Osteoarthritis and Cartilage

Activation of TRPV4 by mechanical, osmotic or pharmaceutical stimulation is anti-inflammatory blocking IL-1β mediated articular cartilage matrix destruction
--Manuscript Draft--

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Abstract:	Objective: Cartilage health is maintained in response to a range of mechanical stimuli including compressive, shear and tensile strains and associated alterations in osmolality. The osmotic-sensitive ion channel Transient Receptor Potential Vanilloid 4 (TRPV4) is required for mechanotransduction. Mechanical stimuli inhibit interleukin-1β (IL-1β) mediated inflammatory signalling, however the mechanism is unclear. This study aims to clarify the role of TRPV4 in this response. Design: TRPV4 activity was modulated (GSK205 antagonist or GSK1016790A (GSK101) agonist) in articular chondrocytes and cartilage explants in the presence or absence of IL-1β, mechanical (10% cyclic tensile strain (CTS), 0.33Hz, 24hrs) or osmotic loading (200mOsm, 24hrs). Nitric oxide (NO), prostaglandin E$_2$ (PGE$_2$) and sulphated glycosaminoglycan (sGAG) release and cartilage biomechanics were analysed. Alterations in post-translational tubulin modifications and primary cilia length regulation were examined. Results : In isolated chondrocytes, mechanical loading inhibited IL-1β mediated NO and PGE$_2$ release. This response was inhibited by GSK205. Similarly, osmotic loading was anti-inflammatory in cells and explants, this response was abrogated by TRPV4 inhibition. In explants, GSK101 inhibited IL-1β mediated NO release and prevented cartilage degradation and loss of mechanical properties. Upon activation, TRPV4 cilia localisation was increased resulting in HDAC6-dependent modulation of soluble tubulin and altered cilia length regulation. Conclusion: Mechanical, osmotic or pharmaceutical activation of TRPV4 regulates HDAC6-dependent modulation of ciliary tubulin and is anti-inflammatory. This study reveals for the first time, the potential of TRPV4 manipulation as a novel therapeutic mechanism to supress pro-inflammatory signalling and cartilage degradation.

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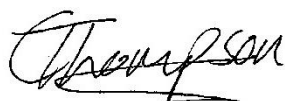
Re: Article resubmission OAC10500

To Joel A Block,

We would like to thank both yourself and the reviewers once again for their careful review of our research article and the constructive feedback they have provided. We have edited the manuscript for clarity and compliance with the word count, included further experimental detail in the methods section and made the corrections identified by reviewer 2.

As discussed via email, we have been unable to incorporate the suggested experimental validation of the TRPV4 antagonist/agonist used in this study within the required timeframe due to the current COVID situation and issues with lab access for our research staff. While we accept these data would add further rigor to our study, it is not expected to change our overall conclusions. The discussion section of the manuscript has been amended to acknowledge this and highlight numerous published studies validating the efficacy of these compounds in chondrocytes.

We hope that with these additional changes you will support publication of this article in Osteoarthritis & Cartilage.

We look forward to your response,

Yours sincerely,

Clare Thompson

Reviewer #2:

1. Although the authors describe that to clarify TRPV4 activation and inactivation by GSK101 and GSK205 is not necessary because these methods using the agonist and antagonist are well established in multiple cell types including chondrocytes, all figures in this paper are based on the activation and inactivation of TRPV4 by using GSK101 and GSK205, respectively. Therefore, the authors must show TRPV4 activation and inactivation by GSK101 and GSK205 as positive and negative control, respectively, using your isolated chondrocytes and cartilage implant.

Response:

Reviewer 2 has asked that we conduct a Ca^{2+} signalling analysis of isolated chondrocytes and cartilage explants in the presence of the TRPV4 agonist GSK1016790A (GSK101) and the antagonist (GSK205) in order to confirm the efficacy of these compounds in modulating channel activity in our hands.

Although our study does not demonstrate the efficacy of these compounds we do provide evidence that they are able to modulate the process of mechanotransduction which is itself dependent on TRPV4 Ca^{2+} channel activity (O'Conor, Leddy et al. 2014). While we accept that confirmation of the efficacy of the TRPV4 agonists/antagonists will add to the rigor of this study however the addition of this data would not change our overall conclusions as significant work demonstrating their specificity/efficacy has been conducted previously (see references below).

The experiments suggested are quite extensive to optimise and then conduct in both isolated cells and cartilage explants. Unfortunately we are unable to perform them in the timeframe offered due to the current covid situation and associated issues with lab access for the researchers on this study. Therefore we have added the additional statement below to the discussion to highlight that GSK205 and GSK101 modulation of Ca^{2+} signalling has not been analysed in this study but has been shown in numerous previous studies in chondrocytes.

Line 344:

'…Previous studies demonstrate that GSK101 activates Ca^{2+} signalling in isolated chondrocytes [17, 48, 56], while GSK205 inhibits this response and blocks Ca^{2+} signalling in response to mechanical or osmotic loading [18, 57, 58]. While Ca^{2+} signalling was not assessed in the current study, we hypothesise that elevated Ca^{2+} levels may regulate HDAC activity…'

Extensive evidence in the literature confirming the efficacy of GSK205 and GSK101 in chondrocytes as used in the present study:

- Demonstrated that 10 µM GSK205 inhibits ligand-gated activation of TRPV4 by ruthenium red or the TRPV4 agonist TRPV4 agonist 4αPDD in porcine articular chondrocytes (Phan, Leddy et al. 2009).
- Demonstrated activation with 5nM GSK101 by patch clamp electrophysiology could be diminished by 5 µM GSK205 inhibitor in porcine articular chondrocytes (Kanju, Chen et al. 2016).
- Demonstrated that in healthy human chondrocytes and chondrocytes isolated from patients with metatropic dysplasia 1 µM GSK1016790A activated Ca2+ signalling which could be blocked by 50 µM GSK205 (Hurd, Kirwin et al. 2015).
- Demonstrated 100nM GSK101 activated Ca2+ signalling in isolate murine chondrocytes (Servin-Vences, Moroni et al. 2017).
- Demonstrated that 1 nM GSK101 or osmotic loading activated Ca2+ signalling which could be inhibited by 10 µM GSK205 in porcine articular chondrocytes (O'Conor, Leddy et al. 2014).

2. There is no explain in Fig. 2D.
    Response:
    The figure legend for Fig.2D reads:
    '(D) confocal microscopy of explants stained with Calcein-AM (live cells, green) and ethidium homodimer (dead cells, red).'

    We have edited the manuscript for clarity, line 188 now reads:
    'Chondrocyte viability was maintained throughout the experiment as determined by live/dead staining.'

    Furthermore, we have added further details of this analysis to the methods section, line 88:
    'For live imaging, cell viability was assessed by live/dead staining at the end of the experimental period. Explants were incubated for 45 min at 37°C with 5 µM Ethidium homodimer-1 (EthD-1) to label dead cells and 5 µM Calcein AM, a marker for live cells.'

3. Line 189 Fig. 3D ------- > Fig. 2D Is it OK?
    Response:
    We apologise for this error, we have altered the text to the correct figure reference.

4. Fig. 3B: GSK205 ------ > GSK101 Is it OK?
    Response:
    We apologise for this error, this has been corrected in the new figure.

Activation of TRPV4 by mechanical, osmotic or pharmaceutical stimulation is anti-inflammatory blocking IL-1β mediated articular cartilage matrix destruction

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Abstract

Objective: Cartilage health is maintained in response to a range of mechanical stimuli including compressive, shear and tensile strains and associated alterations in osmolality. The osmotic-sensitive ion channel Transient Receptor Potential Vanilloid 4 (TRPV4) is required for mechanotransduction. Mechanical stimuli inhibit interleukin-1β (IL-1β) mediated inflammatory signalling, however the mechanism is unclear. This study aims to clarify the role of TRPV4 in this response.

Design: TRPV4 activity was modulated (GSK205 antagonist or GSK1016790A (GSK101) agonist) in articular chondrocytes and cartilage explants in the presence or absence of IL-1β, mechanical (10% cyclic tensile strain (CTS), 0.33Hz, 24hrs) or osmotic loading (200mOsm, 24hrs). Nitric oxide (NO), prostaglandin E_2 (PGE_2) and sulphated glycosaminoglycan (sGAG) release and cartilage biomechanics were analysed. Alterations in post-translational tubulin modifications and primary cilia length regulation were examined.

Results: In isolated chondrocytes, mechanical loading inhibited IL-1β mediated NO and PGE_2 release. This response was inhibited by GSK205. Similarly, osmotic loading was anti-inflammatory in cells and explants, this response was abrogated by TRPV4 inhibition. In explants, GSK101 inhibited IL-1β mediated NO release and prevented cartilage degradation and loss of mechanical properties. Upon

activation, TRPV4 cilia localisation was increased resulting in HDAC6-dependent modulation of soluble tubulin and altered cilia length regulation.

Conclusion: Mechanical, osmotic or pharmaceutical activation of TRPV4 regulates HDAC6-dependent modulation of ciliary tubulin and is anti-inflammatory. This study reveals for the first time, the potential of TRPV4 manipulation as a novel therapeutic mechanism to supress pro-inflammatory signalling and cartilage degradation.

Key words

Running headline

TRPV4 activation suppresses inflammation

1 Introduction

2 Osteoarthritis (OA) effects over 4.4 million people in the UK alone representing significant

3 economic cost [1]. Cartilage health is maintained in response to mechanical stimuli, articular

4 cartilage is routinely exposed to a wide array of dynamic mechanical loading consisting of

5 compressive, shear and tensile strains as well as associated alterations in fluid shear and

6 osmolality [2]. Mechanical loading in the form of compression or tensile strain is anti-

7 inflammatory in chondrocytes and blocks the release of the pro-inflammatory mediator's

8 nitric oxide (NO) and prostaglandin E_2 (PGE_2) in response to interleukin-1β (IL-1β) [3-5].

9 Inflammatory signalling contributes to cartilage degradation in OA thus understanding the

10 link between mechanical loading and inflammation will have significant therapeutic impact.

11 The osmotic-sensitive Ca^{2+} ion channel Transient Receptor Potential Vanilloid 4 (TRPV4) is

12 highly expressed in articular chondrocytes and is activated by mechanical stimuli [6, 7].

13 TRPV4 is required for mechanotransduction in chondrocytes and other cells types [7-9]. It

14 mediates the regulation of pro-anabolic and anti-catabolic genes promoting matrix

15 production and accumulation in agarose-embedded chondrocytes [7, 9]. TRPV4 mutations

16 result in human skeletal dysplasia suggesting a role in bone development (for review see

17 [10]). Indeed, chondrocytes from TRPV4^{-/-} mice exhibit loss of osmosensitivity accompanied

18 by joint degeneration indicating a central role for this channel protein in maintaining joint

19 homeostasis [11, 12]. Pharmaceutical activation of TRPV4 inhibits NO release in response

20 to inflammatory cytokines suggesting a potential mechanistic role in the anti-inflammatory

21 effects of mechanical loading [13, 14]. However, in apparent contradiction of these findings

22 TRPV4 inhibition exerts an anti-inflammatory effect in the cardiovascular system, lung and

23 peripheral nervous system [15-17]. This study therefore aims to clarify the regulatory role of

24 TRPV4 in cartilage inflammatory signalling.

25 TRPV4 localises to the plasma membrane and primary cilium, a small microtubule based

26 signalling compartment present at the cell surface [18, 19]. Primary cilia have been

27   implicated in both chondrocyte mechanotransduction [20-22] and inflammatory signalling

28   [23-26]. The cytoplasmic tubulin deacetylase histone deacetylase 6 (HDAC6) is enriched

29   within the cilium and modulates cilia resorption through de-acetylation and polymerization of

30   ciliary tubulin [27-29]. Post translational modification of ciliary tubulin influences intraflagellar

31   transport (IFT), the microtubule based motility present within the cilium required for cilia-

32   mediated signalling [30, 31]. Previously we report that mechanical loading counteracts

33   inflammatory signalling in response to the pro-inflammatory cytokine interleukin 1β (IL-1β)

34   via HDAC6 activation in association with alterations in IFT/cilia [5]. A role for TRPV4 in this

35   pathway has not previously been identified.

36   In the present study, we demonstrate for the first time that TRPV4 activation by cyclic tensile

37   strain, hypo-osmotic challenge or the TRPV4 agonist GSK1016790A inhibits pro-

38   inflammatory IL-1β signalling and cartilage degradation associated with alterations in primary

39   cilia elongation. TRPV4 may therefore provide a novel target for the treatment of joint

40   disease and other inflammatory pathologies.

41   **Methods**

42   **Antibodies and reagents**

43   Chondrocytes were treated with interleukin-1β (IL-1β, 200-01B; Peprotech, London, UK),

44   TRPV4 antagonist GSK205 (616522; Merck Millipore, London, UK) and agonist

45   GSK1016790A (GSK101, G0798; Sigma Aldrich, Poole, UK). Antibodies for

46   immunocytochemistry: acetylated α-tubulin (1:2000, T7451, Sigma Aldrich, Poole, UK) and

47   TRPV4 (1:200, SAB2104243, Sigma Aldrich). Nuclei were counterstained with 4′,6-

48   diamidino-2-phenylindole (DAPI, Sigma Aldrich). Antibodies for western blotting: acetylated

49   α-tubulin (1:1000, T7451, Sigma Aldrich) and α-tubulin (1:1000, ab4074, Abcam, Cambridge,

50   UK).

51   **Cartilage explant and chondrocyte culture**

52   Bovine cartilage explants and chondrocytes were obtained from 16 month steers as

53   previously described [28]. Full depth articular cartilage was removed from the proximal

54   surface of the metacarpal phalangeal joint and chondrocytes isolated by enzymatic

55   digestion. Explants were harvested using a 5 mm diameter biopsy punch (BP-50F, Selles

56   Medical Ltd, UK). Both were cultured at 37 °C, 5% CO_2 in Dulbeccos Minimal Essential

57   Medial (DMEM, D5921, Sigma-Aldrich, Poole, UK) supplemented with 10% (v/v) foetal calf

58   serum (FCS, F7524, Gibco, Paisley, UK), 1.9 mM L-glutamine (G7513), 96 U/ml penicillin

59   (P4333, All Sigma-Aldrich, Poole, UK). Explants were rested for 2 d prior to experimentation

60   while isolated chondrocytes were cultured to confluence.

61   **Application of cyclic tensile strain**

62   Isolated chondrocytes were cultured on collagen coated flexible elastomeric membranes and

63   subjected to uniform, equibiaxial cyclic tensile strain (CTS) applied using the Flexcell 5000T

64   system (Dunn Labortechnik GMbH).Cells were subjected to 0-10% strain for 24 h at 0.33 Hz.

65   **Application of osmotic loading**

66   Isolated cells were cultured for 24 h without serum in osmotically adjusted media at 200, 315

67   or 400 mOsm, hereafter referred as hypo-, iso- or hyper- osmotic media respectively.

68   Explants were cultured for up to 12 d under similar conditions with the addition of serum to

69   maintain chondrocyte viability resulting in a slightly higher osmolarity of 318 mOsm for the

70   iso-osmotic media. The osmolarity of all solutions was adjusted by adding D-mannitol

71   (M4125, Sigma-Aldrich) or distilled water and measured using a freezing point depression

72   osmometer.

73   **Biochemical analysis of NO, PGE$_2$ and sGAG release**

74   Nitric Oxide (NO) release was assessed using the Griess assay based on quantification of

75   nitrite (NO_2), the stable product of NO degradation. Nitrite content was quantified against a

76   sodium nitrite standard curve using the Galaxy Fluorstar spectrophotometer (BMG Labtech,

77   UK). An immunoassay kit (KGE004B, R&D Systems, UK) was used to quantify PGE_2

78   concentrations in the media according to the manufacturer's instructions. Results were

79   corrected for non-specific binding and calibrated using a PGE_2 standard curve. The release

80   of sGAG into the culture media was quantified using the dimethylmethyleneblue (DMMB)

81   assay against a chondroitin sulphate standard curve (6-sulphate:4-sulphate; 0.33:1; Sigma–

82   Aldrich).

83   **Immunocytochemistry, live imaging and confocal microscopy**

84   For immunocytochemistry, samples were fixed with 4% paraformaldehyde for 10 min,

85   permeabilised for 5 min with 0.5% triton-X100/phosphate buffered saline (PBS) then blocked

86   with 5% goat serum/PBS for 1 h. Primary antibody was incubated at 4°C overnight followed

87   by appropriate Alexa Fluor conjugated secondary antibodies (Molecular Probes) for 1 h at

88   room temperature. Cells were counterstained with 1 µg/ml DAPI for 5 min. For live imaging,

89   cell viability was assessed by live/dead staining. Explants were incubated for 30 min with 5

90   µM Calcein AM and 5 µM Ethidium homodimer-1 (EthD-1) prepared in appropriate osmotic

91   adjusted media, washed and immediately imaged. Samples were imaged using a Zeiss 710

92   ELYRA PS.1 microscope. For cilia analysis, samples were imaged using an x63/1.4 NA

93   objective to generate confocal z-sections made throughout the cell depth (approximately 20

94   sections) using 0.25 µm step size with an image format of 1024 x 1024 yielding a pixel size

95   of 0.13 x 0.13 µm (image size approximately 135 x 135 µm). Cilia length and prevalence was

96   quantified from resulting maximum projection images using Image J software (National

97   Institutes of Health, Maryland, USA).

98   **Western blotting**

99   Cells were lysed in RIPA buffer (R0278, Sigma Aldrich) and total protein quantified by

100   Bicinchoninic acid (BCA) assay. For the fractionation of soluble and polymerized tubulin,

101   extraction buffer A (137 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, and 10% glycerol) was

102   added to cells at 4 °C for 3 min, plates were gently swirled and the buffer removed and

103   saved as the soluble tubulin fraction. Immediately after, extraction buffer B (buffer A + 1%

104   SDS) was added for 1 min, the remaining sample was scraped, incubated on ice for 30 min

105   and saved as the polymerized tubulin fraction.

106   SDS–PAGE was performed under reducing conditions and proteins transferred to

107   nitrocellulose membranes. Membranes were blocked in odyssey blocking buffer (Li-Cor

108   Cambridge, UK) prior to overnight incubation with primary antibodies and infrared secondary

109   antibodies (Li-Cor). Proteins were visualized using the Li-Cor Odyssey and quantified using

110   Image Studio Lite software (Li-Cor).

111   **HDAC6 activity measurement**

112   A commercial fluorometric assay kit (K466-100, Biovision) was used to measure HDAC6

113   activity according to the manufacturer's instructions. This assay determines enzyme activity

114   by exploiting the selectivity of tubacin for HDAC6 in combination with a fluorescent synthetic

115   acetylated-peptide substrate. Cultures were lysed, a 10 µl aliquot was mixed with either

116   acetylated substrate (sample) or with 2 µM tubacin and acetylated substrate (inhibitor

117   control) then incubated for 30 min at 37°C. The deacetylase-dependent release of a 7-

118   amino-4-trifluoromethylcoumarin fluorophore (excitation/emission at 350/490 nm) was then

119   measured on a Galaxy Fluorstar spectrophotometer (BMG Labtech, UK) and HDAC6 activity

120   calculated as [sample-inhibitor control].

121   **Mechanical testing of cartilage explants**

122   The mechanical behaviour of individual cartilage explants was measured using an MTS,

123   Bionix 100. A 2 mm diameter core was cut from the centre of each 5 mm diameter cartilage

124   explant and a tare load of 0.01 N applied to each explant which was then hydrated in culture

125   media. The explants were subjected to a 20% uniaxial unconfined compressive strain (20%

126   /min). This was followed by a stress relaxation period at constant 20% strain in which the

127   load was recorded for a further 300 s. The load was recorded throughout the test at a

128 sampling frequency of 60 Hz. Stress–strain and stress–time curves were generated for each

129 specimen and the following mechanical properties of the cartilage determined:

130 Tangent Modulus (MPa) = $\dfrac{\sigma_{\varepsilon=0.2} - \sigma_{\varepsilon=0.18}}{0.02}$

131 Relaxation Modulus (MPa) = $\dfrac{\sigma_{t=300s}}{0.2}$

132 Percentage Relaxation (%) = $\dfrac{\sigma_{t=0s} - \sigma_{t=300s}}{\sigma_{t=0s}}$

133 The relaxation half-life was calculated as the time from the start of the relaxation phase until

134 stress reduced to half the peak value.

135 **Statistical analyses**

136 The sample size for each experiment was chosen based on previous studies [5, 32] where

137 analysis of cartilage degradation by biochemistry, immunohistochemistry and mechanical

138 testing demonstrated that 6-8 samples/group is sufficient to detect a 25% difference in

139 cartilage matrix catabolism at 80% power and a significance of $p<0.05$. Data analysis was

140 conducted using GraphPad Prism version 8 (GraphPad software, La Jolla California USA,

141 www.graphpad.com). Normality testing (Kolmogorov Smirnov test) was performed to confirm

142 that data exhibited Gaussian distribution. For data sets that were not normally distributed,

143 namely cilia length data, Box Cox transformation ($\lambda=0.5$) was performed prior to statistical

144 analyses. Statistical significance was determined by T-Test, One-way, Two-way or Three-

145 way ANOVA as appropriate with post-hoc Tukey's multiple comparisons performed to

146 identify significant differences between groups. Statistically significant differences were

147 determined based on a threshold of *= $p<0.05$, **=$p<0.01$ and ***=$p=0.001$. Data is

148 presented as mean ± standard deviation (SD) unless otherwise stated.

149   **Results**

150   **TRPV4 activation is required for the anti-inflammatory effects of mechanical loading**

151   **in isolated chondrocytes**

152   IL-1β treatment (24 h) resulted in significant, dose-dependent release of NO and PGE$_2$ (Fig.

153   1AB, S1AB). In response to 1 ng/ml IL-1β isolated chondrocytes exhibited a 3.04-fold

154   increase in nitrite levels indicative of NO release (Fig. 1A), and a 4.84-fold increase in PGE$_2$

155   release (Fig. 1B) which increased to 11.48- and 7.37-fold respectively in response to 10

156   ng/ml IL-1β (Fig. S1A-B). Consistent with previous studies [5] this response was significantly

157   reduced by mechanical loading in the form of CTS. IL-1β induced NO release was abolished

158   by CTS such that there was no statistically significant effect at either 1 or 10 ng/ml (Fig. 1A,

159   S1A). PGE$_2$ release was completely inhibited by CTS at 1 ng/ml IL-1β (Fig. 1B) but only

160   partially suppressed at 10 ng/ml (Fig. S1B).

161   Simultaneous treatment with the TRPV4 antagonist GSK205 (10 µM) abolished the anti-

162   inflammatory effects of mechanical loading (Figure 1, S1). While GSK205 had no effect on

163   NO or PGE$_2$ release in unloaded cells with or without IL-1β, in loaded cells the IL-1β

164   response was restored such that NO (Fig. 1A and S1A) and PGE$_2$ release (Fig. 1B, S1B)

165   were significantly increased by IL-1β. Neither IL-1β nor GSK205 treatment in the presence

166   or absence of CTS influenced TRPV4 protein levels (Fig. S10). These data indicate the anti-

167   inflammatory effects of mechanical loading are mediated by TRPV4 activation.

168   **TRPV4 activation is required for the anti-inflammatory effects of hypo-osmotic loading**

169   **in isolated chondrocytes and cartilage explants**

170   Isolated chondrocytes were treated with hyper-osmotic media (400 mOsm), hypo-osmotic

171   (200 mOsm) or iso-osmotic media (315 mOsm) for 24 h (Figure 2AB, S2). Hyper-osmotic

172   challenge had no significant effect on NO release, with or without IL-1β relative to the iso-

173   osmotic control (Fig. S2, S3). By contrast, hypo-osmotic challenge significantly attenuated

174 the pro-inflammatory response to IL-1β (1 ng/ml), such that the increase in NO release at 24

175 h was significantly reduced (p<0.001, Fig. 2A S2A). Hypo-osmotic challenge had no

176 apparent effect on cell viability compared to control conditions based on brightfield

177 microscopy (Figure 2B). In the presence of GSK205, the anti-inflammatory effect of hypo-

178 osmotic challenge on IL-1β induced NO release was completely inhibited by GSK205 such

179 that the induction of NO release was not significantly different to control conditions (Fig. 2A).

180 In the absence of IL-1β, GSK205 also had no effect on baseline NO or PGE_2 levels (Fig.

181 S2).

182 In cartilage explants, hypo-osmotic challenge significantly reduced IL-1β induced NO release

183 (Fig. 2C, p<0.001) such that there was no significant difference between IL-1β treated and

184 untreated explants (Fig. 2C, S4A). In line with these findings hypo-osmotic challenge

185 blocked the IL-1β mediated release of sGAG into the media, indicative of a reduction in

186 extracellular matrix degradation (Fig. S4B). Chondrocyte viability was maintained throughout

187 the experiment as determined by live/dead staining (Fig. 2D). Consistent with isolated cells,

188 hyper-osmotic challenge (400 mOsm, 12 d) had no effect on NO or sGAG release in the

189 presence or absence of 1 ng/ml IL-1β (Fig. S4). GSK205 treatment restored IL-1β-induced

190 NO release in hypo-osmotic media (Fig. 2C) thus blocking the anti-inflammatory effect of

191 osmotic challenge. Interestingly, GSK205 further increased IL-1β-induced NO release in iso-

192 osmotic, control media a response not seen in isolated cells (Fig. 2C). Together these data

193 indicate the anti-inflammatory effects of hypo-osmotic loading are also mediated by TRPV4

194 activation.

195 **TRPV4 activation is associated with altered primary cilia localisation and regulates**

196 **cilia length**

197 IL-1β induces primary cilia elongation in articular chondrocytes and mediates downstream

198 catabolic NF-κB signalling through regulation of IFT [5, 23, 24]. We therefore examined the

199 involvement of primary cilia in the anti-inflammatory mechanism of TRPV4 activation. TRPV4

200 cilia localisation was observed in isolated chondrocytes (Fig 3A). TRPV4 activation by

201 mechanical loading, hypo-osmotic challenge or the TRPV4 agonist GSK101 (1 nM)

202 increased TRPV4 cilia localisation while not significantly affecting protein expression, as

203 shown by the increased mean intensity of ciliary TRPV4 (Fig. 3B, S10) and altered

204 distribution profile of TRPV4 in proximal and distal regions of the axoneme (Fig. 3C) these

205 data are suggestive of alterations in IFT.

206 In isolated chondrocytes, IL-1β (1 ng/ml) treatment for 24 h induced a significant increase in

207 primary cilia length (p<0.001) from a median value of 2.21 to 2.84 μm. This elongation was

208 abolished by TRPV4 activation with GSK101 (Fig. 3D-E). IL-1β mediated cilia elongation

209 was also blocked by mechanical loading (CTS, 0-10%, 0.33 Hz, Fig. 3G) and hypo-osmotic

210 challenge (Fig 3H). Inhibition of TRPV4 with GSK205 restored IL-1β mediated cilia

211 elongation in the presence of both mechanical loading (p<0.001, Fig. 3G), and hypo-osmotic

212 challenge (p<0.001, Fig. 3H). GSK101, had no effect on cilia length in iso-osmotic conditions

213 with or without IL-1β (Fig. 3E). GSK101 also had no effect on cilia prevalence for any of the

214 treatment groups (Fig. S5A and D).

215 **TRPV4 activation inhibits inflammatory signalling in response to IL-1β through the**

216 **regulation of HDAC6 and ciliary tubulin**

217 We next examined whether direct pharmaceutical activation of TRPV4 would replicate the

218 anti-inflammatory effect of mechanical and osmotic loading. IL-1β (1 ng/ml) induced the

219 characteristic upregulation of NO and PGE$_2$ release in isolated chondrocytes which was

220 abolished by GSK101 (Fig. 4A and B). Similarly IL-1β induced COX2 expression was

221 abolished by GSK101 (Fig. 4C). No effects on cell viability based on bright field microscopy

222 (Fig. S6A) and DNA content were observed although cells appeared to have a more rounded

223 morphology particularly at high concentrations (Fig. S6B).

224 Previously, we identified a mechanistic role for HDAC6 activation and post-transcriptional

225 tubulin modifications in the anti-inflammatory effect of mechanical loading [5]. Similarly,

226   GSK101 resulted in significant upregulation of HDAC6 activity (Fig. 4D) suggesting TRPV4-

227   mediated calcium signalling activates HDAC6. Consistent with this finding we observed

228   significant tubulin deacetylation accompanied by a reduction in the pool of non-polymerized,

229   soluble tubulin (Fig. 4E-F). Furthermore, the HDAC6 specific inhibitor, tubacin (500 nM),

230   restored IL-1β mediated stimulation of NO release in GSK101-treated cells (Fig. 4G). These

231   data suggest that GSK101 mimics the effects of mechanical loading on IL-1β inflammatory

232   signalling, HDAC6 activation and tubulin modification.

233   **TRPV4 activation abolishes IL-1β mediated cartilage degradation and loss of**

234   **mechanical properties**

235   We next determined whether pharmaceutical activation of TRPV4 could prevent cartilage

236   degradation and loss of mechanical properties. Cartilage explants were treated with IL-1β for

237   up to 12 d in the presence of 1 nM or 10 nM GSK101. Cartilage explant viability was

238   maintained at these experimental doses (Fig. S7). In response to IL-1β treatment, significant

239   NO release was observed (Fig. 5A, $P < 0.001$) indicative of activation of inflammatory

240   signalling. This response was accompanied by significant sGAG release indicative of

241   cartilage degradation (Fig. 5B, $P < 0.001$).

242   We measured the viscoelastic properties of cartilage tissue using uniaxial unconfined

243   compression to determine whether GSK101 could prevent the loss of mechanical properties

244   induced by IL-1β. Cartilage explants exhibited a non-linear stress-strain graph represented

245   by a tangent modulus of 15-20MPa (Fig 5C). This was followed by characteristic viscoelastic

246   stress relaxation at 20% strain (Fig 5D) to a relaxation modulus of 2-3 MPa at 300 s

247   representing 80% relaxation and a relaxation half-life of approximately 50 s (Fig. 5E-H). IL-

248   1β treatment resulted in dramatic loss of mechanical stiffness as shown by significant

249   reductions in tangent modulus ($p < 0.001$, Fig. 5E) and relaxation modulus ($p < 0.001$, Fig. 5F),

250   increased percentage relaxation ($p < 0.001$, Fig. 5G) and a reduction in half-life ($p < 0.001$, Fig.

251   5H).

252 GSK10 significantly inhibited the cumulative release of NO from cartilage explants in

253 response to IL-1β treatment (p<0.001, Fig. 5A). Similarly the cumulative release of sGAG

254 was significantly reduced (p<0.001) and loss of mechanical properties in response to IL-1β

255 abolished, such that there was no significant difference in any of the biomechanical

256 parameters with and without IL-1β.

257 **Discussion**

258 This study demonstrates that TRPV4 plays an important mechanistic role in the anti-

259 inflammatory effect of mechanical stimulation. TRPV4 inhibition restores IL-1β mediated pro-

260 inflammatory signalling in the presence of both mechanical and osmotic loading. Conversely,

261 TRPV4 activation by GSK101 blocked the release of pro-inflammatory mediators in the

262 absence of load in isolated cells and prevented cartilage degradation and loss of mechanical

263 properties in an explant model. TRPV4 is activated by mechanical stimulation in the form of

264 cyclic tensile strain or osmotic challenge and functions upstream of HDAC6 to modulate

265 tubulin acetylation and polymerization which regulates IFT thereby suppressing IFT-

266 dependent IL-1β signalling.

267 TRPV4 is expressed in bone marrow stem cells, osteoblasts, osteoclasts and chondrocytes,

268 and is required for skeletal development [10, 33]. TRPV4 belongs to the Transient Receptor

269 Potential (TRP) superfamily which mediate cellular responses to a variety of environmental

270 stimuli including heat [34], cell swelling [35], hypo-osmolality [18, 36] and mechanical

271 loading [7, 9] and results in elevated levels of intracellular Ca^{2+}. Thus, TRPV4 is required for

272 mechanotransduction. It promotes chondrocyte matrix production in response to dynamic

273 compression [7], mediates the fluid shear induced osteogenic response in stem cells [9] and

274 shear stress induced vasodilatation in endothelial cells [8].

275 In other tissues, TRPV4 activation is mostly reported to be pro-inflammatory. In airway

276 epithelial cells, TRPV4 activates NF-κB signalling promoting progression of lung fibrosis [37].

277 Endogenous TRP channel agonists are detected in a lung injury model while TRPV4

278   inhibition suppresses acid-induced pulmonary inflammation [38]. TRPV4 antagonists have

279   been used to treat sepsis in mice by reducing production of TNF-α, IL-1 and IL-6 [16].

280   Moreover loss of TRPV4 function suppresses inflammatory fibrosis in mouse corneas [39].

281   However, Xu et al. report that GSK101 prevents vascular inflammation and atherosclerosis,

282   associated with inhibition of NO synthase and MAPK signalling [14]. TRPV4 is also well-

283   established to mediate inflammatory hyperalgesia (see review [40]) and is regarded as a

284   promising target for novel analgesics.

285   Consistent with our findings, pharmaceutical activation of TRPV4 has been shown to

286   suppress NO release induced by lipopolysaccharide (LPS) in rat temporomandibular

287   chondrocytes, whereas TRPV4 inhibition aggravates the inflammatory response to LPS

288   [13]. Clark et al. report that TRPV4 deficiency induces inflammation and disrupts cartilage

289   matrix homeostasis. As such, TRPV4$^{-/-}$ mice exhibit a severe sex-dependent osteoarthritis

290   (male mice are more susceptible) while the isolated chondrocytes fail to increase Ca^{2+} influx

291   in response to hypo-osmotic challenge [11]. These mice exhibit a more severe obesity-

292   induced osteoarthritis, compared to wild-type mice [12]. However other studies report

293   osmotic challenge to be a pro-inflammatory signal. Hubert et al observed induction of IL-8 in

294   response to both hyper and hypo-osmotic stress [41] while hypo-osmotic stimulation of

295   TRPV4 promoted PGE_2 release in porcine chondrocytes [18] and the expression of IL-1β

296   and IL-6 in bovine intervertebral disc cells [36], suggesting a pro-inflammatory role of

297   TRPV4. We did observe a mild, transient increase in NO release in this study at 3 h hypo-

298   osmotic challenge however this had resolved and was not significantly different to the control

299   at 24 h (Fig. S8). Interestingly we observed dose-dependent cytotoxicity of GSK101 with

300   extended explant culture at concentrations above 10nM (Fig. S7). Low concentrations of

301   GSK101 elicit multiple short peaks of Ca^{2+} signalling, which is more physiological compared

302   with the large, sustained peaks observed at higher concentrations which might explain this

303   [42]. These observations suggest perhaps that only moderate, short-term modulation of

304   TRPV4 will be chondroprotective.

305   Servin-Vences et al suggest TRPV4 mechanosensitivity is dependent upon the type of

306   stimulus applied [6]. Our data supports this hypothesis, complete abolition of NO release in

307   response to IL-1β was observed following application of cyclic tensile strain (Figure 1), while

308   hypo-osmotic challenge merely attenuated the response (Figure 2) suggesting TRPV4

309   activation may be regulated distinct mechanisms and to different extents. Vriens et al report

310   that TRPV4 activation in response to cell swelling is dependent upon arachidonic acid

311   release [43] whereas Servin-Vences et al suggest direct channel gating occurs in response

312   to membrane deflection [6].

313   The mechanosensitive ion channel PIEZO1 reportedly induces TRPV4 channel opening [44].

314   PIEZO1 is activated chondrocytes following injurious loading and is suggested to play a

315   greater role in chondroprotection than TRPV4 [6, 45]. It is possible the more pronounced

316   anti-inflammatory effects of cyclic tensile strain observed in this study are the result of further

317   TRPV4 activation downstream of this channel, which could be explored in future studies.

318   However, while activation of PIEZO1 reportedly influences ciliogenesis [46] studies in

319   osteocytes indicate that it does not interact with TRPV4 in the cilium [47].

320   TRPV4 cilia localisation was observed with greater localisation evident at to the ciliary base

321   (Fig. 3C). TRPV4 activation altered this distribution such that localisation to the base or tip of

322   the axoneme was not significantly different indicative of altered protein trafficking/IFT (Fig.

323   3C). TRPV4 activation is coupled with translocation of TRPV4 to plasma membrane [48], in

324   this study we observed increased TRPV4 labelling in the ciliary membrane (Fig. 3AB).

325   Chemical deletion of primary cilia with chloral hydrate fully abolishes Ca^{2+} signalling in

326   response to TRPV4 activation [18] thus increased ciliary TRPV4 may be important for

327   signalling.

328   HDAC6 is enriched within primary cilia catalysing tubulin de-acetylation and polymerization

329   to regulate cilia resorption [27-29]. In this study, mechanical, hypo-osmotic and

330   pharmaceutical activation of TRPV4 blocked cilia elongation in response to IL-1β. IFT and

331   cilia elongation is required for IL-1β mediated inflammatory signalling and downstream NF-

332   κB signalling [5, 23, 24], therefore we suggest the anti-inflammatory effects of TRPV4

333   activation regulate IFT and associated signalling via HDAC6 dependent modulation of ciliary

334   tubulin. Previous studies demonstrate that GSK101 activates Ca^{2+} signalling in isolated

335   chondrocytes [6, 17, 49], while GSK205 inhibits this response and blocks Ca^{2+} signalling in

336   response to mechanical or osmotic loading [18, 50, 51]. While Ca^{2+} signalling was not

337   assessed in the current study, we hypothesise that Ca^{2+} levels may regulate HDAC activity

338   through activation of upstream kinases such as Ca^{2+}/Calmodulin dependent kinase (CaMK),

339   protein kinase D (PKD) and Aurora A kinase-dependent (AURKA) [27-29, 52-55]. Studies

340   suggest TRPV4 stimulation with GSK101 promotes ERK/MAPK signalling in lung epithelial

341   cells and cancer cells [56] and PKC activity in endothelial cells [57] which phosphorylate

342   HDAC6 resulting in increased deacetylation activity [58, 59]. Indeed, increased HDAC6

343   activity was observed in response to GSK101 (Fig. 4D).

344   In conclusion, this study demonstrates a role for TRPV4 activation in the anti-inflammatory

345   mechanism of loading. In addition to providing new mechanistic understanding of this

346   pathway, this study identifies TRPV4 as a potential therapeutic target and demonstrates that

347   pharmaceutical activation of this protein could regulate inflammation and other IFT-

348   dependent pathways involved in cartilage disease.

351   **Author contributions**

352   All authors aided in revising this manuscript for intellectual content and approved the final

353   version to be published.

354   Study design: Su Fu, Clare L Thompson, Martin M Knight.

355   Data acquisition: Su Fu, Clare L Thompson, Sheetal Inamdar, Huan Meng

356   Data analysis and interpretation: Su Fu, Clare L Thompson, Sheetal Inamdar, Wen Wang,

357   Himadri Gupta, Martin M Knight.

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Conflict of interest

365   The authors have no competing interests.

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figure legends

Figure legends

Figure 1. Cyclic tensile strain inhibits IL-1β mediated NO and PGE2 release via a TRPV4 dependent pathway in isolated chondrocytes.

The TRPV4 antagonist, GSK205 (10 µM), abolishes the anti-inflammatory effects of mechanical loading (CTS, 10%, 0.33 Hz) at 1 ng/ml IL-1β on (A) nitrite and (B) PGE$_2$ release at 24 h. Data represents mean ± SD for n=6 wells per group using cells isolated from 2 different donors. Statistics: Three-way ANOVA and post hoc Tukey's test. # represents statistically significant difference between IL-1β treated and corresponding untreated cells.

Figure 2. Hypo-osmotic challenge inhibits IL-1β mediated NO release via a TRPV4 dependent pathway in isolated chondrocytes and cartilage explants

The TRPV4 antagonist, GSK205, suppresses the anti-inflammatory effects of hypo- osmotic challenge. Nitrite levels measured in the media for (A) isolated cells and (C) cartilage explants in hypo- or iso-osmotic media. Chondrocytes and cartilage explants were treated with and without IL-1β (1 ng/ml) for 24 h and 12 d respectively with and without the TRPV4 inhibitor GSK205 (10 µM). Hypo-osmotic challenge had no effect on cell viability as determined by (B) bright filed images of isolated chondrocytes (D) confocal microscopy of explants stained with Calcein-AM (live cells, green) and ethidium homodimer (dead cells, red). Scale bar represents 100 µm. Data represents mean ± SD for n=6 separate wells (A) or n=8 separate explants (C) using cells/explants isolated from 2 different donors. Statistics: Three-way ANOVA and post hoc Tukey's test. # represents statistically significant difference between IL-1β treated and corresponding untreated cells.

Figure 3. TRPV4 activation increases cilia expression of TRPV4 and inhibits cilia elongation in response to IL-1β
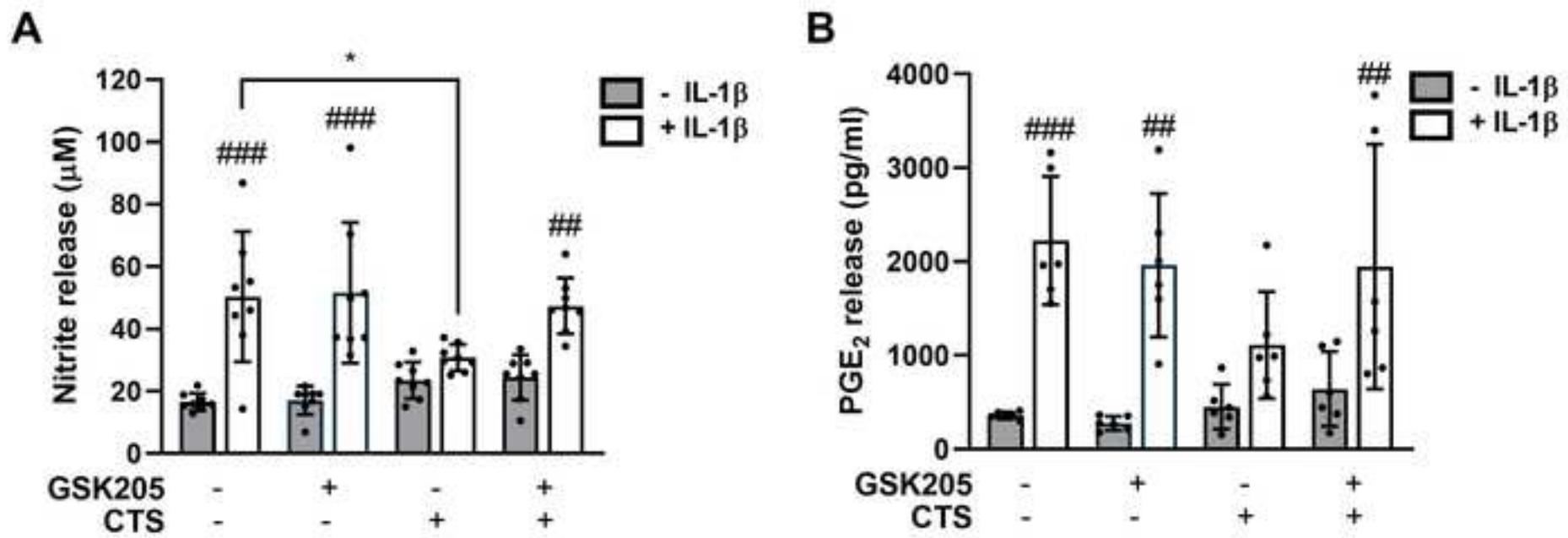
Primary articular chondrocytes were subjected to mechanical loading (CTS, 10%, 0.33 Hz), hypo-osmotic challenge (200 mOsm) or the TRPV4 agonist 1 nM GSK101 for 24 h. (A) Representative maximum intensity projection of confocal images showing co-localisation of Acet-α-tubulin (Red) and TRPV4 (Green). Scale bar represents 1 µm. Pharmaceutical activation of TRPV4 increased the (B) mean intensity of TRPV4 labelling on primary cilia (n=20-30 cilia) and (C) altered the distribution of TRPV4 on the cilium (n=20-30 cilia). (D) Representative maximum intensity projection confocal microscopy images of isolated chondrocytes treated with ± 1 ng/ml IL-1β ±1 nM GSK101 then labelled for acetylated α-tubulin (red) and counter stained with DAPI (blue). Scale bar represents 10 µm. (E) Primary cilia length and (F) associated % elongation were measured at 24 h. % cilia elongation results showing IL-1β induced change in cilia length for cells cultured (G) with and without mechanical loading and in (H) iso- and hypo-osmotic media. For % cilia elongation, data represents cilia length change in the presence of IL-1β (1 ng/ml, 24 h) normalised to median values in corresponding condition without IL-1β. Box plots are displayed as median, with error bars depicting min/max values (for E-H, n=70-130 cilia). Statistics: One-way ANOVA (B) and Two-way ANOVA (D, E, G and H) with post hoc Tukey's test and T-test (C, F).
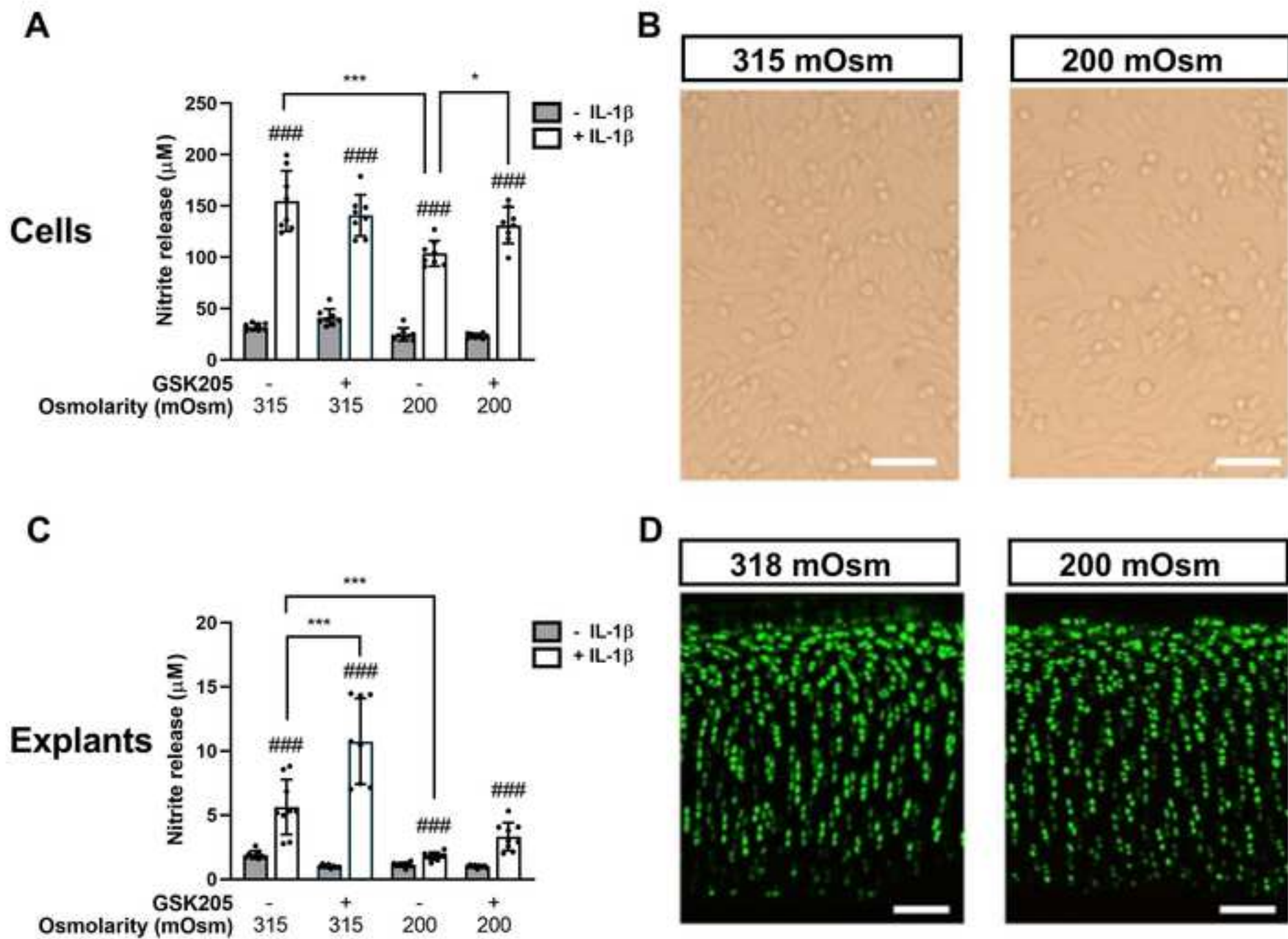
Figure 4. TRPV4 activation abolishes IL-1β inflammatory signalling via HDAC6 activation

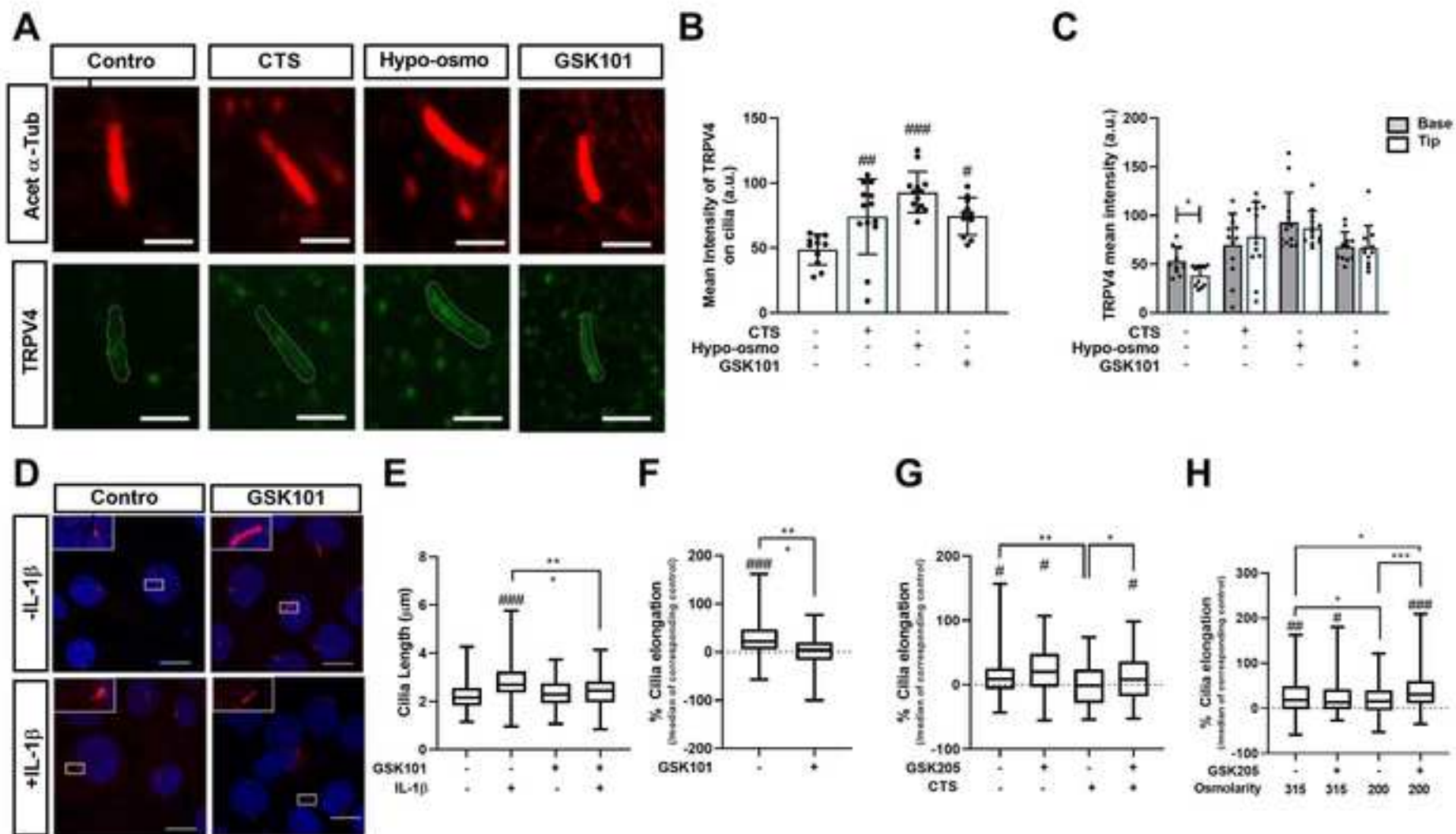Levels of (A) nitrite (B) PGE$_2$ and (C) COX-2 expression associated with isolated chondrocytes ±IL-1β (1 ng/ml) in the presence or absence of GSK101 for 24 hrs. GSK101 promotes (D) HDAC6 activity, induces the (E) de-acetylation and (E) de-polymerization of α-tubulin, as measured by western blot of acetylated α-tubulin (Acet α-Tub), α-tubulin (α-Tub) and non-polymerized α-tubulin. Full western blots can be found in supplementary figure S9. (G) HDAC6 inhibition with tubacin (500 nM) abolished the anti-inflammatory effect of GSK101 on NO release. Data represents mean ± SD for n=6 (A, B, D and G) and n=4 (C, E and F) Statistics: Two-way ANOVA and post hoc Tukey's test (A-C), T-test (D-G. # represents statistically significant difference between IL-1β treated and corresponding untreated cells.
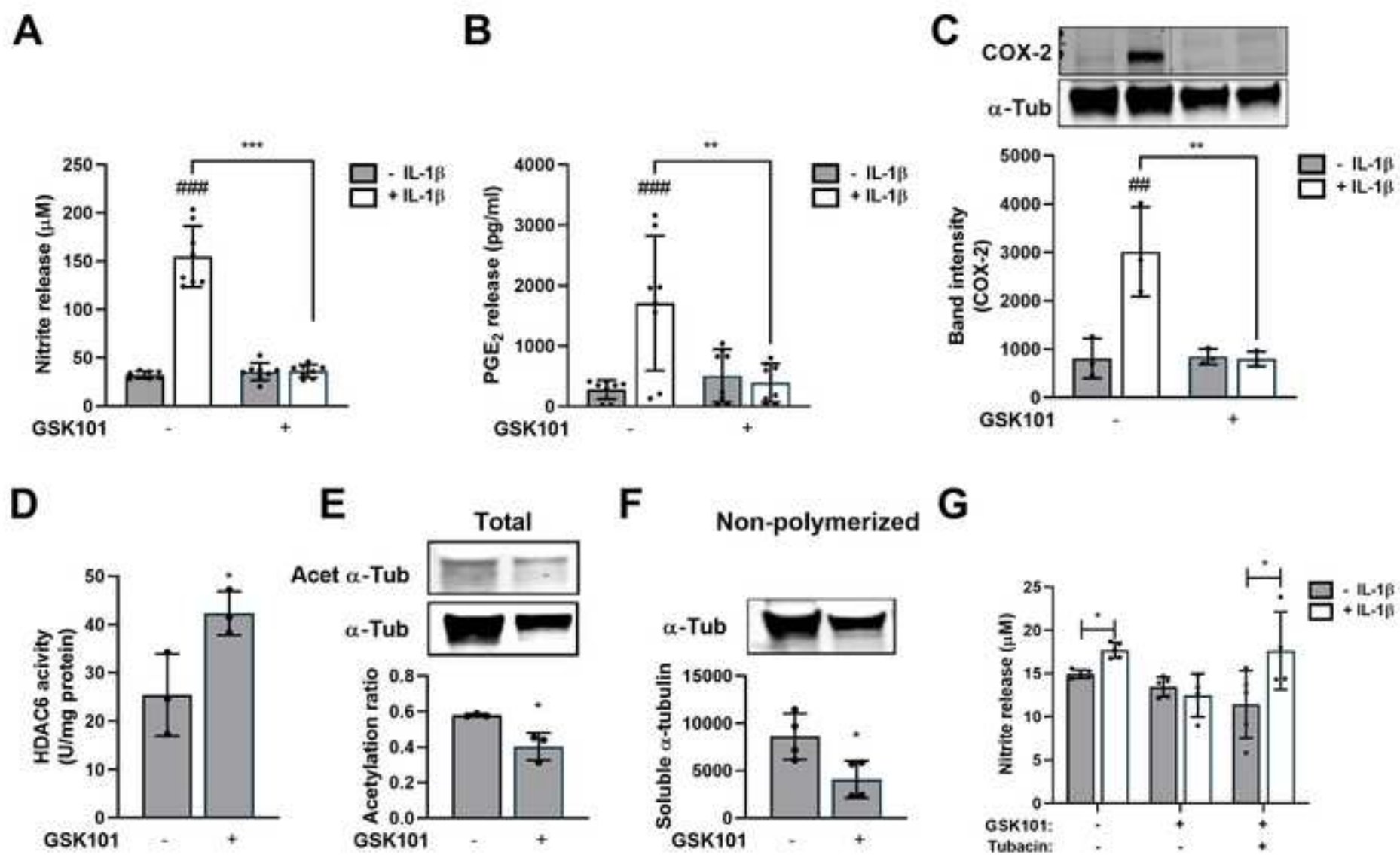
Figure 5. TRPV4 activation suppresses IL-1β induced NO release, matrix degradation and loss of mechanical properties in cartilage explants.

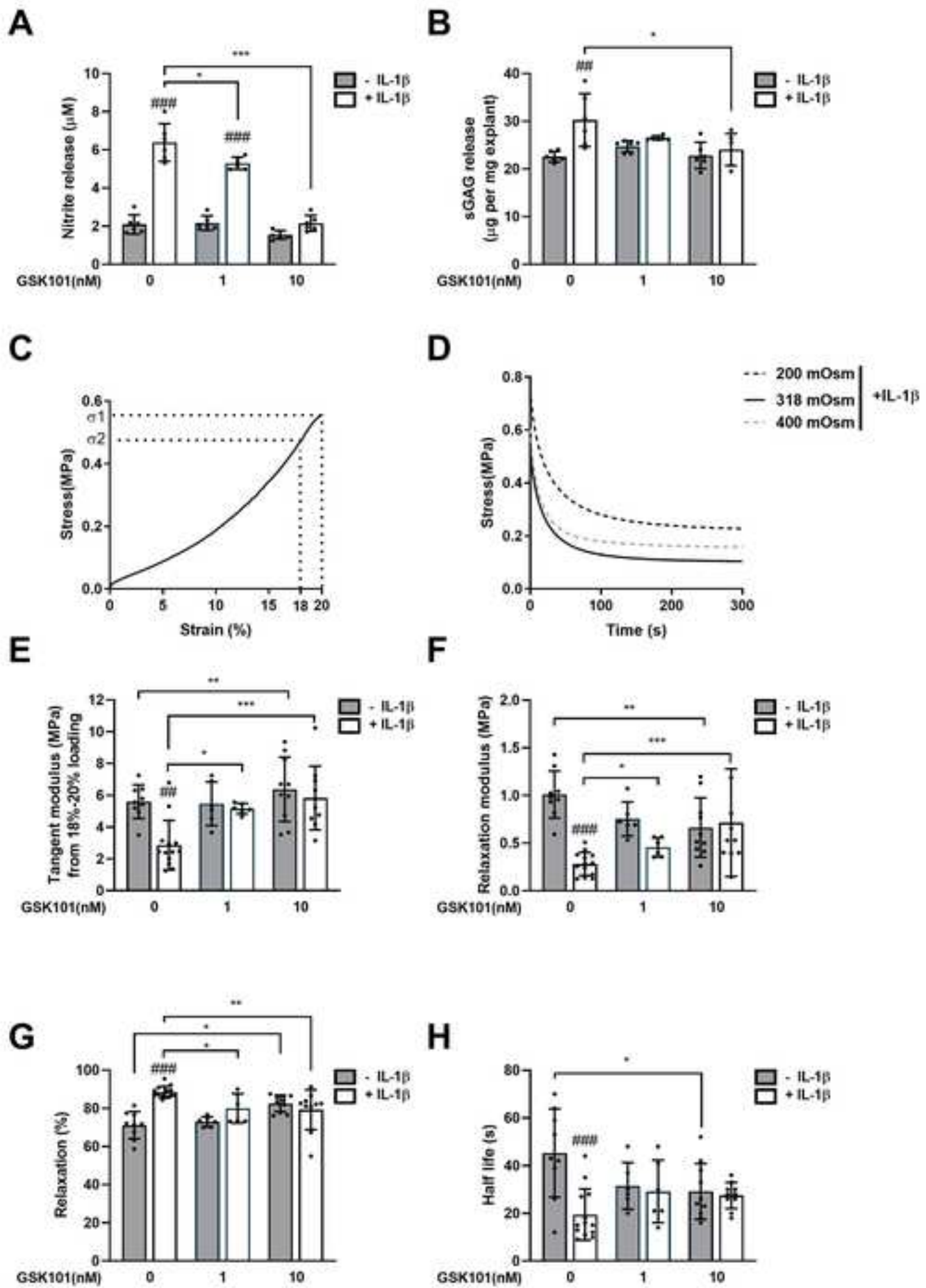Full-depth cartilage explants were treated with the TRPV4 agonist GSK101 (0, 1, 10 nM) in the presence or absence of 1 ng/ml IL-1β for 12 d. The nitrite (A) and sGAG (B) content of the culture media was measured and normalised to wet weight. Cartilage explants were compressed to obtain the stress-strain (C) and stress-relaxation (D) plots, for the calculation of cartilage mechanical properties. The responding mechanical properties of tangent modulus from 18-20% compression (E), relaxation modulus (F), percentage relaxation (G) and half-life (H). Data represents mean ± SD, n=6-12 explants from 4 different donors. Statistics: Two-way ANOVA with post hoc Tukey's test. # represents statistically significant difference between IL-1β treated and corresponding untreated cells.

Figure 1

Figure 2

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Figure 2

Figure 4   Click here to access/download;Figure;Figure 4.jpg

Figure 5

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1   **Introduction**

2   Osteoarthritis (OA) effects over 4.4 million people in the UK alone representing significant

3   economic cost [1]. Cartilage health is maintained in response to mechanical stimuli, articular

4   cartilage is routinely exposed to a wide array of dynamic mechanical loading consisting of

5   compressive, shear and tensile strains as well as associated alterations in fluid shear and

6   osmolality [2]. Mechanical loading in the form of compression or tensile strain is anti-

7   inflammatory in chondrocytes and blocks the release of the pro-inflammatory mediator's

8   nitric oxide (NO) and prostaglandin E_2 (PGE_2) in response to interleukin-1β (IL-1β) [3-5].

9   Inflammatory signalling contributes to cartilage degradation in OA thus understanding the

10   link between mechanical loading and inflammation will have significant therapeutic impact.

11   The osmotic-sensitive Ca^{2+} ion channel Transient Receptor Potential Vanilloid 4 (TRPV4) is

12   highly expressed in articular chondrocytes and is activated by mechanical stimuli [6, 7].

13   TRPV4 is required for mechanotransduction in chondrocytes and other cells types [7-9]. It

14   mediates the regulation of pro-anabolic and anti-catabolic genes promoting matrix

15   production and accumulation in agarose-embedded chondrocytes [7, 9]. TRPV4 mutations

16   result in human skeletal dysplasia suggesting a role in bone development (for review see

17   [10]). Indeed, chondrocytes from TRPV4^{-/-} mice exhibit loss of osmosensitivity accompanied

18   by joint degeneration indicating a central role for this channel protein in maintaining joint

19   homeostasis [11, 12]. Pharmaceutical activation of TRPV4 inhibits NO release in response

20   to inflammatory cytokines suggesting a potential mechanistic role in the anti-inflammatory

21   effects of mechanical loading [13, 14]. However, in apparent contradiction of these findings

22   TRPV4 inhibition exerts an anti-inflammatory effect in the cardiovascular system, lung and

23   peripheral nervous system [15-17]. This study therefore aims to clarify the regulatory role of

24   TRPV4 in cartilage inflammatory signalling.

25   TRPV4 localises to the plasma membrane and primary cilium, a small microtubule based

26   signalling compartment present at the cell surface [18, 19]. Primary cilia have been

27   implicated in both chondrocyte mechanotransduction [20-22] and inflammatory signalling

28   [23-26]. The cytoplasmic tubulin deacetylase histone deacetylase 6 (HDAC6) is enriched

29   within the cilium and modulates cilia resorption through de-acetylation and polymerization of

30   ciliary tubulin [27-29]. Post translational modification of ciliary tubulin influences intraflagellar

31   transport (IFT), the microtubule based motility present within the cilium required for cilia-

32   mediated signalling [30, 31]. Previously we report that mechanical loading counteracts

33   inflammatory signalling in response to the pro-inflammatory cytokine interleukin 1β (IL-1β)

34   via HDAC6 activation in association with alterations in IFT/cilia [5]. A role for TRPV4 in this

35   pathway has not previously been identified.

36   In the present study, we demonstrate for the first time that TRPV4 activation by cyclic tensile

37   strain, hypo-osmotic challenge or the TRPV4 agonist GSK1016790A inhibits pro-

38   inflammatory IL-1β signalling and cartilage degradation associated with alterations in primary

39   cilia elongation. TRPV4 may therefore provide a novel target for the treatment of joint

40   disease and other inflammatory pathologies.

41   **Methods**

42   **Antibodies and reagents**

43   Chondrocytes were treated with interleukin-1β (IL-1β, 200-01B; Peprotech, London, UK),

44   TRPV4 antagonist GSK205 (616522; Merck Millipore, London, UK) and agonist

45   GSK1016790A (GSK101, G0798; Sigma Aldrich, Poole, UK). Antibodies for

46   immunocytochemistry: acetylated α-tubulin (1:2000, T7451, Sigma Aldrich, Poole, UK) and

47   TRPV4 (1:200, SAB2104243, Sigma Aldrich). Nuclei were counterstained with 4′,6-

48   diamidino-2-phenylindole (DAPI, Sigma Aldrich). Antibodies for western blotting: acetylated

49   α-tubulin (1:1000, T7451, Sigma Aldrich) and α-tubulin (1:1000, ab4074, Abcam, Cambridge,

50   UK).

51   **Cartilage explant and chondrocyte culture**

52   Bovine cartilage explants and chondrocytes were obtained from 16 month steers as

53   previously described [28]. Full depth articular cartilage was removed from the proximal

54   surface of the metacarpal phalangeal joint and chondrocytes isolated by enzymatic

55   digestion. Explants were harvested using a 5 mm diameter biopsy punch (BP-50F, Selles

56   Medical Ltd, UK). Both were cultured at 37 °C, 5% CO_2 in Dulbeccos Minimal Essential

57   Medial (DMEM, D5921, Sigma-Aldrich, Poole, UK) supplemented with 10% (v/v) foetal calf

58   serum (FCS, F7524, Gibco, Paisley, UK), 1.9 mM L-glutamine (G7513), 96 U/ml penicillin

59   (P4333, All Sigma-Aldrich, Poole, UK). Explants were rested for 2 d prior to experimentation

60   while isolated chondrocytes were cultured to confluence.

61   **Application of cyclic tensile strain**

62   Isolated chondrocytes were cultured on collagen coated flexible elastomeric membranes and

63   subjected to uniform, equibiaxial cyclic tensile strain (CTS) applied using the Flexcell 5000T

64   system (Dunn Labortechnik GMbH).Cells were subjected to 0-10% strain for 24 h at 0.33 Hz.

65   **Application of osmotic loading**

66   Isolated cells were cultured for 24 h without serum in osmotically adjusted media at 200, 315

67   or 400 mOsm, hereafter referred as hypo-, iso- or hyper- osmotic media respectively.

68   Explants were cultured for up to 12 d under similar conditions with the addition of serum to

69   maintain chondrocyte viability resulting in a slightly higher osmolarity of 318 mOsm for the

70   iso-osmotic media. The osmolarity of all solutions was adjusted by adding D-mannitol

71   (M4125, Sigma-Aldrich) or distilled water and measured using a freezing point depression

72   osmometer.

73   **Biochemical analysis of NO, PGE_2 and sGAG release**

74   Nitric Oxide (NO) release was assessed using the Griess assay based on quantification of

75   nitrite (NO_2), the stable product of NO degradation. Nitrite content was quantified against a

76   sodium nitrite standard curve using the Galaxy Fluorstar spectrophotometer (BMG Labtech,

77 UK). An immunoassay kit (KGE004B, R&D Systems, UK) was used to quantify PGE_2

78 concentrations in the media according to the manufacturer's instructions. Results were

79 corrected for non-specific binding and calibrated using a PGE_2 standard curve. The release

80 of sGAG into the culture media was quantified using the dimethylmethyleneblue (DMMB)

81 assay against a chondroitin sulphate standard curve (6-sulphate:4-sulphate; 0.33:1; Sigma–

82 Aldrich).

83 **Immunocytochemistry, live imaging and confocal microscopy**

84 For immunocytochemistry, samples were fixed with 4% paraformaldehyde for 10 min,

85 permeabilised for 5 min with 0.5% triton-X100/phosphate buffered saline (PBS) then blocked

86 with 5% goat serum/PBS for 1 h. Primary antibody was incubated at 4°C overnight followed

87 by appropriate Alexa Fluor conjugated secondary antibodies (Molecular Probes) for 1 h at

88 room temperature. Cells were counterstained with 1 µg/ml DAPI for 5 min. For live imaging,

89 cell viability was assessed by live/dead staining. Explants were incubated for 30 min with 5

90 µM Calcein AM and 5 µM Ethidium homodimer-1 (EthD-1) prepared in appropriate osmotic

91 adjusted media, washed and immediately imaged. Samples were imaged using a Zeiss 710

92 ELYRA PS.1 microscope. For cilia analysis, samples were imaged using an x63/1.4 NA

93 objective to generate confocal z-sections made throughout the cell depth (approximately 20

94 sections) using 0.25 µm step size with an image format of 1024 x 1024 yielding a pixel size

95 of 0.13 x 0.13 µm (image size approximately 135 x 135 µm). Cilia length and prevalence was

96 quantified from resulting maximum projection images using Image J software (National

97 Institutes of Health, Maryland, USA).

98 **Western blotting**

99 Cells were lysed in RIPA buffer (R0278, Sigma Aldrich) and total protein quantified by

100 Bicinchoninic acid (BCA) assay. For the fractionation of soluble and polymerized tubulin,

101 extraction buffer A (137 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, and 10% glycerol) was

102 added to cells at 4 °C for 3 min, plates were gently swirled and the buffer removed and

103   saved as the soluble tubulin fraction. Immediately after, extraction buffer B (buffer A + 1%

104   SDS) was added for 1 min, the remaining sample was scraped, incubated on ice for 30 min

105   and saved as the polymerized tubulin fraction.

106   SDS–PAGE was performed under reducing conditions and proteins transferred to

107   nitrocellulose membranes. Membranes were blocked in odyssey blocking buffer (Li-Cor

108   Cambridge, UK) prior to overnight incubation with primary antibodies and infrared secondary

109   antibodies (Li-Cor). Proteins were visualized using the Li-Cor Odyssey and quantified using

110   Image Studio Lite software (Li-Cor).

111   **HDAC6 activity measurement**

112   A commercial fluorometric assay kit (K466-100, Biovision) was used to measure HDAC6

113   activity according to the manufacturer's instructions. This assay determines enzyme activity

114   by exploiting the selectivity of tubacin for HDAC6 in combination with a fluorescent synthetic

115   acetylated-peptide substrate. Cultures were lysed, a 10 µl aliquot was mixed with either

116   acetylated substrate (sample) or with 2 µM tubacin and acetylated substrate (inhibitor

117   control) then incubated for 30 min at 37°C. The deacetylase-dependent release of a 7-

118   amino-4-trifluoromethylcoumarin fluorophore (excitation/emission at 350/490 nm) was then

119   measured on a Galaxy Fluorstar spectrophotometer (BMG Labtech, UK) and HDAC6 activity

120   calculated as [sample-inhibitor control].

121   **Mechanical testing of cartilage explants**

122   The mechanical behaviour of individual cartilage explants was measured using an MTS,

123   Bionix 100. A 2 mm diameter core was cut from the centre of each 5 mm diameter cartilage

124   explant and a tare load of 0.01 N applied to each explant which was then hydrated in culture

125   media. The explants were subjected to a 20% uniaxial unconfined compressive strain (20%

126   /min). This was followed by a stress relaxation period at constant 20% strain in which the

127   load was recorded for a further 300 s. The load was recorded throughout the test at a

128   sampling frequency of 60 Hz. Stress–strain and stress–time curves were generated for each

129   specimen and the following mechanical properties of the cartilage determined:

130   Tangent Modulus (MPa) = $\dfrac{\sigma_{\varepsilon=0.2} - \sigma_{\varepsilon=0.18}}{0.02}$

131   Relaxation Modulus (MPa) = $\dfrac{\sigma_{t=300s}}{0.2}$

132   Percentage Relaxation (%) = $\dfrac{\sigma_{t=0s} - \sigma_{t=300s}}{\sigma_{t=0s}}$

133   The relaxation half-life was calculated as the time from the start of the relaxation phase until

134   stress reduced to half the peak value.

135   **Statistical analyses**

136   The sample size for each experiment was chosen based on previous studies [5, 32] where

137   analysis of cartilage degradation by biochemistry, immunohistochemistry and mechanical

138   testing demonstrated that 6-8 samples/group is sufficient to detect a 25% difference in

139   cartilage matrix catabolism at 80% power and a significance of $p<0.05$. Data analysis was

140   conducted using GraphPad Prism version 8 (GraphPad software, La Jolla California USA,

141   www.graphpad.com). Normality testing (Kolmogorov Smirnov test) was performed to confirm

142   that data exhibited Gaussian distribution. For data sets that were not normally distributed,

143   namely cilia length data, Box Cox transformation (λ=0.5) was performed prior to statistical

144   analyses. Statistical significance was determined by T-Test, One-way, Two-way or Three-

145   way ANOVA as appropriate with post-hoc Tukey's multiple comparisons performed to

146   identify significant differences between groups. Statistically significant differences were

147   determined based on a threshold of *= $p<0.05$, **=$p<0.01$ and ***=$p=0.001$. Data is

148   presented as mean ± standard deviation (SD) unless otherwise stated.

149   **Results**

150   **TRPV4 activation is required for the anti-inflammatory effects of mechanical loading**

151   **in isolated chondrocytes**

152   IL-1β treatment (24 h) resulted in significant, dose-dependent release of NO and PGE$_2$ (Fig.

153   1AB, S1AB). In response to 1 ng/ml IL-1β isolated chondrocytes exhibited a 3.04-fold

154   increase in nitrite levels indicative of NO release (Fig. 1A), and a 4.84-fold increase in PGE$_2$

155   release (Fig. 1B) which increased to 11.48- and 7.37-fold respectively in response to 10

156   ng/ml IL-1β (Fig. S1A-B). Consistent with previous studies [5] this response was significantly

157   reduced by mechanical loading in the form of CTS. IL-1β induced NO release was abolished

158   by CTS such that there was no statistically significant effect at either 1 or 10 ng/ml (Fig. 1A,

159   S1A). PGE$_2$ release was completely inhibited by CTS at 1 ng/ml IL-1β (Fig. 1B) but only

160   partially suppressed at 10 ng/ml (Fig. S1B).

161   Simultaneous treatment with the TRPV4 antagonist GSK205 (10 µM) abolished the anti-

162   inflammatory effects of mechanical loading (Figure 1, S1). While GSK205 had no effect on

163   NO or PGE$_2$ release in unloaded cells with or without IL-1β, in loaded cells the IL-1β

164   response was restored such that NO (Fig. 1A and S1A) and PGE$_2$ release (Fig. 1B, S1B)

165   were significantly increased by IL-1β. Neither IL-1β nor GSK205 treatment in the presence

166   or absence of CTS influenced TRPV4 protein levels (Fig. S10). These data indicate the anti-

167   inflammatory effects of mechanical loading are mediated by TRPV4 activation.

168   **TRPV4 activation is required for the anti-inflammatory effects of hypo-osmotic loading**

169   **in isolated chondrocytes and cartilage explants**

170   Isolated chondrocytes were treated with hyper-osmotic media (400 mOsm), hypo-osmotic

171   (200 mOsm) or iso-osmotic media (315 mOsm) for 24 h (Figure 2AB, S2). Hyper-osmotic

172   challenge had no significant effect on NO release, with or without IL-1β relative to the iso-

173   osmotic control (Fig. S2, S3). By contrast, hypo-osmotic challenge significantly attenuated

174   the pro-inflammatory response to IL-1β (1 ng/ml), such that the increase in NO release at 24

175   h was significantly reduced (p<0.001, Fig. 2A S2A). Hypo-osmotic challenge had no

176   apparent effect on cell viability compared to control conditions based on brightfield

177   microscopy (Figure 2B). In the presence of GSK205, the anti-inflammatory effect of hypo-

178   osmotic challenge on IL-1β induced NO release was completely inhibited by GSK205 such

179   that the induction of NO release was not significantly different to control conditions (Fig. 2A).

180   In the absence of IL-1β, GSK205 also had no effect on baseline NO or PGE$_2$ levels (Fig.

181   S2).

182   In cartilage explants, hypo-osmotic challenge significantly reduced IL-1β induced NO release

183   (Fig. 2C, p<0.001) such that there was no significant difference between IL-1β treated and

184   untreated explants (Fig. 2C, S4A). In line with these findings hypo-osmotic challenge

185   blocked the IL-1β mediated release of sGAG into the media, indicative of a reduction in

186   extracellular matrix degradation (Fig. S4B). Chondrocyte viability was maintained throughout

187   the experiment as determined by live/dead staining (Fig. 2D). Consistent with isolated cells,

188   hyper-osmotic challenge (400 mOsm, 12 d) had no effect on NO or sGAG release in the

189   presence or absence of 1 ng/ml IL-1β (Fig. S4). GSK205 treatment restored IL-1β-induced

190   NO release in hypo-osmotic media (Fig. 2C) thus blocking the anti-inflammatory effect of

191   osmotic challenge. Interestingly, GSK205 further increased IL-1β-induced NO release in iso-

192   osmotic, control media a response not seen in isolated cells (Fig. 2C). Together these data

193   indicate the anti-inflammatory effects of hypo-osmotic loading are also mediated by TRPV4

194   activation.

195   **TRPV4 activation is associated with altered primary cilia localisation and regulates**

196   **cilia length**

197   IL-1β induces primary cilia elongation in articular chondrocytes and mediates downstream

198   catabolic NF-κB signalling through regulation of IFT [5, 23, 24]. We therefore examined the

199   involvement of primary cilia in the anti-inflammatory mechanism of TRPV4 activation. TRPV4

200   cilia localisation was observed in isolated chondrocytes (Fig 3A). TRPV4 activation by

201   mechanical loading, hypo-osmotic challenge or the TRPV4 agonist GSK101 (1 nM)

202   increased TRPV4 cilia localisation while not significantly affecting protein expression, as

203   shown by the increased mean intensity of ciliary TRPV4 (Fig. 3B, S10) and altered

204   distribution profile of TRPV4 in proximal and distal regions of the axoneme (Fig. 3C) these

205   data are suggestive of alterations in IFT.

206   In isolated chondrocytes, IL-1β (1 ng/ml) treatment for 24 h induced a significant increase in

207   primary cilia length (p<0.001) from a median value of 2.21 to 2.84 µm. This elongation was

208   abolished by TRPV4 activation with GSK101 (Fig. 3D-E). IL-1β mediated cilia elongation

209   was also blocked by mechanical loading (CTS, 0-10%, 0.33 Hz, Fig. 3G) and hypo-osmotic

210   challenge (Fig 3H). Inhibition of TRPV4 with GSK205 restored IL-1β mediated cilia

211   elongation in the presence of both mechanical loading (p<0.001, Fig. 3G), and hypo-osmotic

212   challenge (p<0.001, Fig. 3H). GSK101, had no effect on cilia length in iso-osmotic conditions

213   with or without IL-1β (Fig. 3E). GSK101 also had no effect on cilia prevalence for any of the

214   treatment groups (Fig. S5A and D).

215   **TRPV4 activation inhibits inflammatory signalling in response to IL-1β through the**

216   **regulation of HDAC6 and ciliary tubulin**

217   We next examined whether direct pharmaceutical activation of TRPV4 would replicate the

218   anti-inflammatory effect of mechanical and osmotic loading. IL-1β (1 ng/ml) induced the

219   characteristic upregulation of NO and PGE$_2$ release in isolated chondrocytes which was

220   abolished by GSK101 (Fig. 4A and B). Similarly IL-1β induced COX2 expression was

221   abolished by GSK101 (Fig. 4C). No effects on cell viability based on bright field microscopy

222   (Fig. S6A) and DNA content were observed although cells appeared to have a more rounded

223   morphology particularly at high concentrations (Fig. S6B).

224   Previously, we identified a mechanistic role for HDAC6 activation and post-transcriptional

225   tubulin modifications in the anti-inflammatory effect of mechanical loading [5]. Similarly,

226 GSK101 resulted in significant upregulation of HDAC6 activity (Fig. 4D) suggesting TRPV4-

227 mediated calcium signalling activates HDAC6. Consistent with this finding we observed

228 significant tubulin deacetylation accompanied by a reduction in the pool of non-polymerized,

229 soluble tubulin (Fig. 4E-F). Furthermore, the HDAC6 specific inhibitor, tubacin (500 nM),

230 restored IL-1β mediated stimulation of NO release in GSK101-treated cells (Fig. 4G). These

231 data suggest that GSK101 mimics the effects of mechanical loading on IL-1β inflammatory

232 signalling, HDAC6 activation and tubulin modification.

233 **TRPV4 activation abolishes IL-1β mediated cartilage degradation and loss of**

234 **mechanical properties**

235 We next determined whether pharmaceutical activation of TRPV4 could prevent cartilage

236 degradation and loss of mechanical properties. Cartilage explants were treated with IL-1β for

237 up to 12 d in the presence of 1 nM or 10 nM GSK101. Cartilage explant viability was

238 maintained at these experimental doses (Fig. S7). In response to IL-1β treatment, significant

239 NO release was observed (Fig. 5A, P<0.001) indicative of activation of inflammatory

240 signalling. This response was accompanied by significant sGAG release indicative of

241 cartilage degradation (Fig. 5B, P<0.001).

242 We measured the viscoelastic properties of cartilage tissue using uniaxial unconfined

243 compression to determine whether GSK101 could prevent the loss of mechanical properties

244 induced by IL-1β. Cartilage explants exhibited a non-linear stress-strain graph represented

245 by a tangent modulus of 15-20MPa (Fig 5C). This was followed by characteristic viscoelastic

246 stress relaxation at 20% strain (Fig 5D) to a relaxation modulus of 2-3 MPa at 300 s

247 representing 80% relaxation and a relaxation half-life of approximately 50 s (Fig. 5E-H). IL-

248 1β treatment resulted in dramatic loss of mechanical stiffness as shown by significant

249 reductions in tangent modulus (p<0.001, Fig. 5E) and relaxation modulus (p<0.001, Fig. 5F),

250 increased percentage relaxation (p<0.001, Fig. 5G) and a reduction in half-life (p<0.001, Fig.

251 5H).

252 GSK10 significantly inhibited the cumulative release of NO from cartilage explants in

253 response to IL-1β treatment (p<0.001, Fig. 5A). Similarly the cumulative release of sGAG

254 was significantly reduced (p<0.001) and loss of mechanical properties in response to IL-1β

255 abolished, such that there was no significant difference in any of the biomechanical

256 parameters with and without IL-1β.

257 **Discussion**

258 This study demonstrates that TRPV4 plays an important mechanistic role in the anti-

259 inflammatory effect of mechanical stimulation. TRPV4 inhibition restores IL-1β mediated pro-

260 inflammatory signalling in the presence of both mechanical and osmotic loading. Conversely,

261 TRPV4 activation by GSK101 blocked the release of pro-inflammatory mediators in the

262 absence of load in isolated cells and prevented cartilage degradation and loss of mechanical

263 properties in an explant model. TRPV4 is activated by mechanical stimulation in the form of

264 cyclic tensile strain or osmotic challenge and functions upstream of HDAC6 to modulate

265 tubulin acetylation and polymerization which regulates IFT thereby suppressing IFT-

266 dependent IL-1β signalling.

267 TRPV4 is expressed in bone marrow stem cells, osteoblasts, osteoclasts and chondrocytes,

268 and is required for skeletal development [10, 33]. TRPV4 belongs to the Transient Receptor

269 Potential (TRP) superfamily which mediate cellular responses to a variety of environmental

270 stimuli including heat [34], cell swelling [35], hypo-osmolality [18, 36] and mechanical

271 loading [7, 9] and results in elevated levels of intracellular Ca^{2+}. Thus, TRPV4 is required for

272 mechanotransduction. It promotes chondrocyte matrix production in response to dynamic

273 compression [7], mediates the fluid shear induced osteogenic response in stem cells [9] and

274 shear stress induced vasodilatation in endothelial cells [8].

275 In other tissues, TRPV4 activation is mostly reported to be pro-inflammatory. In airway

276 epithelial cells, TRPV4 activates NF-κB signalling promoting progression of lung fibrosis [37].

277 Endogenous TRP channel agonists are detected in a lung injury model while TRPV4

278   inhibition suppresses acid-induced pulmonary inflammation [38]. TRPV4 antagonists have

279   been used to treat sepsis in mice by reducing production of TNF-α, IL-1 and IL-6 [16].

280   Moreover loss of TRPV4 function suppresses inflammatory fibrosis in mouse corneas [39].

281   However, Xu et al. report that GSK101 prevents vascular inflammation and atherosclerosis,

282   associated with inhibition of NO synthase and MAPK signalling [14]. TRPV4 is also well-

283   established to mediate inflammatory hyperalgesia (see review [40]) and is regarded as a

284   promising target for novel analgesics.

285   Consistent with our findings, pharmaceutical activation of TRPV4 has been shown to

286   suppress NO release induced by lipopolysaccharide (LPS) in rat temporomandibular

287   chondrocytes, whereas TRPV4 inhibition aggravates the inflammatory response to LPS

288   [13]. Clark et al. report that TRPV4 deficiency induces inflammation and disrupts cartilage

289   matrix homeostasis. As such, TRPV4$^{-/-}$ mice exhibit a severe sex-dependent osteoarthritis

290   (male mice are more susceptible) while the isolated chondrocytes fail to increase Ca^{2+} influx

291   in response to hypo-osmotic challenge [11]. These mice exhibit a more severe obesity-

292   induced osteoarthritis, compared to wild-type mice [12]. However other studies report

293   osmotic challenge to be a pro-inflammatory signal. Hubert et al observed induction of IL-8 in

294   response to both hyper and hypo-osmotic stress [41] while hypo-osmotic stimulation of

295   TRPV4 promoted PGE_2 release in porcine chondrocytes [18] and the expression of IL-1β

296   and IL-6 in bovine intervertebral disc cells [36], suggesting a pro-inflammatory role of

297   TRPV4. We did observe a mild, transient increase in NO release in this study at 3 h hypo-

298   osmotic challenge however this had resolved and was not significantly different to the control

299   at 24 h (Fig. S8). Interestingly we observed dose-dependent cytotoxicity of GSK101 with

300   extended explant culture at concentrations above 10nM (Fig. S7). Low concentrations of

301   GSK101 elicit multiple short peaks of Ca^{2+} signalling, which is more physiological compared

302   with the large, sustained peaks observed at higher concentrations which might explain this

303   [42]. These observations suggest perhaps that only moderate, short-term modulation of

304   TRPV4 will be chondroprotective.

305   Servin-Vences et al suggest TRPV4 mechanosensitivity is dependent upon the type of

306   stimulus applied [6]. Our data supports this hypothesis, complete abolition of NO release in

307   response to IL-1β was observed following application of cyclic tensile strain (Figure 1), while

308   hypo-osmotic challenge merely attenuated the response (Figure 2) suggesting TRPV4

309   activation may be regulated distinct mechanisms and to different extents. Vriens et al report

310   that TRPV4 activation in response to cell swelling is dependent upon arachidonic acid

311   release [43] whereas Servin-Vences et al suggest direct channel gating occurs in response

312   to membrane deflection [6].

313   The mechanosensitive ion channel PIEZO1 reportedly induces TRPV4 channel opening [44].

314   PIEZO1 is activated chondrocytes following injurious loading and is suggested to play a

315   greater role in chondroprotection than TRPV4 [6, 45]. It is possible the more pronounced

316   anti-inflammatory effects of cyclic tensile strain observed in this study are the result of further

317   TRPV4 activation downstream of this channel, which could be explored in future studies.

318   However, while activation of PIEZO1 reportedly influences ciliogenesis [46] studies in

319   osteocytes indicate that it does not interact with TRPV4 in the cilium [47].

320   TRPV4 cilia localisation was observed with greater localisation evident at to the ciliary base

321   (Fig. 3C). TRPV4 activation altered this distribution such that localisation to the base or tip of

322   the axoneme was not significantly different indicative of altered protein trafficking/IFT (Fig.

323   3C). TRPV4 activation is coupled with translocation of TRPV4 to plasma membrane [48], in

324   this study we observed increased TRPV4 labelling in the ciliary membrane (Fig. 3AB).

325   Chemical deletion of primary cilia with chloral hydrate fully abolishes Ca^{2+} signalling in

326   response to TRPV4 activation [18] thus increased ciliary TRPV4 may be important for

327   signalling.

328   HDAC6 is enriched within primary cilia catalysing tubulin de-acetylation and polymerization

329   to regulate cilia resorption [27-29]. In this study, mechanical, hypo-osmotic and

330   pharmaceutical activation of TRPV4 blocked cilia elongation in response to IL-1β. IFT and

331 cilia elongation is required for IL-1β mediated inflammatory signalling and downstream NF-

332 κB signalling [5, 23, 24], therefore we suggest the anti-inflammatory effects of TRPV4

333 activation regulate IFT and associated signalling via HDAC6 dependent modulation of ciliary

334 tubulin. Previous studies demonstrate that GSK101 activates Ca^{2+} signalling in isolated

335 chondrocytes [6, 17, 49], while GSK205 inhibits this response and blocks Ca^{2+} signalling in

336 response to mechanical or osmotic loading [18, 50, 51]. While Ca^{2+} signalling was not

337 assessed in the current study, we hypothesise that Ca^{2+} levels may regulate HDAC activity

338 through activation of upstream kinases such as Ca^{2+}/Calmodulin dependent kinase (CaMK),

339 protein kinase D (PKD) and Aurora A kinase-dependent (AURKA) [27-29, 52-55]. Studies

340 suggest TRPV4 stimulation with GSK101 promotes ERK/MAPK signalling in lung epithelial

341 cells and cancer cells [56] and PKC activity in endothelial cells [57] which phosphorylate

342 HDAC6 resulting in increased deacetylation activity [58, 59]. Indeed, increased HDAC6

343 activity was observed in response to GSK101 (Fig. 4D).

344 In conclusion, this study demonstrates a role for TRPV4 activation in the anti-inflammatory

345 mechanism of loading. In addition to providing new mechanistic understanding of this

346 pathway, this study identifies TRPV4 as a potential therapeutic target and demonstrates that

347 pharmaceutical activation of this protein could regulate inflammation and other IFT-

348 dependent pathways involved in cartilage disease.

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Author contributions

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356   Data analysis and interpretation: Su Fu, Clare L Thompson, Sheetal Inamdar, Wen Wang,

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Conflict of interest

365   The authors have no competing interests.

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OSTEOARTHRITIS AND CARTILAGE

AUTHORS' DISCLOSURE

Manuscript title _____ Activation of TRPV4 by mechanical, osmotic or pharmaceutical stimulation is anti-inflammatory blocking IL-1β mediated articular cartilage matrix destruction._____

Corresponding author Clare l Thompson

Manuscript number _____

Authorship
All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted. By signing below each author also verifies that he (she) confirms that neither this manuscript, nor one with substantially similar content, has been submitted, accepted or published elsewhere (except as an abstract). Each manuscript must be accompanied by a declaration of contributions relating to sections (1), (2) and (3) above. This declaration should also name one or more authors who take responsibility for the integrity of the work as a whole, from inception to finished article. These declarations will be included in the published manuscript.

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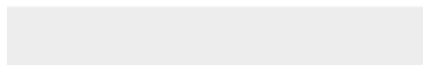**ICMJE COI form**
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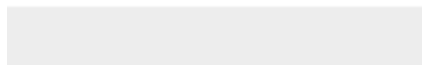**ICMJE COI form**
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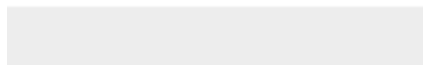**ICMJE COI form**
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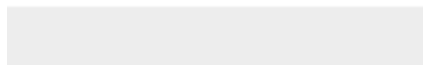Click here to access/download
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