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Characterization of diabetic osteoarthritic cartilage and role of high glucose environment on chondrocyte activation: toward pathophysiological delineation of diabetes mellitus-related osteoarthritis

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1 **Characterization of diabetic osteoarthritic cartilage and role of high glucose**
2 **environment on chondrocyte activation: toward pathophysiological delineation**
3 **of diabetes mellitus-related osteoarthritis**

4
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8 **osteoarthritis**

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36 **ABSTRACT**
37

38 **Objective:** To examine the relationship between osteoarthritis (OA) and type 2
39 diabetes mellitus (DM).

40 **Methods:** OA cartilage from DM and non-DM patients undergoing knee replacement
41 were stimulated by IL-1 β for 24 h and release of interleukin-6 (IL-6) and
42 prostaglandin E₂ (PGE₂) was measured. Primary cultured murine chondrocytes were
43 stimulated for 24 and 72 h with or without IL-1 β (5 ng/mL) under normal-glucose (5.5
44 mM) or high-glucose (25 mM) conditions. The expression and release of pro-
45 inflammatory mediators (IL-6, cyclooxygenase 2 [COX2]/PGE₂) were analyzed by
46 quantitative RT-PCR and ELISA/EIA. Glucose uptake was assessed with (¹⁴C)-2-
47 deoxyglucose. Reactive oxygen species (ROS) and nitric oxide (NO) production were
48 measured. To analyze the mechanism of IL-1 β -induced inflammation, cells were
49 pretreated or treated with inhibitors of glucose transport (cytochalasin B), the polyol
50 pathway (epalrestat), mitochondrial oxidative stress (MitoTEMPO) or nitric oxide
51 synthase (L-NAME).

52 **Results:** With IL-1 β stimulation, IL-6 and PGE₂ release was greater in human DM
53 than non-DM OA cartilage (2.7- and 3-fold, respectively) (p<0.05). *In vitro*, with IL-1 β
54 stimulation, IL-6 and COX2 mRNA expression, IL-6 and PGE₂ release, and ROS and
55 NO production were greater under high- than normal-glucose conditions in cultured
56 chondrocytes. IL-1 β -increased IL-6 release was reduced with cytochalasin B,
57 epalrestat, L-NAME or MitoTEMPO treatment (-45%, -62%, -38% and -40%,
58 respectively).

59 **Conclusion:** OA cartilages from DM patients showed increased responsiveness to
60 IL-1 β -induced inflammation. Accordingly, high glucose enhanced IL-1 β -induced

- 61 inflammation in cultured chondrocytes *via* oxidative stress and the polyol pathway.
- 62 High glucose and diabetes may thus participate in the increased inflammation in OA.

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1 INTRODUCTION

2 Osteoarthritis (OA) is the most frequent age-related joint disease affecting all tissues
3 in the joint (1). The incidence and prevalence of the disease are increased with
4 specific risk factors (2), with overweight and trauma at the forefront. These 2 risk
5 factors may be involved in the pathophysiology of the disease by specific and
6 independent pathways. Trauma and overweight on weight-bearing joints provoke
7 local biomechanical stresses; however, recent studies suggest that a low-grade
8 systemic inflammation process may be involved in some cases such as hand OA, in
9 which obesity increases the risk by two-fold (3, 4). More recently, epidemiological
10 studies have suggested that the OA risk increases with the number of cardio-
11 metabolic factors, including type 2 diabetes mellitus (DM) (5-8). Moreover, DM
12 independently alters the prognosis by increasing the risk of total joint replacement (9)
13 and could be a specific OA risk factor (10, 11).

14 The inflammatory status of OA joint tissues in the setting of DM has never been
15 investigated. Overall, explanations regarding any links between DM and OA remain
16 scarce. Streptozotocin-induced diabetic rats with insulin deficiency and severe
17 hyperglycemia showed progressive loss of type II collagen and proteoglycans in
18 cartilage (12). At the molecular level, both diseases feature increased content of
19 interleukin 1 β (IL-1 β) along with other low-grade inflammatory mediators such as IL-
20 6, IL-8, prostaglandin E₂ (PGE₂) as well as reactive oxygen species (ROS).

21 Despite the role of glucose in energy metabolism in all cell types, including
22 chondrocytes (13, 14), high glucose concentration may have a detrimental effect *per*
23 *se* because excessive intracellular glucose concentration can saturate the glycolytic
24 pathway, thereby activating other secondary pathways involved in glucose
25 metabolism such as polyol, hexosamine, protein kinase C or pentose phosphate

26 pathways and producing advanced glycation end products (AGEs) (15). All these
27 pathways are responsible for inducing oxidative stress, also incriminated in the OA
28 process (16). Increased ROS production generated by a high-glucose environment is
29 linked to mitochondrial dysfunction, which may affect cartilage homeostasis (16-20).
30 Nitric oxide (NO), the main nitrogen active species known to have pro-inflammatory
31 and pro-degradative effects on chondrocytes, has been implicated in the
32 pathophysiology of both DM and OA (21-23).

33 Finally, OA chondrocytes exposed to high glucose were found unable to down
34 regulate the glucose transporter 1 (GLUT-1), one of the main glucose transporters
35 expressed in chondrocytes, which led to enhanced glucose uptake and ROS
36 generation (24). However, the role of high extracellular glucose level on activation of
37 articular chondrocytes has never been investigated.

38 To further elucidate the link between DM, chronic hyperglycemia and OA, we aimed
39 to 1) characterize the inflammatory profile of human OA cartilage in patients with and
40 without type 2 DM and 2) investigate the effect and mechanism of action of high
41 glucose on chondrocyte activation.

42

43 **METHODS**

44 **Collection of OA human cartilage**

45 Human OA knee explants were obtained from patients undergoing total joint
46 replacement because of OA at Saint-Antoine Hospital (Paris) shortly after surgery.
47 Informed consent for the use of tissue and clinical data was obtained from each
48 patient before surgery. Experiments with human samples were approved by a French
49 institutional review board (Comité de Protection des Personnes, Paris Ile de France
50 5). The diagnosis of knee OA was based on the American College of Rheumatology

51 criteria (25). We pre-operatively screened patients about their diabetic status using
52 the medical file, drugs prescription, and patient interview. For each diabetic patient
53 included, we matched a non-diabetic patient undergoing total joint replacement
54 because of OA by age and body mass index (BMI), to avoid confounding factors. All
55 explants from each patient were manually dissected from all remaining cartilage
56 zones (i.e., tibial plateaus and femoral condyles) and mixed to obtain homogenous
57 isolated cartilage samples and managed as previously described (26). Briefly, the
58 cartilage explants were cut into small pieces (~5 mm³), washed several times with
59 phosphate buffered saline (PBS) and incubated in DMEM culture medium containing
60 25 mM glucose necessary for human explant maintenance, and supplemented with
61 100 U/mL penicillin, 100 µg/mL streptomycin, and 4 mM glutamine for 24 h at 37°C
62 with or without IL-1β (5 ng/mL). Conditioned media (CM) were then collected,
63 centrifuged (1600 g for 6 min) and stored at -20°C. Each volume of medium was
64 normalized to wet weight of explants (6 mL/ g tissue) (27).

65

66 **Primary culture of murine articular chondrocytes**

67 Mouse primary chondrocytes were isolated from articular cartilage of 5- to 6-day-old
68 newborn C57Bl6 mice from Janvier (St Berthevin, France) and seeded at 8×10^3
69 cells per cm as described (28). Articular chondrocytes obtained from newborn mice
70 using this protocol are considered as relevant to study mature chondrocytes since
71 type II collagen and aggrecan mRNA levels are highly expressed while expression of
72 type I collagen mRNA expression remains low (29). After 1 week of amplification in
73 DMEM with 5.5 mM glucose, cells were incubated in serum-free DMEM (with 5.5 mM
74 glucose) containing 0.1% bovine serum albumin (BSA) for 24 h before treatment
75 (basal experimental medium). Each litter of mice was used for one experiment. All

76 experiments with murine articular chondrocytes were performed according to
77 protocols approved by the French and European ethics committees (Comité Régional
78 d'Ethique en Expérimentation Animale N°3 de la région Ile de France).

79

80 **Treatment of primary culture of murine chondrocytes**

81 After 24-h incubation in basal experimental medium, murine chondrocytes were
82 stimulated for 24 or 72 h with IL-1 β (5 ng/mL) (PeproTech, Rocky Hill, NJ, USA)
83 under normal-glucose (5.5 mM) or high-glucose (25 mM) conditions. Cell lysates
84 were collected for mRNA extractions and supernatants for assays. The dose of IL-1 β
85 was considered efficient after a dose-effect experiment (data not shown).

86 To investigate whether the effect of high glucose was related to its osmotic effect,
87 cells were incubated with mannitol (19.5 mM) (Sigma-Aldrich, Lyon, France) instead
88 of glucose (25 mM) for 24 and 72 h, with or without IL-1 β (5 ng/mL) in 4 separate
89 experiments.

90 For mechanistic studies, chondrocytes cultured with normal or high glucose for 72 h
91 with or without IL-1 β (5 ng/mL) were pretreated or treated with inhibitors: cytochalasin
92 B (1 μ M [Sigma-Aldrich]), a glucose transporter inhibitor; epalrestat (10 μ M [Sigma-
93 Aldrich]), a specific inhibitor of aldose reductase, the rate limiting enzyme of the
94 polyol pathway; MitoTEMPO (50 μ M [Santa Cruz Biotechnology, Heidelberg,
95 Germany]), a specific scavenger of mitochondrial ROS; or L-NAME, a non-specific
96 inhibitor of NO synthase (5 mM [Sigma-Aldrich]). The doses of these inhibitors were
97 chosen according to dose-effect experiment (data not shown) and literature data.

98 To analyze the cytotoxic effects of the treatments, we analyzed cell viability by the
99 Cytotoxicity Detection Kit for Lactate Dehydrogenase (LDH) (Roche, Mannheim,
100 Germany). All measurements were performed in duplicate and the mean of

101 duplicates from one litter of mice was considered as one experiment, for each
102 condition. This method of measurement was used for all experiments in this study.

103

104 **RNA extraction and quantitative RT-PCR (qRT-PCR)**

105 Total RNA was extracted from chondrocytes with use of the ReliaPrep RNA Cell
106 Miniprep System kit (Promega, Madison, WI, USA) and concentrations were
107 determined by spectrophotometry (Eppendorf, Le Pecq, France). Reverse
108 transcription involved 500 ng total RNA with the Omniscript RT kit (Qiagen). mRNA
109 levels of IL-6 and cyclooxygenase 2 (COX2) were quantified with Light Cycler LC480
110 (Roche Diagnostics, Indianapolis, IN, USA). PCR amplification conditions were: initial
111 denaturation for 5 min at 95°C followed by 40 cycle s consisting of 10 s at 95°C, 15 s
112 at 60°C and 10 s at 72°C. Product formation was detected at 72°C in the fluorescein
113 isothiocyanate channel. Levels of mRNA were normalized to those of murine
114 hypoxanthine guanine phosphoribosyltransferase (HPRT). Specific mouse primer
115 sequences were for IL-6, forward 5'-GTCACAGAAGGAGTGGCTA-3', and reverse
116 5'-AGAGAACAACATAAGTCAGATAACC-3'; COX2, forward 5'-
117 GTGCCTGGTCTGATGATGTA-3', and reverse 5'-AGTTTGAAGTGGTAACCGC-3';
118 and HPRT, forward 5'-AGGACCTCTCGAAGTGT-3', and reverse 5'-
119 ATTCAAATCCCTGAAGTACTCAT-3'. All measurements were performed in
120 duplicate.

121

122 **IL-6 and PGE₂ production**

123 Murine IL-6 concentration was measured with the Quantikine enzyme-linked
124 immunosorbent assay (ELISA) kit (R&D systems, Lille, France). Human IL-6
125 concentration was measured in human CM with the Pelikine compact kit (Sanquin,

126 Amsterdam, Netherlands). Murine and human PGE₂ concentrations in CM were
127 measured with the enzymatic immunoassay (EIA) kit (Cayman Chemical, Ann Arbor,
128 MI, USA). The limits of detection were 7.8, 0.6 and 7.8 pg/mL, respectively. All
129 measurements were performed in duplicate.

130

131 **Glucose uptake**

132 Chondrocytes were seeded and cultured as described above. After 24- or 72-h
133 stimulation with or without IL-1 β under normal- or high-glucose conditions, cells were
134 washed in PBS and incubated for 2 h in a KRP pyruvate buffer 1 mM, and pH 7.6
135 (Hepes 12.5 mM, NaCl 120 mM, KCl 5 mM, MgSO₄ 7 H₂O 1.2 mM, CaCl₂ 1 mM,
136 Na₂HPO₄ 1 mM, sodium pyruvate 2 mM, BSA 2%). To analyse the role of
137 intracellular glucose, glucose transport was inhibited by cytochalasin B (50 μ M)
138 added for 20 min at 37°C. Then, the radioactive 2DG* mixture (2-deoxy-D-[1-
139 ¹⁴C]glucose [0.25 μ Ci/well], cold 2-deoxy-D-glucose [48 mg/mL] diluted in KRP
140 pyruvate buffer) was added for incubation for 8 min at 37°C. Cells were washed twice
141 in PBS and incubated in 0.1% SDS for 30 min at 22°C. Cells lysates were collected
142 in a counting vial and rinsed in 500 μ L H₂O. A volume of 50 μ L was collected for
143 assay of intracellular protein concentration with the Protein Assay kit (Bio-Rad), and
144 radioactivity (disintegration per minute) was measured in duplicates by use of a beta
145 counter (Hidex, Turku, Finland). Data are expressed as disintegrations per min
146 (DPM) reported for 1 μ g intracellular protein. Three separate experiments were
147 performed with three different litters of mice.

148

149 **Cellular ROS production**

150 Chondrocytes were seeded and cultured as described above in 96-well plates at
151 1×10^4 per well. After 24- and 72-h treatment, ROS production was measured by
152 fluorometric assay with dichlorodihydrofluorescein diacetate (DCFDA) (Molecular
153 Probes, Life Technologies) and colorimetric assay with nitroblue tetrazolium (NBT)
154 (Sigma-Aldrich). For DCFDA assay, which is directly oxidized by ROS such as
155 superoxide ion, hydrogen peroxide and hydroxyl, chondrocytes were incubated in
156 obscurity with 17 μ M DCFDA for 60 min at 37°C, then washed in PBS, and
157 fluorescence was measured with the Fluostar Galaxy reader (BMG Labtech,
158 Ortenberg, Germany) at 485 nm excitation and 520 nm emission and analysed by
159 use of Biolise (Labsystems, Helsinki, Finland). For NBT assay, which detects the
160 activity of oxidoreductases, chondrocytes were incubated with 2 mg/mL NBT for 2
161 hours at 37°C, in obscurity, then disrupted with KOH-DMSO, and the amount of
162 coloration was measured by a spectrophotometer at 560 nm. To normalize the
163 results, intracellular proteins were collected with NaOH (0.5 M) and concentrations
164 were measured with a spectrophotometer and the Protein Assay kit (Bio-Rad). ROS
165 concentrations are given as fold induction from that of the control and by microgram
166 protein. All measurements were performed in triplicate.

167

168 **NO release**

169 Nitrite (NO_2^-) production was measured by Griess reaction with the DetectX Nitric
170 Oxide Detection Kit (Arbor Assays, Eisenhower, Michigan, USA) in CM from
171 chondrocytes treated or not with IL-1 β in normal or high glucose with or without the
172 inhibitor L-NAME. The nitrite production reflects NO production. The sample reacts
173 with sulphanilamide and N-(1-Naphtyl) ethylenediamine and the concentration is
174 determined by comparing the optical density at 540 nm in a standard range with

175 minimum detection limit 3.125 μM . Nitrite measurements were performed in
176 duplicate.

177

178 **Statistical analysis**

179 All data are reported as points representing one single experiment from one litter of
180 mice or one patient with 95% ci. All tests were non-parametric and were analyzed by
181 use of GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The Wilcoxon
182 paired test was used for all mice experiments and for the human experiments
183 comparing the induction of IL-6 and PGE_2 w/o IL-1 β . Mann-Whitney unpaired test
184 was used for the comparison between non diabetic and diabetic patients. To
185 compare clinical characteristics of diabetic versus non-diabetic patients, Chi-square
186 test and Mann-Whitney was used for categorical and quantitative variables,
187 respectively. $P \leq 0.05$ was considered statistically significant.

188

189 **RESULTS**

190 **Increased IL-1 β -induced IL-6 and PGE_2 production in OA cartilage from** 191 **diabetic versus non-diabetic patients**

192 We used 5 cartilage explants from OA type 2 DM patients and 5 from OA non-
193 diabetic patients matched on age and BMI. The diabetic and non-diabetic OA
194 patients did not differ in other characteristics, including sex ratio, hypertension,
195 dyslipidemia and creatininemia (**Table 1**).

196 To characterize the inflammatory features of OA cartilage, we analysed the capacity
197 of explants from diabetic and non-diabetic patients to release IL-6 and PGE_2 in CM
198 after 24-h IL-1 β stimulation (5 ng/mL), in similar glucose concentration. First, in the
199 absence of IL-1 β , there was a non-significant trend for a spontaneous increased

200 production of IL-6 and PGE₂ in cartilage from diabetic and non-diabetic patients
201 (p=0.15 and 0.27, respectively) (**Figure 1**). Second, after IL-1 β stimulation, the
202 release of IL-6 and PGE₂ was significantly increased in DM and non-DM patients,
203 with higher levels in diabetic than non-diabetic patients (3-fold for non-DM against
204 4.5-fold for DM for IL-6; 1.5-fold for non-DM against 2.7-fold for DM for PGE₂)
205 (**Figure 1**).

206

207 **High glucose enhances IL-1 β -induced IL-6 and PGE₂ production by murine** 208 **chondrocytes**

209 Because human OA cartilage explants from DM patients showed a stronger
210 inflammatory phenotype than non-DM cartilage especially after IL-1 β stimulation, we
211 speculated that sustained extracellular high glucose exposure could be one of the
212 actors in this responsiveness. Thus, we explored *in vitro* the mechanistic impact of
213 high glucose on murine chondrocyte activation induced by IL-1 β . Murine cells were
214 treated or not with IL-1 β (5 ng/mL) for 24 or 72 h with normal glucose (5.5 mM) or
215 high glucose (25 mM) (**Figure 2**). As expected, IL-1 β increased IL-6 mRNA
216 expression and protein release as well as COX2 mRNA expression and PGE₂
217 release, regardless of glucose concentration (**Figure 2A and 2B**). However, with IL-
218 1 β stimulation at 72 h, IL-6 mRNA and protein levels and PGE₂ release were higher
219 with high than normal glucose (4- to 7-fold increase). At 24 h with IL-1 β stimulation,
220 IL-6 mRNA and protein levels and PGE₂ release were higher but not significantly,
221 except for COX2 expression which was significantly higher with high than normal
222 glucose. Conversely, high glucose alone (without IL-1 β stimulation) did not change
223 the expression or production of the two pro-inflammatory mediators as compared
224 with normal glucose at 24 and 72 h. Thus, a high-glucose environment enhanced the

225 chondrocyte response to IL-1 β stimulation after 72 h. Because the effects of high
226 glucose and IL-1 β stimulation were higher at 72 than 24 h, we chose the longer
227 incubation time for mechanistic experiments.

228

229 **Role of glucose uptake and the polyol pathway in the increased IL-1 β -activated** 230 **pro-inflammatory phenotype induced by high glucose**

231 As a control experiment, we first measured glucose uptake in murine chondrocytes
232 by using 2-deoxyglucose, a non-metabolized analogue of glucose, with the same
233 experimental schedule (**Figure 3A**). At 24 h, and even more so at 72 h, glucose
234 uptake was increased with IL-1 β treatment under normal-glucose conditions.
235 Additionally, high glucose further increased glucose uptake, which was at its peak
236 rate at 72 h (p for trend = 0.12). Changes in glucose concentration without IL-1 β
237 moderately changed glucose uptake, which suggests a role for IL-1 β in glucose
238 transport variation.

239 To analyse the possibility that the effect of high glucose level was due to osmotic
240 stress, we replaced glucose excess (19.5 mM) by mannitol at the same molar
241 concentration, as a control for the hyperosmotic effects of high glucose level (**Figure**
242 **3B**). Chondrocytes stimulated with IL-1 β in the presence of mannitol released
243 amounts of IL-6 similar to that under normal glucose conditions ($p=0.12$) at 24 and 72
244 h, but we found a trend for a difference between expression under high glucose and
245 mannitol ($p=0.06$). Therefore, we could rule out the impact of osmotic stress in the
246 pro-inflammatory effect of high glucose environment.

247 To determine the direct role of glucose uptake, cells were pre-treated for 30 min with
248 cytochalasin B (1 μ M), a glucose transport inhibitor (**Figure 4A**), and IL-6 release
249 was measured. Under normal-glucose conditions, the IL-1 β -increased IL-6 level was

250 not modified by cytochalasin B. Conversely, under high-glucose conditions, the
251 enhanced IL-6 production was completely blunted as compared with the normal-
252 glucose condition, which suggests that the enhanced pro-inflammatory phenotype
253 acquired by the chondrocytes could result from high glucose uptake.

254 With increased intracellular glucose, the glycolytic pathway is saturated, which leads
255 the activated secondary polyol metabolic pathway (15). Aldose reductase and
256 sorbitol dehydrogenase are the two rate-limiting enzymes in this pathway and
257 transform excessive glucose into sorbitol and fructose. To address the involvement of
258 the polyol pathway in the pro-inflammatory phenotype of chondrocytes with high
259 glucose, we incubated cells with or without IL-1 β in normal or high glucose for 72 h
260 with a specific inhibitor of aldose reductase, epalrestat (10 μ M) (**Figure 4B**). Under
261 normal glucose, epalrestat significantly decreased the inflammatory effect of IL-1 β (-
262 28%, $p < 0.05$). This inhibition was stronger under high glucose, with a decrease of
263 62% in IL-6 release with IL-1 β stimulation, thus completely erasing the potentiating
264 effect of high glucose. With epalrestat treatment, PGE₂ production by IL-1 β -
265 stimulated chondrocytes was similarly reduced (-47% and -88% under normal and
266 high glucose, respectively) (**Supplementary Figure S1**).

267

268 **High glucose increases the oxidative stress induced by IL-1 β**

269 To determine whether excess glucose may potentiate chondrocyte activation by
270 producing ROS and reactive nitrogen species, we measured the production of ROS
271 by fluorescent DCFDA (**Figure 5A**) and NBT (**Figure 5B**) and the production of
272 nitrite, as a reflection of NO, by the Griess reaction (**Figure 5C**). ROS production was
273 not modified with high glucose or IL-1 β stimulation separately, but with IL-1 β
274 stimulation and high glucose, ROS production was enhanced at 24 h and especially

275 at 72 h (**Figures 5A and B**). IL-1 β stimulated nitrite production whatever the glucose
276 concentration at 24 and 72 h (**Figure 5C**). Interestingly, with IL-1 β stimulation, nitrite
277 generation was significantly higher under high- than normal-glucose conditions,
278 especially at 72 h. These results stressed the role of IL-1 β and high glucose
279 combined in the formation of oxidative stress derivatives.

280 To investigate whether these findings could be responsible for the pro-inflammatory
281 effect in chondrocytes, cells were treated with MitoTEMPO (50 μ M), a specific
282 mitochondrial ROS scavenger, or L-NAME (5 mM), an inhibitor of NO-synthase. LDH
283 measurement revealed no cytotoxic effects at these concentrations (data not shown).
284 Under normal glucose, neither MitoTEMPO nor L-NAME modified IL-1 β -increased IL-
285 6 production (**Figures 6A and 6D**). However, under high glucose, IL-1 β -increased IL-
286 6 production was inhibited with MitoTEMPO (-40%, $p=0.06$) and L-NAME (-38%;
287 $p<0.05$). In addition, L-NAME but not MitoTempo could inhibit IL-1 β -increased PGE₂
288 production under high glucose (-78%; $p<0.05$) (**Supplementary Figure S2**).

289

290 **DISCUSSION**

291 To understand the epidemiological link between diabetes mellitus and OA, we
292 phenotyped OA cartilage from diabetic and non-diabetic patients according to
293 responsiveness to IL-1 β -induced inflammatory stress, as assessed by IL-6 and PGE₂
294 release. Such increased reactivity was corroborated by sensitization of articular
295 chondrocytes to a high-glucose environment with IL-1 β stimulation. This enhanced
296 pro-inflammatory response under high glucose conditions was related, at least in
297 part, to increased glucose uptake, oxidative stress and activation of the polyol
298 pathway and resulted in a sustained chondrocytic pro-inflammatory phenotype
299 **(Figure 7)**.

300 First, we aimed to assess the spontaneous release of IL-6 and PGE₂ by human
301 cartilage from diabetic *versus* non-diabetic patients exposed to a high glucose level
302 in the years before arthroplasty. And, because the low-grade inflammation observed
303 in type 2 DM and OA involves IL-1 β , we investigated the inflammatory response of
304 diabetic and non-diabetic OA cartilage to IL-1 β . We carefully selected OA cartilage
305 explants from humans matched on age and BMI to diabetic patients and found no
306 differences in sex ratio and other metabolic co-morbidities. All diabetic patients
307 received at least one anti-diabetic therapy. Unfortunately, we could not analyse the
308 role of specific drugs because of lack of statistical power. The mean values of HbA1c
309 fraction in our patients reflects a good glycemia control for the last 3 months before
310 surgery, but does not reflect all the diabetes history and not preclude previous
311 uncontrolled DM periods with chronic hyperglycemia. Finally, cartilage was not
312 histologically scored, but because all patients underwent total knee replacement, we
313 assume that OA tissular lesions were at an advanced stage in all cases.

314 *In vitro*, we demonstrated increased glucose uptake by articular chondrocytes under
315 a high-glucose condition, especially with IL-1 β stimulation. IL-1 β was already found
316 to increase the synthesis of the inducible glucose transporters GLUT-1 and -9 in
317 articular chondrocytes (30). We found similar results for GLUT-1 (data not shown).
318 Thus, under normal-glucose conditions, glucose uptake was moderately stimulated
319 by IL-1 β , despite IL-1 β -increased expression of GLUT-1. This uptake was markedly
320 increased when cells were cultured under high-glucose conditions. Treating articular
321 chondrocytes with cytochalasin B abolished the additive pro-inflammatory effect of
322 high glucose in IL-1 β -stimulated chondrocytes, which confirms the role of glucose
323 uptake in chondrocyte activation. Although cytochalasin B is usually used as a
324 glucose transport inhibitor (31), this compound can be responsible for damages of
325 the microfilament network, and thus can alter mechanical environment of the cell.
326 However, we first can observe that cytochalasin B treatment in normal glucose
327 condition, under IL-1 β stimulation, has no effect on IL-6 production, suggesting that
328 cytochalasin B reverses specifically the potentiating effect of high glucose. Second,
329 2DG* experiments have confirmed that, concomitantly to chondrocytes activation,
330 there is an increased glucose entrance in high glucose condition in presence of IL-
331 1 β . We then can speculate that the effect of cytochalasin B is related here to glucose
332 uptake effect, not to a global disruption of the microfilament network.

333 Under high-glucose conditions, the glycolytic pathway may be saturated, for
334 increased flux of available glucose through the alternative polyol pathway (15).
335 Increased activity of the polyol pathway, which occurs especially when the glycolytic
336 pathway is saturated by glucose excess, is an important pathogenic factor in diabetic
337 complications that are beyond glycemic control, because of sorbitol accumulation
338 (32-35). Considering OA as such, we aimed to address the involvement of this

339 pathway in the potentiation of chondrocyte activation induced by high glucose. To
340 that end, we treated articular chondrocytes with epalrestat, a specific inhibitor of
341 aldose reductase, which has had promising results in preventing neuropathy
342 progression and is currently used in Japan as an oral drug in clinical practice for this
343 complication (36-38). We found a significant inhibition of the potentiating effect of
344 high glucose for both IL-6 and PGE₂ production. The production of both mediators
345 was decreased to a lesser extent under normal glucose conditions when
346 chondrocytes were treated with epalrestat, which suggests that the polyol pathway
347 may participate in IL-1 β stimulation of chondrocytes by high glucose.

348 Finally, we studied the impact of high-glucose conditions on direct synthesis of ROS
349 by articular chondrocytes using 2 different methods, as well as its potentiating effect
350 on NO production induced by IL-1 β . At 72h, IL-1 β behaved differently under high and
351 normal glucose conditions and may be responsible for early oxidative stress only
352 under high glucose, for subsequent IL-6 production.

353 We used the antioxidant MitoTEMPO and an inhibitor of NO-synthase, L-NAME. In
354 healthy chondrocytes, the production of energy from glucose metabolism is mainly
355 from the glycolytic pathway and mitochondria are poorly solicited (39). In a pro-
356 inflammatory and high-glucose environment, the mitochondrial respiratory chain
357 could be more activated and thus increase mitochondrial ROS production. Moreover,
358 superoxide ion (O₂^{•-}), mainly produced by mitochondria, is the main product
359 responsible for the formation of other ROS (19). Thus, we treated chondrocytes with
360 a specific scavenger of mitochondrial ROS, MitoTEMPO, and found inhibition of IL-6
361 production (p=0.06), which supports that ROS production induced by high glucose
362 was responsible at least in part for the production of pro-inflammatory mediators.
363 Additionally, the use of the inhibitor L-NAME suggested the involvement of NO in the

364 potentiating effect of high glucose in pro-inflammatory effects because we found a
365 significant inhibition of IL-6 and PGE₂ production.

366 Excessive production of both superoxide ion and NO can lead to peroxynitrite
367 formation, an extremely reactive compound responsible for DNA lesions and lipid
368 peroxidation and thus deleterious for tissues (40, 41). This molecule is involved in a
369 wide range of diseases, including diabetes and OA (42, 43).

370 This study has some limitations. First, in our human study, we cannot exclude that
371 some patients from the non-diabetic group were undiagnosed. However, including
372 diabetic patients in the non-diabetic group may underestimate the difference in
373 release of inflammatory mediators.

374 Second, we performed our mechanistic experiments on primary cultures of murine
375 but not human chondrocytes. Indeed, to investigate the effect of glucose and/or IL-1 β
376 on chondrocyte activation, we needed to use normal chondrocytes to rule out the
377 potential impact of diabetic status. Because of the challenge in obtaining normal
378 human cartilage explants, we used murine cells. We previously compared the
379 phenotype of murine and human chondrocytes and showed that the main markers of
380 differentiation were similar (28-29). Moreover, the similarity in terms of IL-6 and PGE₂
381 release by IL-1 β -stimulated murine chondrocytes under high glucose and by diabetic
382 cartilage explants strengthen the use of murine cells for mechanistic experiments.

383 Finally, we investigated some pathways involved in the molecular consequences of
384 glucose excess (i.e., ROS, glucose uptake, polyol pathway). However, glucose may
385 also act through numerous other pathways such as the hexosamine pathway, protein
386 kinase C activation or the production of AGEs, also involved in the glucotoxicity
387 process seen in DM, but not explored in the present study (15). ROS blockade and
388 polyol inhibition could prevent reactivity to high glucose, which illustrates their crucial

389 role in chondrocyte activation due to hyperglycemia. While the use of antioxidants
390 has led to deceptive results in OA, epalrestat may be useful in this articular
391 complication of diabetes. The enhanced degradation of extracellular matrix by the
392 polyol pathway in intervertebral discs of diabetic rats supports such a hypothesis
393 (44).

394 Moreover, beyond the effect of high glucose, other molecules might be involved to
395 explain higher responsiveness of human cartilage from diabetic patients to
396 inflammatory stress. Indeed, type 2 DM is characterized by hyperglycemia,
397 hyperinsulinemia with insulin resistance and free fatty acids increase (45, 46). Some
398 studies have examined the role of insulin in joint tissues and have shown an anabolic
399 role of this hormone with the induction of the synthesis of type II collagen and
400 proteoglycans by chondrocytes (47-49). However, the effect of insulin on joint cells
401 and tissues or of insulin resistance in OA needs to be addressed. Activity of the
402 insulin receptor was found lower in human OA than healthy chondrocytes, which
403 limits the beneficial effects of this hormone on cells during OA (50).

404 In conclusion, OA cartilages from diabetic patients are more reactive than non-
405 diabetic to pro-inflammatory stress, thus displaying an inflammatory phenotype. This
406 phenotype may be due to glucotoxicity combined with IL1- β stress and then
407 responsible for sustained inflammatory chondrocyte activation involving increased
408 GLUT expression, glucose uptake, oxidative stress and the polyol pathway. These
409 results strengthen the hypothesis that diabetes could be a trigger for the initiation
410 and/or the severity of metabolic OA and open up the opportunity to prevent OA
411 initiation and/or OA progression by optimal control of glycemia in this subset of OA
412 patients.

413

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418

419 **Author contributions** M-CL, AC, XH, MA, JC, BF, FB and JS were responsible for
420 the study design, manuscript preparation, and interpretation of the data. AS collected
421 human tissue samples and participated in the study design of experiments with
422 human tissue and in the interpretation of the data. M-CL and AC performed the
423 experiments. MA contributed to the *in vitro* experiments of glucose uptake. All
424 authors reviewed and approved the final manuscript.

425

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432

433 **Conflict of interests** None.

434

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581
582

583 **TABLE**
584

585 **Table 1:** Clinical details and pathological characteristics of patients with osteoarthritis
586 of the knee with or without type 2 diabetes mellitus.

Clinical or pathological characteristics	Type 2 diabetes mellitus (n= 5)	Non-type 2 diabetes mellitus (n=5)	P value
Age (years)	62 ± 7.8	69,6 ± 8.6	0.2934
Female	2 (33%)	3 (60%)	0.5271
Weight (kg)	87.6 ± 15.43	82.6 ± 18.71	>0.99
BMI (kg/m²)	30.6 ±5.95	29.4 ± 5.46	0.90
Obesity (BMI ≥30 kg/m²)	2 (40%)	2 (40%)	>0.99
Hypertension	3 (60%)	3 (60%)	>0.99
Dyslipidemia	1 (20%)	1 (20%)	>0.99
Creatinine level (µmol/L)	69 ± 4.6	74.6 ± 24.9	0.88
Fasting plasma glucose (mmol/L)	7.5 ± 2.0	NA	NA
HbA1c (%)	6.27 ± 0.7	NA	NA
Treatments	Metformin (n=5), Sitagliptin (n=1), Glimepiride (n=1), Gliclazide (n=1), Vildagliptin (n=1)	NA	NA

587 Data are mean ± SD or n (%). BMI = body mass index. HbA1c = hemoglobin A1c. NA
588 = not applicable. All patients had osteoarthritis. Chi-square and Mann-Whitney tests
589 were used for categorical and continuous variables, respectively.

590

591

592 **FIGURE LEGENDS**

593 **Figure 1: Responsiveness of cartilage explants from osteoarthritic (OA)**
594 **diabetic and non-diabetic patients to interleukin 1 β (IL-1 β) stimulation.** IL-6 and
595 prostaglandin E₂ (PGE₂) release by cartilage explants with or without IL-1 β
596 stimulation (5 ng/mL). Each symbol represents a patient, non-diabetic (ND) n=5 (○)
597 or diabetic (D) n=5 (●). The bar represents the mean for each condition and 95%
598 confidence interval is represented. † $P=0.016$, * $P=0.03$, ‡ $P=0.048$.

599
600 **Figure 2: Potentiating effect of high glucose on pro-inflammatory induction of**
601 **murine chondrocytes stimulated with IL-1 β .** Induction of pro-inflammatory
602 mediators with normal glucose (5 mM) and high glucose (25 mM) in murine
603 chondrocytes treated with IL-1 β (5 ng/mL) at 24 and 72 h. **A**, Quantitative RT-PCR
604 (qRT-PCR) analysis of mRNA levels of IL-6 and cyclooxygenase 2 (COX2) relative to
605 that of hypoxanthine guanine phosphoribosyltransferase (HPRT), n=5 for each
606 condition. **B**, IL-6 and PGE₂ release, n=5 for each condition. Each symbol represents
607 an experiment from one litter of mice in normal glucose (○) and high glucose (●)
608 conditions. The bar represents the mean for each condition and 95% confidence
609 interval is represented. * $P=0.031$.

610
611 **Figure 3: Effect of high glucose on radioactive 2-deoxyglucose uptake and of**
612 **osmotic stress in murine chondrocytes stimulated with IL-1 β .** **A**, Assay of
613 glucose uptake by murine chondrocytes. Measurement of disintegration per minute
614 (DPM) of the intracellular radioactive 2-deoxyglucose for total intracellular protein,
615 n=3 for each condition. **B**, Impact of osmotic stress on the expression of IL-6 by
616 murine chondrocytes with or without IL-1 β stimulation. Cells were incubated with

617 normal glucose (5.5 mM, ○), high glucose (25 mM, ●) or mannitol (19.5 mM, ⊗). qRT-
618 PCR analysis of mRNA levels of IL-6 relative to that of HPRT, n=4 for each condition.
619 Each symbol represents an experiment from one litter of mice. The bar represents
620 the mean for each condition.

621

622 **Figure 4: Involvement of high glucose uptake and polyol pathway activation in**
623 **murine chondrocytes stimulated with IL-1 β .** **A**, Involvement of glucose uptake on
624 pro-inflammatory chondrocyte activation. Cells were pre-treated for 30 min with
625 cytochalasin B (Cyt; 1 μ M). Protein release of IL-6, n=5 for each condition. **B**,
626 Involvement of polyol pathway activation in chondrocyte activation with normal or
627 high glucose in IL-1 β -stimulated murine chondrocytes. Cells were treated with
628 epalrestat (Epal, 10 μ M). Protein release of IL-6, n=5 for each condition. Each
629 symbol represents an experiment from one litter of mice in normal glucose (○) and
630 high glucose (●) conditions. The bar represents the mean for each condition and
631 95% ci is represented.* $P=0.031$.

632

633 **Figure 5: Effect of high glucose and pro-inflammatory stimulation with IL-1 β on**
634 **generation of oxidative stress reagents by murine chondrocytes.** Measurement
635 of ROS production by fluorimetric assay of DCFDA (**A**) and by colorimetric assay of
636 NBT (**B**), n=5 for each condition. Data are fold induction compared to the control
637 condition without IL-1 β at 24 h and at 72 h, for intracellular protein quantity. **C**, Assay
638 of extracellular nitrite production, n=5 for each condition. Each symbol represents an
639 experiment from one litter of mice in normal glucose (○) and high glucose (●)
640 conditions. The bar represents the mean for each condition and 95% confidence
641 interval is represented.* $P=0.031$.

642

643 **Figure 6: Involvement of high glucose and IL-1 β -induced oxidative stress in the**
644 **pro-inflammatory profile induction of murine chondrocytes.** Protein release of
645 IL-6. Cells were treated with a specific mitochondrial ROS scavenger, MitoTEMPO
646 (50 μ M) (**A**, n=4 for each condition) and an inhibitor of NO-synthase, L-NAME (**B**,
647 n=5 for each condition). Each symbol represents an experiment from one litter of
648 mice in normal glucose (\circ) and high glucose (\bullet) conditions. The bar represents the
649 mean for each condition and 95% confidence interval is represented.* $P=0.031$.

650

651 **Figure 7: Hypothetical scheme of high-glucose conditions potentiating the pro-**
652 **inflammatory effect of IL-1 β on articular chondrocytes.** Under high glucose,
653 glucose uptake is increased when cells are in a pro-inflammatory condition (i.e., IL-1 β
654 stimulation) induced by increased expression of glucose transporters GLUT-1 and -9.
655 Intracellular glucose increase is responsible for the potentiation of the pro-
656 inflammatory effect of IL-1 β by activating the alternative polyol pathway, the
657 production of mitochondrial ROS and inducible nitric oxide synthase (iNOS) for the
658 production of NO. This activation leads to IL-6 and PGE₂ release.

659 **Supplementary file**

660 **Figure S1: Polyol pathway involvement in murine chondrocytes stimulated with**
661 **IL-1 β .** Cells were treated with epalrestat (Epal, 10 μ M). Protein release of PGE₂, n=5
662 for each condition. Each symbol represents an experiment from one litter of mice in
663 normal glucose (\circ) and high glucose (\bullet) conditions. The bar represents the mean for
664 each condition and 95% ci is represented.* $P=0.031$.

665

666 **Figure S2: Involvement of nitric oxide in the pro-inflammatory profile induction**
667 **of high-glucose and IL-1 β -stimulated chondrocytes.** Cells were treated with an
668 inhibitor of NO-synthase, L-NAME (D). Protein release of PGE₂, n=5 for each
669 condition. Each symbol represents an experiment from one litter of mice in normal
670 glucose (\circ) and high glucose (\bullet) conditions. The bar represents the mean for each
671 condition and 95% ci is represented.* $P=0.031$.

Figure 1

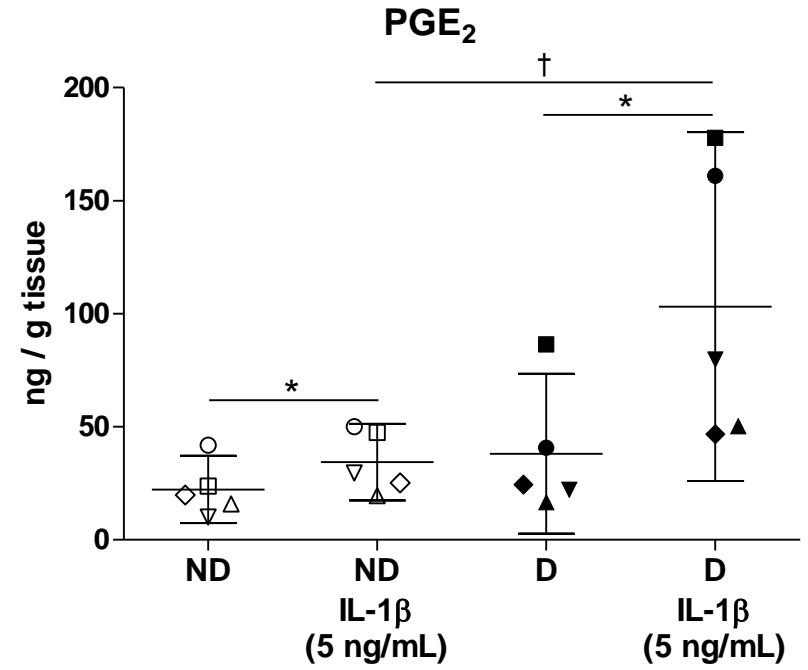
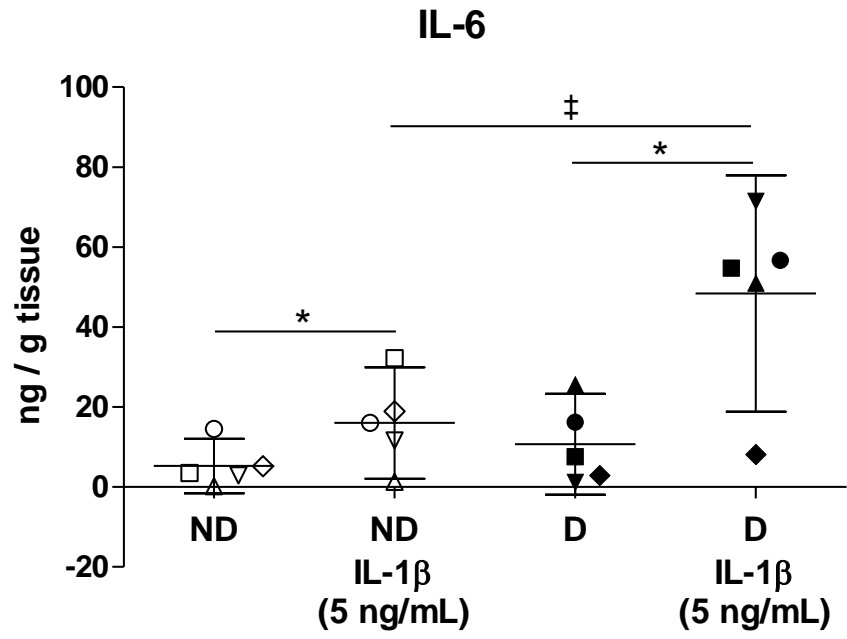
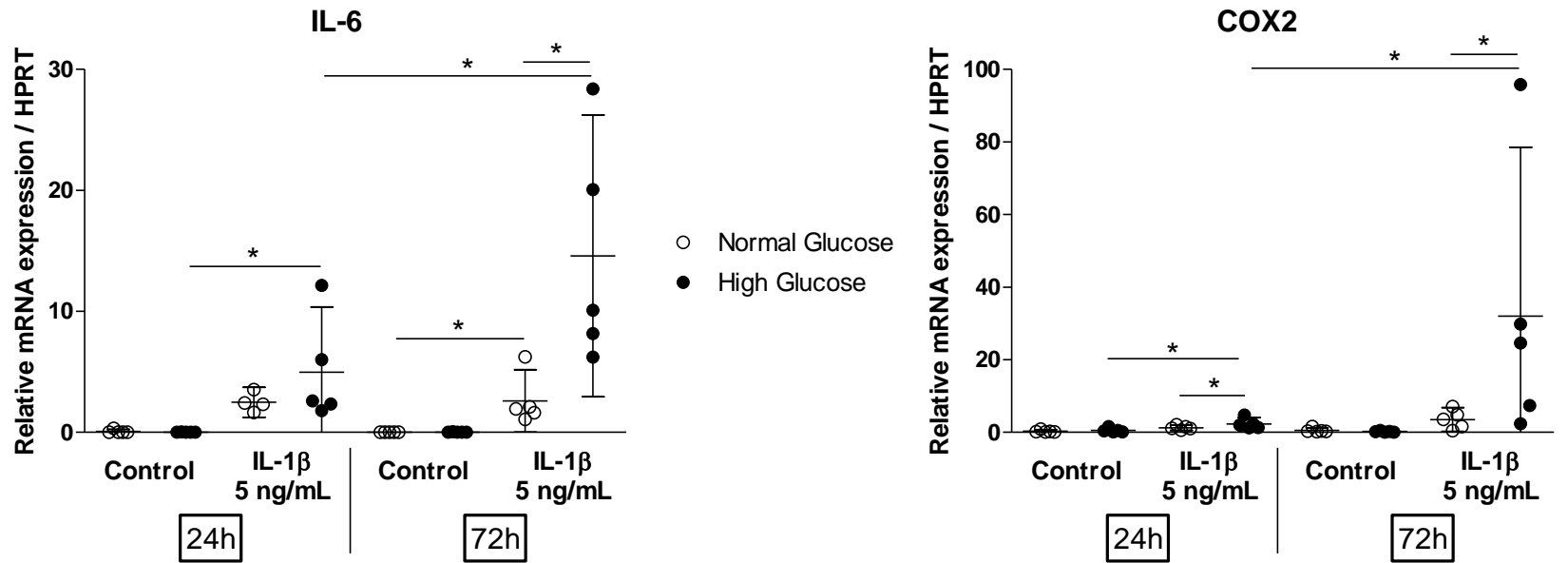


Figure 2

A



B

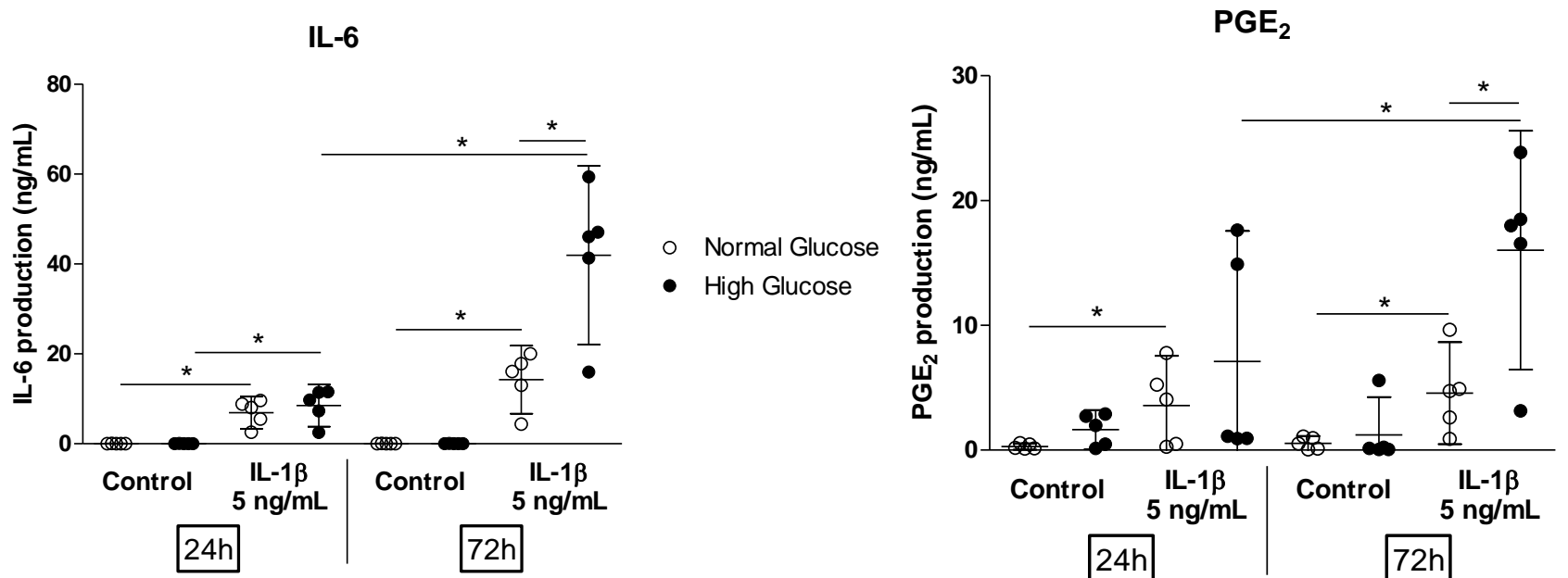


Figure 3

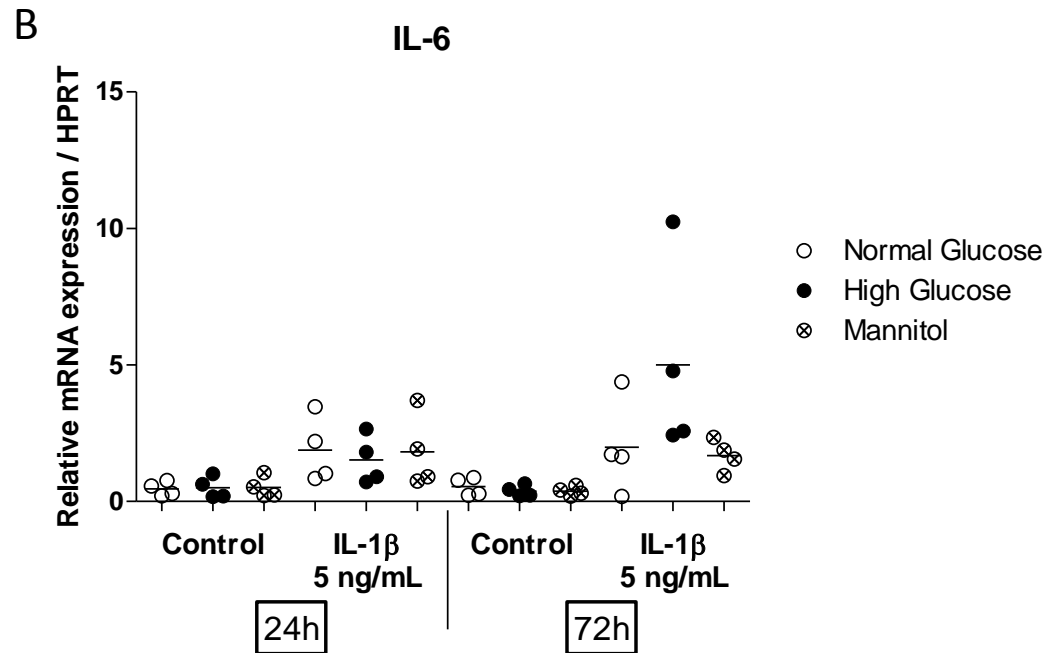
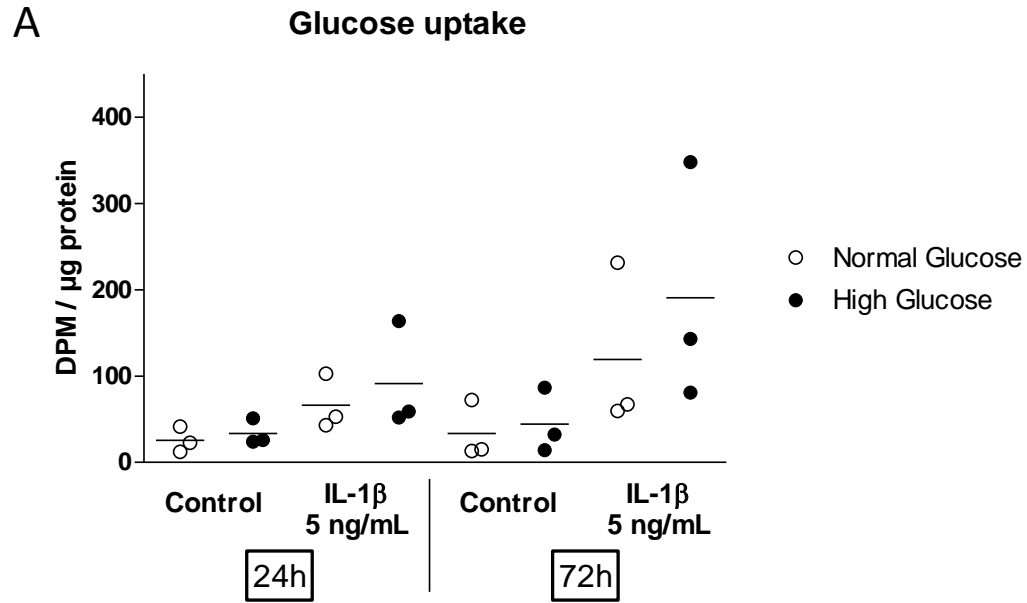
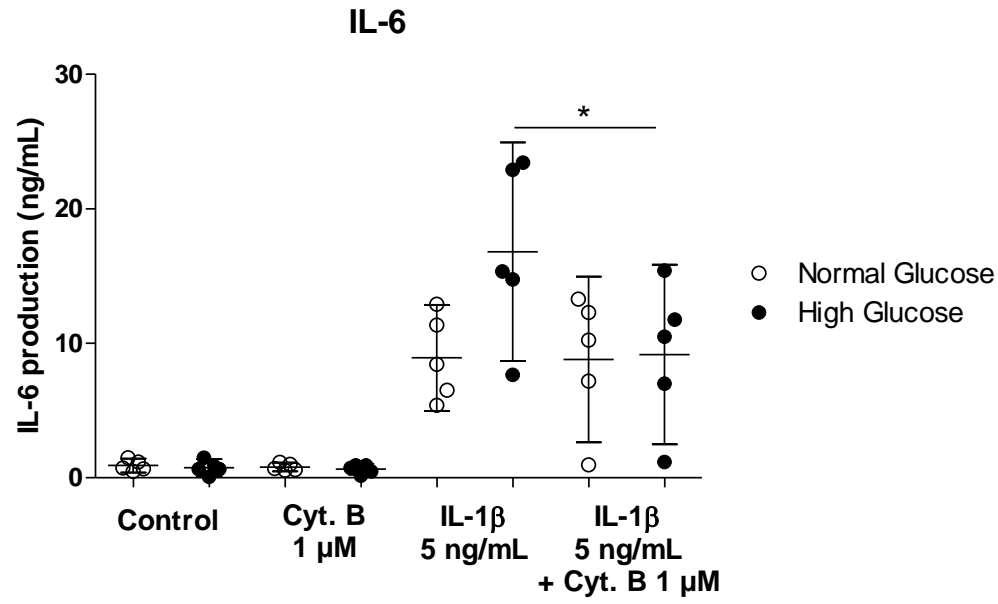


Figure 4

A



B

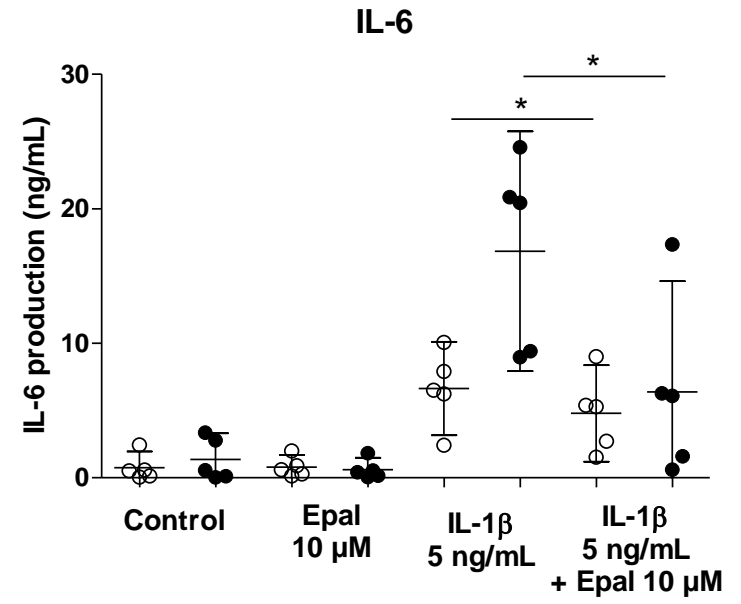
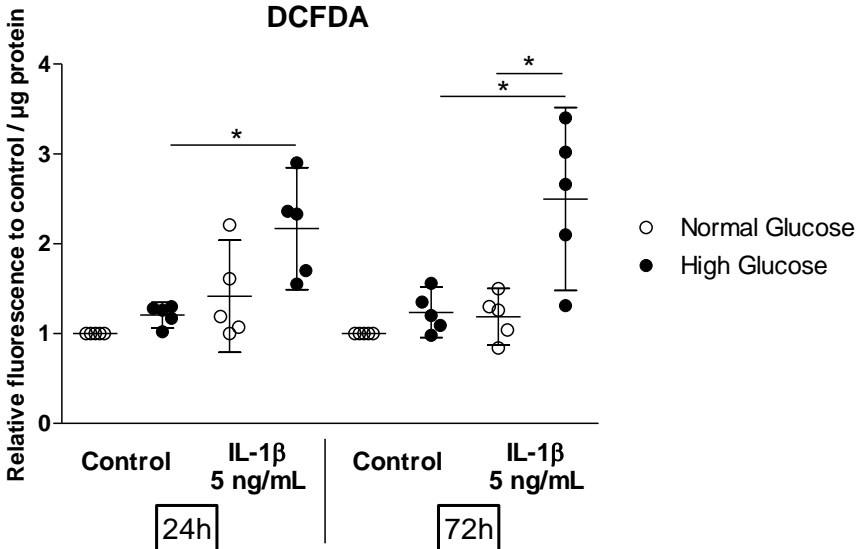
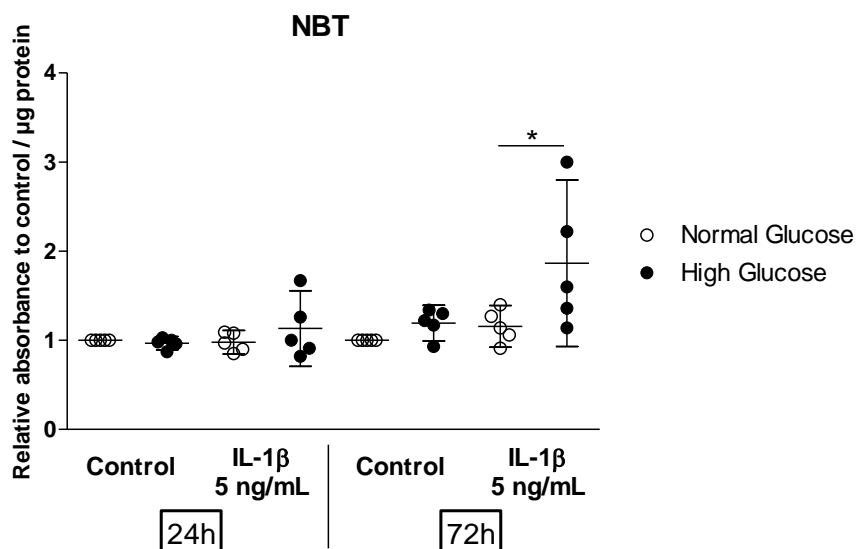


Figure 5

A



B



C

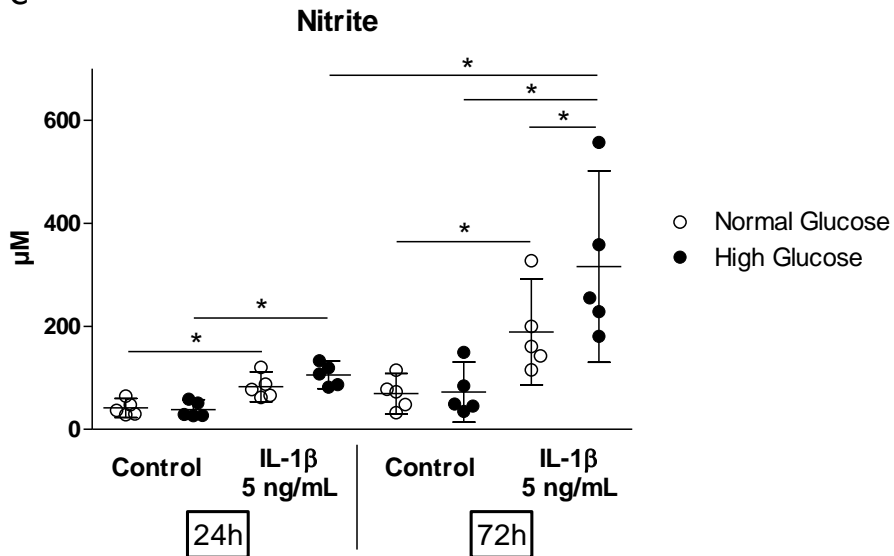
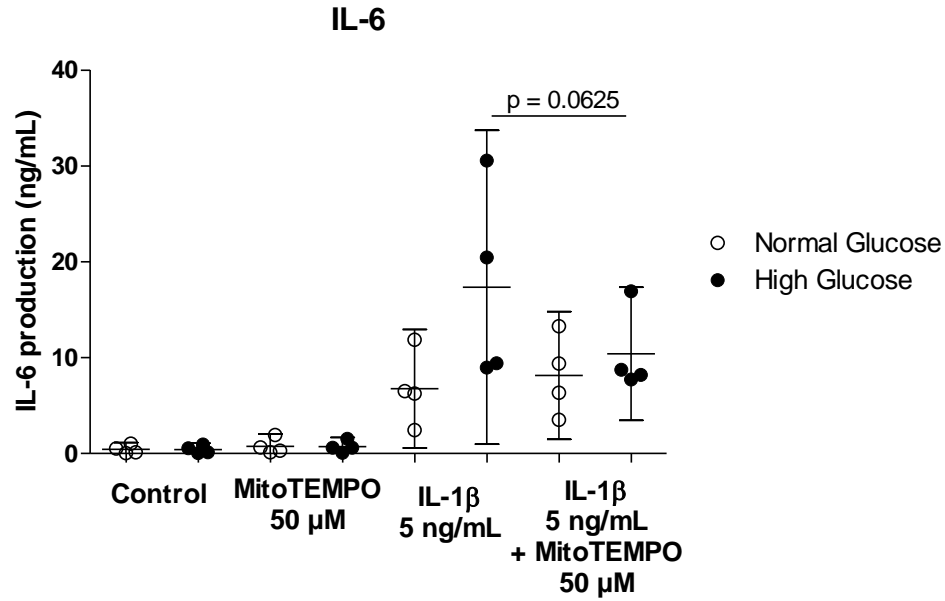


Figure 6

A



B

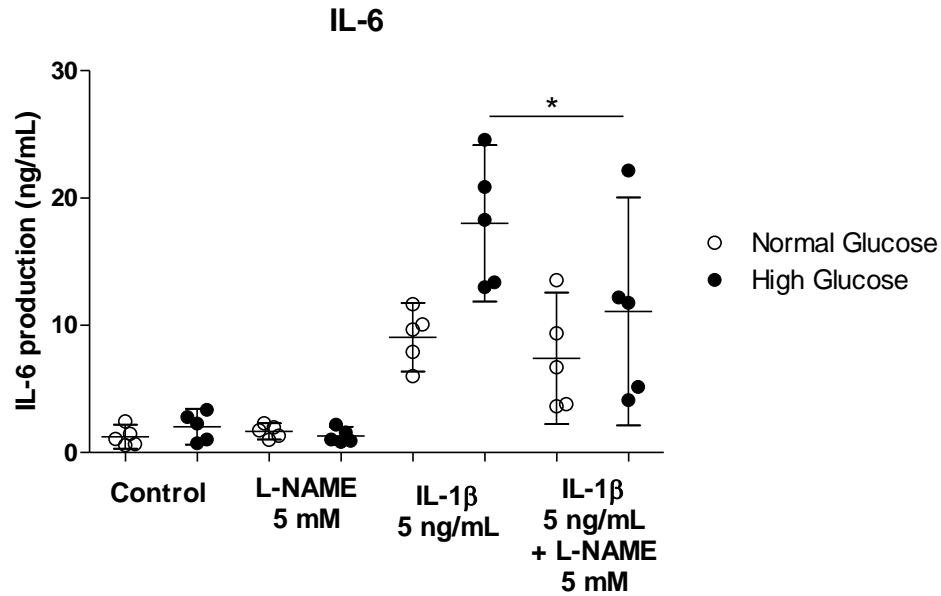


Figure 7

High glucose

Low-grade inflammation

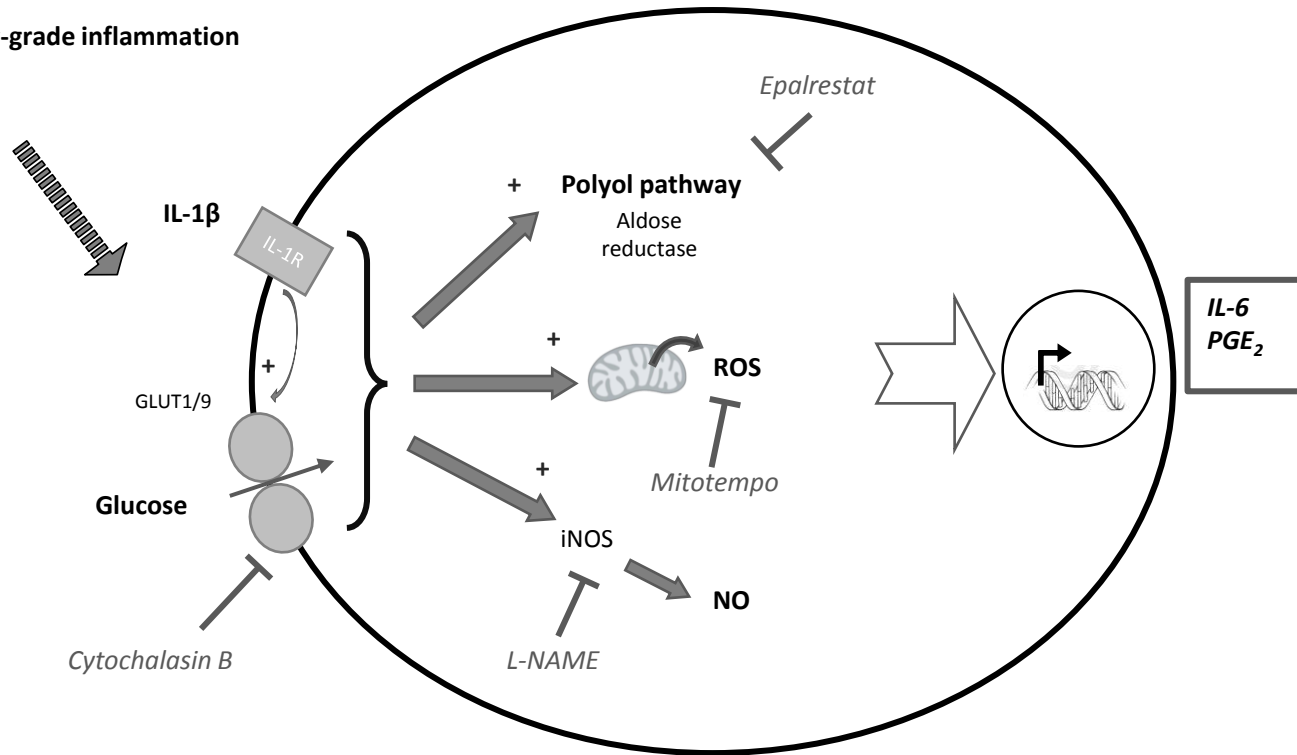


Figure S1

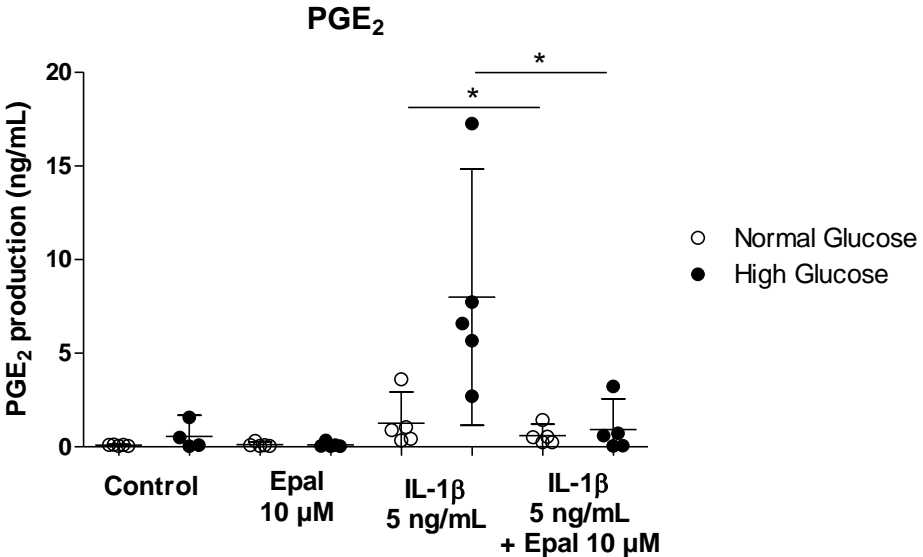


Figure S2

