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C-type natriuretic peptide signalling drives homeostatic effects in human chondrocytes

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ABSTRACT

Signals induced by mechanical loading and C-type natriuretic peptide (CNP) represent chondroprotective routes that may potentially prevent osteoarthritis (OA). We examined whether CNP will reduce hyaluronan production and export via members of the multidrug resistance protein (MRP) and diminish pro-inflammatory effects in human chondrocytes. The presence of interleukin-1β (IL-1β) increased HA production and export via MRP5 that was reduced with CNP and/or loading. Treatment with IL-1β conditioned medium increased production of catabolic mediators and the response was reduced with the hyaluronan inhibitor, Pep-1. The induction of pro-inflammatory cytokines by the conditioned medium was reduced by CNP and/or Pep-1, αCD44 or αTLR4 in a cytokine-dependent manner, suggesting that the CNP pathway is protective and should be exploited further.

1. Introduction

The importance of the C-type natriuretic peptide (CNP) pathway is evident in regulating joint homeostasis [1–3]. In chondrocytes, CNP binds to natriuretic peptide receptor (Npr) 2 leading to synthesis of cyclic guanosine-3′,5′-monophosphate (cGMP) and homeostasis. CNP also binds to Npr3 that degrades the peptide and reduces beneficial signalling via Npr2 [4]. CNP treatment of cell lines, monolayers or pellet culture increased chondrocyte differentiation, proliferation and matrix synthesis, mediated by Npr2/cGMP [5–9]. In the chondrocyte/agarose model, the protective effects of CNP were enhanced by mechanical loading [10,11]. Taken together, the evidence indicates that the effects of CNP are mediated via Npr2 and its antagonism inhibits the protective actions of CNP in cartilage.

We previously reported that CNP or mechanical loading upregulates intracellular and extracellular levels of cGMP [11], but the way in which it is exported in chondrocytes is unclear. In fibroblasts, the multidrug resistance protein 5 (MRP5) was reported to export both HA and cGMP [12,13]. However, agents designed to inhibit HA export by increasing cGMP levels prevent proteoglycan and collagen loss in OA affected cartilage explants [14,15]. Since we reported that cGMP levels were also influenced by CNP, IL-1β and mechanical loading [11], the action of cGMP on preventing HA export could potentially be chondroprotective and might reduce the downstream actions of HA signalling and its breakdown. However, HA fragmentation is influenced by mechanical loading or the presence of hyaluronidases or free radicals typically found in the OA joint [16–18]. It was reported that the HA binding receptors, CD44 or Toll-Like Receptor 4 (TLR4) promotes inflammatory effects induced by hyaluronidase treatment [19–24]. We hypothesised that induction of cGMP by CNP and mechanical loading will reduce HA production and export that prevents the catabolic effects induced by HA fragmentation. These impacts may alter both quantity and size distribution of HA, and play an important role in balancing the protective and inflammatory effects mediated by CNP and HA fragmentation.

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2. Materials and methods

2.1. Cell isolation and 3D agarose culture

Human cartilage was obtained from sixteen patients with ethical approval (East London and The City Research Ethics Committee) and informed patient consent, undergoing total knee arthroplasty at the Royal London Hospital, Barts Health NHS Trust, London, UK. Cartilage was removed from the femoral condyles and tibial plateaux with experiments repeated with cells from 3 to 4 donors. Chondrocytes were isolated from tissue explants and resuspended in DMEM +20% FCS at a cell concentration of 8×10^6 cells/ml using well-established protocols [10,11]. The cell suspension was added to an equal volume of molten 6% (wt/vol) agarose type VII in Earle Balanced Salt Solutions (EBSS) to yield a final cell concentration of 4×10^6 cells/ml in 3% (wt/vol) agarose (Sigma–Aldrich, Poole, UK). The chondrocyte/agarose suspension was allowed to gel at 4 °C for 20 min in a sterile stainless steel mould, containing holes 5 mm in diameter and 5 mm in height. Constructs were equilibrated in culture in DMEM +10% FCS at 37 °C in 5% CO_2 for 24 h (Sigma–Aldrich, Poole, UK).

2.2. Effect of agents that interfere with the MRP/Npr pathway

The effect of IL-1β and CNP on HA production was investigated in free-swelling culture. Equilibrated constructs were cultured with 0 or 10 ng/mL IL-1β and/or 100 nM CNP or 1 μM MK571 (inhibits MRP5), 0.5 μM cyclic gly-24-ser (P19, selective Npr2 antagonist, Gentaur Molecular Products, Whetsone, UK) or 1 μM c-Atrial Natriuretic Factor ($cANF^{4\text{-}23}$, selective Npr3 agonist, Bachem AG, Bubendorf, Switzerland) [11]. In separate experiments, constructs were subjected to 15% dynamic compression at 1 Hz frequency (10 min every 6 h) using well-established protocols [10,11] for 48 h. Media was supplemented with 0 or 10 ng/ml IL-1β and/or 100 nM CNP and/or 0.5 μM P19 and/or 1 μM $cANF^{4\text{-}23}$. Controls were unstrained in the bioreactor and cultured for the same time period.

2.3. The effect of conditioned media and agents that influence the HA pathway

The role of HA and its fragments on promoting inflammatory effects was investigated with conditioned medium, and/or specific pharmacological agents that inhibit downstream effects on two HA receptors, CD44 and TLR4. Constructs were cultured with 0 or 10 ng/ml IL-1β and/or 100 nM CNP for 48 h 300 μL of the supernatant was used as conditioned medium to treat patient-matched constructs for the following conditions:

- Untreated conditioned media (UT^{CM})
- CNP conditioned media (CNP^{CM})
- IL-1β conditioned media ($IL\text{-}1\beta^{CM}$)
- IL-1β + CNP conditioned media ($IL\text{-}1\beta + CNP^{CM}$)

Conditioned medium was supplemented with HA binding peptide Pep-1 (30 μM, AnaSpec), which blocks binding of small HA fragments and prevents downstream signalling [25,26]. For analysis of the HA binding mechanism, conditioned media was treated with specific antibodies that blocked HA binding to CD44 (clone Bu52, AbDSerotec, Kidlington, UK) or TLR4 (anti-CD284 clone HTA125, AbDSerotec, Kidlington, UK). Both antibodies were used at 2 μg/mL and were shows to block activity [26,27]. Constructs were cultured with conditioned media with P19 and cANF for a further 48 h.

2.4. Gene expression analysis

Total RNA was isolated with the QIAquick Spin gel extraction and RNeasy kits (Qiagen, West Sussex, UK), reverse transcribed (200 ng) with Enhanced Avian RT First Strand cDNA synthesis kit (Sigma Genosys, Cambridge, UK) and real-time PCR reactions performed on the Mx3000P quantitative PCR instrument (Stratagene, Amsterdam, The Netherlands) using well established protocols [28]. The following specific primer sequences were used: MRP2 sense: 5′CGTTGTTGCCATCTTAGG-3′, antisense: 5′-CAAACATCATTGCTGGGTAA-3′; MRP4 sense: 5′-GCAGTTCTAATCATTCTC-3′, antisense: 5′-AAATCTCCTTCTTTCTCA-3′; MRP5 sense: 5′-CTTGTCCTGGAAGATGTT-3′, antisense: 5′-GAAGATGTCA TTCACTAGC-3′ (Sigma Genosys, Cambridge, UK). The real-time PCR efficiencies (E) of amplification for each target were defined according to the relation, $E = 10^{[-1/slope]}$, and revealed efficiency values ranging from 1.94 to 2.03 for optimal primer pairs concentrations (0.3 μM) derived from standard curves ($n = 3$). The C_t values for GAPDH remained stable, with no changes detected under all culture conditions, suggesting its suitability as a reference gene. Relative quantification of MRPs were normalized to the target ΔCt and reference GAPDH ΔCt, and to the calibrator sample by a comparative C_t approach, as described [28].

2.5. Biochemical analysis

NO, PGE_2 and HA production were determined in supernatant by Griess, EIA (GE Healthcare, Buckinghamshire, UK) or ELISA (R&D Systems, UK), using well established methods [10,11]. Total MMP activity was measured with fluorogenic MMP substrate at excitation and emission values of 340 and 440 nm, respectively (Enzo Life Sciences, Exeter, UK). GAG synthesis was measured by DMMB assay and normalised to DNA values using Hoescht 33258. Cytokines were measured with Human Th1/Th2 10-plex tissue culture MSD plates according to manufacturer's instructions (Meso Scale Discovery, Rockville, USA).

2.6. Statistics

Statistical analysis was performed by a two-way analysis of variance (ANOVA) and the multiple post hoc Bonferroni-corrected t-tests to compare differences between the various treatment groups as indicated in the figure legend. For gene-expression data, ratio values were log transformed before analysis by a two-way ANOVA and a *post hoc* Bonferroni-corrected *t* test. In all cases, a level of 5% was considered statistically significant ($p < 0.05$).

3. Results

3.1. MRP transporters are regulated by IL-1β and CNP

MRP5 gene expression was reduced with CNP when compared to untreated controls ($p < 0.05$, Fig. 1A). IL-1β enhanced MRP5 ($p < 0.001$) but was reduced with CNP ($p < 0.01$). In the absence of IL-1β, the pattern of MRP2 and MRP4 gene expression was similar to MRP5, with a significant downregulation with CNP when compared to untreated controls (both $p < 0.001$). In the presence of IL-1β, MRP2 and MRP4 expression was increased (both $p < 0.05$) but reduced with CNP (Fig. 1B and C).

3.2. IL-1β increased HA and the response was inhibited by CNP and/or dynamic compression

MRP5 mediates export of cGMP, but is also a HA exporter, an activity inhibited by cGMP. We therefore considered whether the