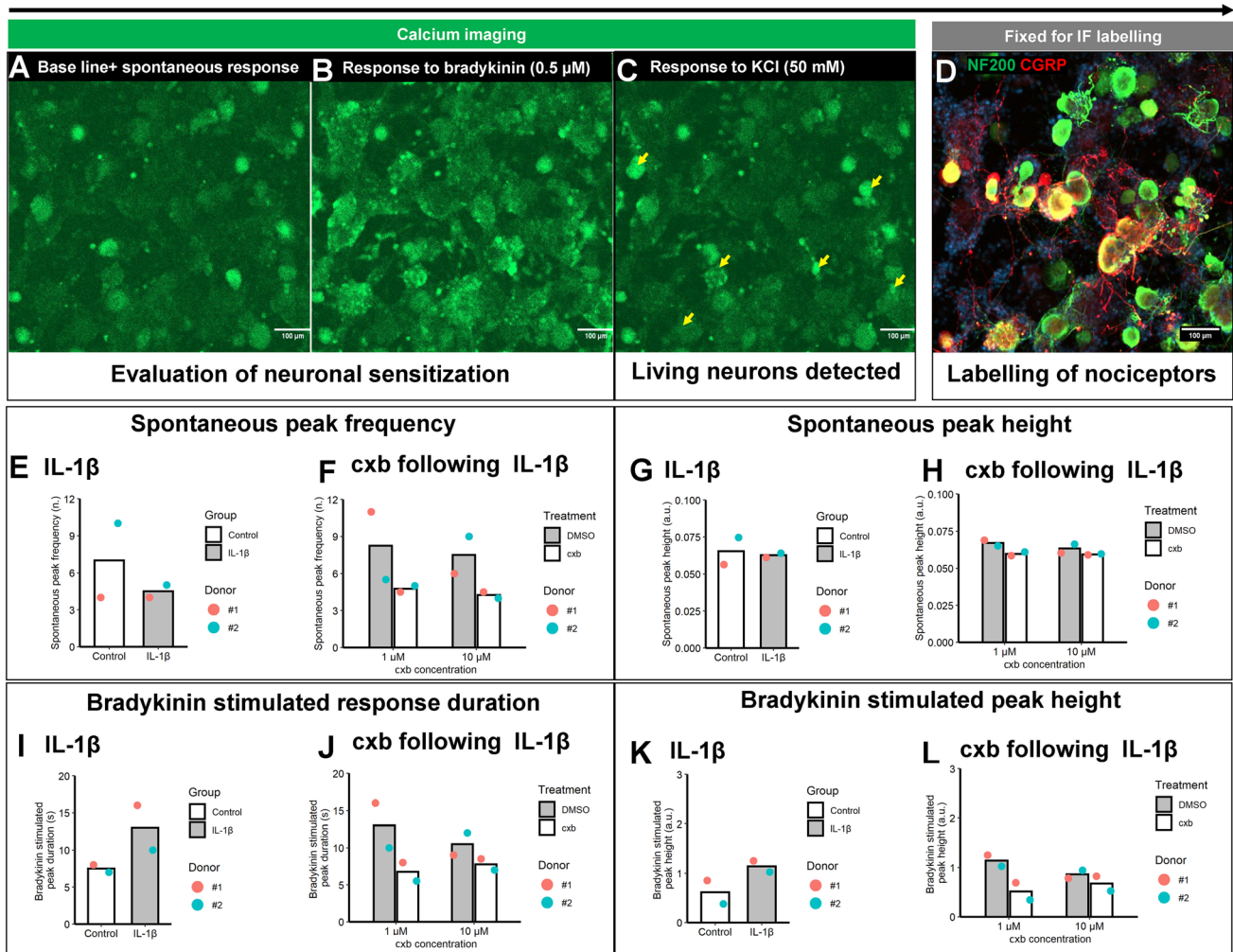


Fig. 7 Calcium imaging of CGRP(+) nociceptors. (**A–C**) Calcium imaging of primary bovine dorsal root ganglion at baseline, bradykinin stimulation, and potassium stimulation. (**D**) Nociceptors were labeled using immunostaining of CGRP from the same field of calcium imaging. (**E–H**) Neurons activate and produce calcium signals at baseline level spontaneously without any stimulation. Celecoxib

at both concentrations reduced spontaneous calcium peak frequencies. (**I–L**) Celecoxib at both concentrations reduced the duration and intensity of bradykinin-stimulated response in CGRP(+) nociceptors. For E–L, $n=2$ human donors of annulus fibrosus cells with 3 technical replicates each. Data points are the respective median levels of each annulus fibrosus cell donor

DRG cell culture used to investigate nociceptor sensitization

The rodent DRG cell line ND7/23 has previously been used as a model to study DRG nociceptor plasticity. This cell line displays higher spontaneous and bradykinin-induced intracellular calcium oscillation following hypoxic and low pH conditions suggesting a sensitization status following stress [15, 22]. In our results, the cell line model was comparable to the primary large animal DRG cell culture in terms of cxb's effect on the bradykinin-stimulated response, but the cell line is less sensible in detecting cxb's influence on spontaneous calcium response.

IL-1 β which was used to stimulate hAFCs, could also directly sensitize DRG neurons [23]. To exclude this effect,

a wash of hAFCs was performed before cxb treatment, and CM collection to remove manually added recombinant IL-1 β . Interestingly, TNF- α is associated with inflammatory IVD degeneration but is different from IL-1 β that did not increase PGE-2 release from the hAFCs [24].

Chronic pain is associated with elevated sensitivity of DRG neurons to multiple noxious stimuli. An increased response to bradykinin, a well-known pain mediator, may suggest an underlying mechanism associated with pain. The bradykinin-triggered calcium influx in DRG neurons is correlated with neuronal discharge and is necessary for neurotransmitter release responsible for pain transmission [17, 25].

The strength of the *in vitro* model is the potential to reduce animal models in line with 3R. The *in vitro* model

is cheap and is with higher throughput. The human AF and large animal DRG may add additional value when translating rodent model findings to human applications. We acknowledge the limitations of our study in using different species for the model, including human annulus fibrosus (AF) cells and rodent/bovine DRG cells. It is important to interpret the results with caution. Although using mouse and bovine DRG cells was necessary due to limited access to human DRG cells, PGE-2 is conserved among different species. Therefore, using AF and DRG cells from different species should not affect the results, at least in terms of PGE-2-dependent mechanisms of celecoxib's effects. However, we cannot exclude the possibility that celecoxib may function through an alternative mechanism that relies on the release of a protein in the human AF cell conditioned medium. In this case, a human protein produced by AF cells may still cross react with rodent/bovine DRG cells. According to a previous study, cytokines tend to cross-react only if they have a 60% or greater amino acid identity [26]. Furthermore, we did not analyze the effect of celecoxib on gene expression in DRG nociceptors in this study, but this has been explored by other research studies. For example, in a chronic constriction injury rat model, a single intravenous injection of a nanoemulsion containing celecoxib (0.24 mg/kg) reduced the expression of chemokines and cytokines in the DRG that are associated with macrophage recruitment, as determined by RNA sequencing [27]. Additionally, in another study using an in vitro model, celecoxib exhibited neuroprotective properties when applied to DRG cells, which were partly mediated through the upregulation of miR-155 antioxidant-associated genes [28]. Another significant limitation of our study is that the in vitro model findings require validation in an in vivo model to confirm their relevance and applicability to real-world scenarios. In addition, to gain a better understanding of the physiology of neurons, a more sophisticated evaluation of electrophysiology (such as patch-clamp techniques) should be employed in future studies.

Conclusion

Cxb has the potential to counteract the DRG cell sensitization induced by IL-1 β -stimulated hAFCs. These results could contribute to a better understanding of IVD-nerve communication in the context of inflammation.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00586-023-07672-x>.

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Authors' contributions All authors contributed to the study's conception and design. Material preparation, data collection and analysis were performed by JM, SH, SH, JE, JP, LMB, SH, and GL. The first draft of the manuscript was written by JM and SH. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no conflicts of interest to declare relevant to this article's content.

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