HAL
open science

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

Marie-Charlotte Laiguillon, Alice Courties, Xavier Houard, Martine Auclair, Alain Sautet, Jacqueline Capeau, Bruno Fève, Francis Berenbaum, Jérémie Sellam

To cite this version:

Marie-Charlotte Laiguillon, Alice Courties, Xavier Houard, Martine Auclair, Alain Sautet, et al.. Characterization of diabetic osteoarthritic cartilage and role of high glucose environment on chondrocyte activation: toward pathophysiological delineation of diabetes mellitus-related osteoarthritis. Osteoarthritis and Cartilage, 2015, 23 (9), pp.1513-1522. 10.1016/j.joca.2015.04.026 . hal-01153393

HAL Id: hal-01153393

https://hal.sorbonne-universite.fr/hal-01153393

Submitted on 19 May 2015

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

ACCEPTED MANUSCRIPT

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

Keywords: osteoarthritis, glucose, diabetes mellitus, metabolic osteoarthritis, oxidative stress

Running headline: Diabetes mellitus or hyperglycemia-related inflammation in osteoarthritis

Marie-Charlotte Laiguillon, PhD[1], Alice Courties, MD[1,2], Xavier Houard, PhD[1], Martine Auclair[1], Alain Sautet MD[3], Jacqueline Capeau, MD, PhD[1], Bruno Fève, MD, PhD[1], Francis Berenbaum, MD, PhD[1,2], Jérémie Sellam, MD, PhD[1,2]

1. Inserm UMRS_938, Centre de Recherche St-Antoine, Sorbonne Université UPMC Univ Paris 06, Paris, France
2. Rheumatology department, Assistance Publique – Hôpitaux de Paris (AP-HP), Inflammation-Immunopathology-Biotherapy Department (DHU i2B), Paris, France
3. Orthopedic surgery department, AP-HP, Sorbonne Université UPMC Univ Paris 06, Paris, France

Number of pages: 27
Number of figures: 7
Number of tables: 1
Supplementary files: 2

Correspondance:

Prof. Francis Berenbaum
Saint-Antoine Hospital, Department of Rheumatology

ACCEPTED MANUSCRIPT

184 rue du Faubourg Saint-Antoine, 75012 Paris, France
e-mail : francis.berenbaum@sat.aphp.fr
tel : + 33 1 49 28 25 20
fax : + 33 1 49 28 25 13

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

ABSTRACT

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

Methods: OA cartilage from DM and non-DM patients undergoing knee replacement were stimulated by IL-1β for 24 h and release of interleukin-6 (IL-6) and prostaglandin E_2 (PGE_2) was measured. Primary cultured murine chondrocytes were stimulated for 24 and 72 h with or without IL-1β (5 ng/mL) under normal-glucose (5.5 mM) or high-glucose (25 mM) conditions. The expression and release of pro-inflammatory mediators (IL-6, cyclooxygenase 2 [COX2]/PGE_2) were analyzed by quantitative RT-PCR and ELISA/EIA. Glucose uptake was assessed with (^{14}C)-2-deoxyglucose. Reactive oxygen species (ROS) and nitric oxide (NO) production were measured. To analyze the mechanism of IL-1β-induced inflammation, cells were pretreated or treated with inhibitors of glucose transport (cytochalasin B), the polyol pathway (epalrestat), mitochondrial oxidative stress (MitoTEMPO) or nitric oxide synthase (L-NAME).

Results: With IL-1β stimulation, IL-6 and PGE_2 release was greater in human DM than non-DM OA cartilage (2.7- and 3-fold, respectively) ($p<0.05$). *In vitro*, with IL-1β stimulation, IL-6 and COX2 mRNA expression, IL-6 and PGE_2 release, and ROS and NO production were greater under high- than normal-glucose conditions in cultured chondrocytes. IL-1β–increased IL-6 release was reduced with cytochalasin B, epalrestat, L-NAME or MitoTEMPO treatment (-45%, -62%, -38% and -40%, respectively).

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

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

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

INTRODUCTION

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

The inflammatory status of OA joint tissues in the setting of DM has never been investigated. Overall, explanations regarding any links between DM and OA remain scarce. Streptozotocin-induced diabetic rats with insulin deficiency and severe hyperglycemia showed progressive loss of type II collagen and proteoglycans in cartilage (12). At the molecular level, both diseases feature increased content of interleukin 1β (IL-1β) along with other low-grade inflammatory mediators such as IL-6, IL-8, prostaglandin E_2 (PGE_2) as well as reactive oxygen species (ROS).

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

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

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

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

METHODS

Collection of OA human cartilage

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

criteria (25). We pre-operatively screened patients about their diabetic status using the medical file, drugs prescription, and patient interview. For each diabetic patient included, we matched a non-diabetic patient undergoing total joint replacement because of OA by age and body mass index (BMI), to avoid confounding factors. All explants from each patient were manually dissected from all remaining cartilage zones (i.e., tibial plateaus and femoral condyles) and mixed to obtain homogenous isolated cartilage samples and managed as previously described (26). Briefly, the cartilage explants were cut into small pieces (~5 mm^3), washed several times with phosphate buffered saline (PBS) and incubated in DMEM culture medium containing 25 mM glucose necessary for human explant maintenance, and supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 4 mM glutamine for 24 h at 37℃ with or without IL-1β (5 ng/mL). Conditioned media (CM) were then collected, centrifuged (1600 g for 6 min) and stored at -20℃. Each volume of medium was normalized to wet weight of explants (6 mL/ g tissue) (27).

Primary culture of murine articular chondrocytes

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

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

Treatment of primary culture of murine chondrocytes

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

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

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

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

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

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

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

IL-6 and PGE_2 production

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

Amsterdam, Netherlands). Murine and human PGE_2 concentrations in CM were measured with the enzymatic immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA). The limits of detection were 7.8, 0.6 and 7.8 pg/mL, respectively. All measurements were performed in duplicate.

Glucose uptake

Chondrocytes were seeded and cultured as described above. After 24- or 72-h stimulation with or without IL-1β under normal- or high-glucose conditions, cells were washed in PBS and incubated for 2 h in a KRP pyruvate buffer 1 mM, and pH 7.6 (Hepes 12.5 mM, NaCl 120 mM, KCl 5 mM, $MgSO_4$ 7 H_2O 1.2 mM, $CaCl_2$ 1 mM, Na_2HPO_4 1 mM, sodium pyruvate 2 mM, BSA 2%). To analyse the role of intracellular glucose, glucose transport was inhibited by cytochalasin B (50 µM) added for 20 min at 37℃. Then, the radioactive 2DG * mixture (2-deoxy-D-[1-^{14}C]glucose [0.25 µCi/well], cold 2-deoxy-D-glucose [48 mg/mL] diluted in KRP pyruvate buffer) was added for incubation for 8 min at 37℃. Cells were washed twice in PBS and incubated in 0.1% SDS for 30 min at 22℃. Cells lysates were collected in a counting vial and rinsed in 500 µL H_2O. A volume of 50 µL was collected for assay of intracellular protein concentration with the Protein Assay kit (Bio-Rad), and radioactivity (disintegration per minute) was measured in duplicates by use of a beta counter (Hidex, Turku, Finland). Data are expressed as disintegrations per min (DPM) reported for 1 µg intracellular protein. Three separate experiments were performed with three different litters of mice.

Cellular ROS production

Chondrocytes were seeded and cultured as described above in 96-well plates at $1x10^4$ per well. After 24- and 72-h treatment, ROS production was measured by fluorometric assay with dichlorodihydrofluorescein diacetate (DCFDA) (Molecular Probes, Life Technologies) and colorimetric assay with nitroblue tetrazolium (NBT) (Sigma-Aldrich). For DCFDA assay, which is directly oxidized by ROS such as superoxide ion, hydrogen peroxide and hydroxyl, chondrocytes were incubated in obscurity with 17 µM DCFDA for 60 min at 37℃, then washed in PBS, and fluorescence was measured with the Fluostar Galaxy reader (BMG Labtech, Ortenberg, Germany) at 485 nm excitation and 520 nm emission and analysed by use of Biolise (Labsystems, Helsinki, Finland). For NBT assay, which detects the activity of oxidoreductases, chondrocytes were incubated with 2 mg/mL NBT for 2 hours at 37℃, in obscurity, then disrupted with KOH-DMSO, and the amount of coloration was measured by a spectrophotometer at 560 nm. To normalize the results, intracellular proteins were collected with NaOH (0.5 M) and concentrations were measured with a spectrophotometer and the Protein Assay kit (Bio-Rad). ROS concentrations are given as fold induction from that of the control and by microgram protein. All measurements were performed in triplicate.

NO release

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

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

Statistical analysis

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

RESULTS

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

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

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

production of IL-6 and PGE_2 in cartilage from diabetic and non-diabetic patients (p=0.15 and 0.27, respectively) (**Figure 1**). Second, after IL-1β stimulation, the release of IL-6 and PGE_2 was significantly increased in DM and non-DM patients, with higher levels in diabetic than non-diabetic patients (3-fold for non-DM against 4.5-fold for DM for IL-6; 1.5-fold for non-DM against 2.7-fold for DM for PGE_2) (**Figure 1**).

High glucose enhances IL-1β-induced IL-6 and PGE_2 production by murine chondrocytes

Because human OA cartilage explants from DM patients showed a stronger inflammatory phenotype than non-DM cartilage especially after IL-1β stimulation, we speculated that sustained extracellular high glucose exposure could be one of the actors in this responsiveness. Thus, we explored *in vitro* the mechanistic impact of high glucose on murine chondrocyte activation induced by IL-1β. Murine cells were treated or not with IL-1β (5 ng/mL) for 24 or 72 h with normal glucose (5.5 mM) or high glucose (25 mM) (**Figure 2**). As expected, IL-1β increased IL-6 mRNA expression and protein release as well as COX2 mRNA expression and PGE_2 release, regardless of glucose concentration (**Figure 2A and 2B**). However, with IL-1β stimulation at 72 h, IL-6 mRNA and protein levels and PGE_2 release were higher with high than normal glucose (4- to 7-fold increase). At 24 h with IL-1β stimulation, IL-6 mRNA and protein levels and PGE_2 release were higher but not significantly, except for COX2 expression which was significantly higher with high than normal glucose. Conversely, high glucose alone (without IL-1β stimulation) did not change the expression or production of the two pro-inflammatory mediators as compared with normal glucose at 24 and 72 h. Thus, a high-glucose environment enhanced the

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

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

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

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

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

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

With increased intracellular glucose, the glycolytic pathway is saturated, which leads the activated secondary polyol metabolic pathway (15). Aldose reductase and sorbitol deshydrogenase are the two rate-limiting enzymes in this pathway and transform excessive glucose into sorbitol and fructose. To address the involvement of the polyol pathway in the pro-inflammatory phenotype of chondrocytes with high glucose, we incubated cells with or without IL-1β in normal or high glucose for 72 h with a specific inhibitor of aldose reductase, epalrestat (10 µM) (**Figure 4B**). Under normal glucose, epalrestat significantly decreased the inflammatory effect of IL-1β (-28%, $p<0.05$). This inhibition was stronger under high glucose, with a decrease of 62% in IL-6 release with IL-1β stimulation, thus completely erasing the potentiating effect of high glucose. With epalrestat treatment, PGE_2 production by IL-1β-stimulated chondrocytes was similarly reduced (-47% and -88% under normal and high glucose, respectively) (**Supplementary Figure S1**).

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

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

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

To investigate whether these findings could be responsible for the pro-inflammatory effect in chondrocytes, cells were treated with MitoTEMPO (50 µM), a specific mitochondrial ROS scavenger, or L-NAME (5 mM), an inhibitor of NO-synthase. LDH measurement revealed no cytotoxic effects at these concentrations (data not shown). Under normal glucose, neither MitoTEMPO nor L-NAME modified IL-1β-increased IL-6 production (**Figures 6A and 6D**). However, under high glucose, IL-1β-increased IL-6 production was inhibited with MitoTEMPO (-40%, $p=0.06$) and L-NAME (-38%; $p<0.05$). In addition, L-NAME but not MitoTempo could inhibit IL-1β–increased PGE_2 production under high glucose (-78%; $p<0.05$) (**Supplementary Figure S2**).

ACCEPTED MANUSCRIPT

DISCUSSION

To understand the epidemiological link between diabetes mellitus and OA, we phenotyped OA cartilage from diabetic and non-diabetic patients according to responsiveness to IL-1β-induced inflammatory stress, as assessed by IL-6 and PGE_2 release. Such increased reactivity was corroborated by sensitization of articular chondrocytes to a high-glucose environment with IL-1β stimulation. This enhanced pro-inflammatory response under high glucose conditions was related, at least in part, to increased glucose uptake, oxidative stress and activation of the polyol pathway and resulted in a sustained chondrocytic pro-inflammatory phenotype (**Figure 7**).

First, we aimed to assess the spontaneous release of IL-6 and PGE_2 by human cartilage from diabetic *versus* non-diabetic patients exposed to a high glucose level in the years before arthroplasty. And, because the low-grade inflammation observed in type 2 DM and OA involves IL-1β, we investigated the inflammatory response of diabetic and non-diabetic OA cartilage to IL-1β. We carefully selected OA cartilage explants from humans matched on age and BMI to diabetic patients and found no differences in sex ratio and other metabolic co-morbidities. All diabetic patients received at least one anti-diabetic therapy. Unfortunately, we could not analyse the role of specific drugs because of lack of statistical power. The mean values of HbA1c fraction in our patients reflects a good glycemia control for the last 3 months before surgery, but does not reflect all the diabetes history and not preclude previous uncontrolled DM periods with chronic hyperglycemia. Finally, cartilage was not histologically scored, but because all patients underwent total knee replacement, we assume that OA tissular lesions were at an advanced stage in all cases.

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

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

pathway in the potentiation of chondrocyte activation induced by high glucose. To that end, we treated articular chondrocytes with epalrestat, a specific inhibitor of aldose reductase, which has had promising results in preventing neuropathy progression and is currently used in Japan as an oral drug in clinical practice for this complication (36-38). We found a significant inhibition of the potentiating effect of high glucose for both IL-6 and PGE_2 production. The production of both mediators was decreased to a lesser extent under normal glucose conditions when chondrocytes were treated with epalrestat, which suggests that the polyol pathway may participate in IL-1β stimulation of chondrocytes by high glucose.

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

We used the antioxidant MitoTEMPO and an inhibitor of NO-synthase, L-NAME. In healthy chondrocytes, the production of energy from glucose metabolism is mainly from the glycolytic pathway and mitochondria are poorly solicited (39). In a pro-inflammatory and high-glucose environment, the mitochondrial respiratory chain could be more activated and thus increase mitochondrial ROS production. Moreover, superoxide ion ($O_2^{\circ-}$), mainly produced by mitochondria, is the main product responsible for the formation of other ROS (19). Thus, we treated chondrocytes with a specific scavenger of mitochondrial ROS, MitoTEMPO, and found inhibition of IL-6 production ($p=0.06$), which supports that ROS production induced by high glucose was responsible at least in part for the production of pro-inflammatory mediators. Additionally, the use of the inhibitor L-NAME suggested the involvement of NO in the

potentiating effect of high glucose in pro-inflammatory effects because we found a significant inhibition of IL-6 and PGE_2 production.

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

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

Second, we performed our mechanistic experiments on primary cultures of murine but not human chondrocytes. Indeed, to investigate the effect of glucose and/or IL-1β on chondrocyte activation, we needed to use normal chondrocytes to rule out the potential impact of diabetic status. Because of the challenge in obtaining normal human cartilage explants, we used murine cells. We previously compared the phenotype of murine and human chondrocytes and showed that the main markers of differentiation were similar (28-29). Moreover, the similarity in terms of IL-6 and PGE_2 release by IL-1β-stimulated murine chondrocytes under high glucose and by diabetic cartilage explants strengthen the use of murine cells for mechanistic experiments.

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

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

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

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

Acknowledgements The authors thank Martine Caron-Debarle (Inserm UMRS_938, Centre de Recherche St-Antoine, Sorbonne Université UPMC Univ Paris 06, Paris, France) for advice about glucose uptake and oxidative stress experiments and Laura Smales (BioMedEditing, Toronto, Canada) for editing the manuscript.

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

Funding The present work was supported by French state Transimmunom funds managed by the ANR within the Investissements d'Avenir program (ANR-11-IDEX-0004-02) and ROAD network (Fondation Arthritis Jacques Courtin). M-CL was supported by doctoral fellowships from the French Ministère de l'Education Nationale, de la Recherche et de la Technologie. AC was supported by the Année Recherche program (AP-HP, Paris, France).

Conflict of interests None.

REFERENCES

1. Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: a disease of the joint as an organ. Arthritis Rheum 2012;64(6):1697-707.

2. Bijlsma JW, Berenbaum F, Lafeber FP. Osteoarthritis: an update with relevance for clinical practice. Lancet 2011;377(9783):2115-26.

3. Yusuf E, Ioan-Facsinay A, Bijsterbosch J, Klein-Wieringa I, Kwekkeboom J, Slagboom PE, et al. Association between leptin, adiponectin and resistin and long-term progression of hand osteoarthritis. Ann Rheum Dis 2011;70(7):1282-4.

4. Sellam J, Berenbaum F. The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. Nat Rev Rheumatol 2010;6(11):625-35.

5. Sowers M, Karvonen-Gutierrez CA, Palmieri-Smith R, Jacobson JA, Jiang Y, Ashton-Miller JA. Knee osteoarthritis in obese women with cardiometabolic clustering. Arthritis Rheum 2009;61(10):1328-36.

6. Yoshimura N, Muraki S, Oka H, Tanaka S, Kawaguchi H, Nakamura K, et al. Accumulation of metabolic risk factors such as overweight, hypertension, dyslipidaemia, and impaired glucose tolerance raises the risk of occurrence and progression of knee osteoarthritis: a 3-year follow-up of the ROAD study. Osteoarthritis Cartilage 2012;20(11):1217-26.

7. Dahaghin S, Bierma-Zeinstra SM, Koes BW, Hazes JM, Pols HA. Do metabolic factors add to the effect of overweight on hand osteoarthritis? The Rotterdam Study. Ann Rheum Dis 2007;66(7):916-20.

8. Sellam J, Berenbaum F. Is osteoarthritis a metabolic disease? Joint Bone Spine 2013;80(6):568-73.

9. Schett G, Kleyer A, Perricone C, Sahinbegovic E, Iagnocco A, Zwerina J, et al. Diabetes is an independent predictor for severe osteoarthritis: results from a longitudinal cohort study. Diabetes Care 2013;36(2):403-9.

10. Hart DJ, Doyle DV, Spector TD. Association between metabolic factors and knee osteoarthritis in women: the Chingford Study. J Rheumatol 1995;22(6):1118-23.

11. Berenbaum F. Diabetes-induced osteoarthritis: from a new paradigm to a new phenotype. Ann Rheum Dis 2011;70(8):1354-6.

12. Atayde SA, Yoshinari NH, Nascimento DP, Catanozi S, Andrade PC, Velosa AP, et al. Experimental diabetes modulates collagen remodelling of joints in rats. Histol Histopathol 2012;27(11):1471-9.

13. Mobasheri A, Neama G, Bell S, Richardson S, Carter SD. Human articular chondrocytes express three facilitative glucose transporter isoforms: GLUT1, GLUT3 and GLUT9. Cell Biol Int 2002;26(3):297-300.

14. Mobasheri A, Vannucci SJ, Bondy CA, Carter SD, Innes JF, Arteaga MF, et al. Glucose transport and metabolism in chondrocytes: a key to understanding chondrogenesis, skeletal development and cartilage degradation in osteoarthritis. Histol Histopathol 2002;17(4):1239-67.

15. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001;414(6865):813-20.

16. Henrotin YE, Bruckner P, Pujol JP. The role of reactive oxygen species in homeostasis and degradation of cartilage. Osteoarthritis Cartilage 2003;11(10):747-55.

17. Nelson KK, Melendez JA. Mitochondrial redox control of matrix metalloproteinases. Free Radic Biol Med 2004;37(6):768-84.

18. Goodwin W, McCabe D, Sauter E, Reese E, Walter M, Buckwalter JA, et al. Rotenone prevents impact-induced chondrocyte death. J Orthop Res 2010;28(8):1057-63.

19. Vaamonde-Garcia C, Riveiro-Naveira RR, Valcarcel-Ares MN, Hermida-Carballo L, Blanco FJ, Lopez-Armada MJ. Mitochondrial dysfunction increases inflammatory responsiveness to cytokines in normal human chondrocytes. Arthritis Rheum 2012;64(9):2927-36.

20. Yu SM, Kim SJ. Production of reactive oxygen species by withaferin A causes loss of type collagen expression and COX-2 expression through the PI3K/Akt, p38, and JNK pathways in rabbit articular chondrocytes. Exp Cell Res 2013;319(18):2822-34.

21. Murrell GA, Jang D, Williams RJ. Nitric oxide activates metalloprotease enzymes in articular cartilage. Biochem Biophys Res Commun 1995;206(1):15-21.

22. Pelletier JP, Mineau F, Ranger P, Tardif G, Martel-Pelletier J. The increased synthesis of inducible nitric oxide inhibits IL-1ra synthesis by human articular chondrocytes: possible role in osteoarthritic cartilage degradation. Osteoarthritis Cartilage 1996;4(1):77-84.

23. Pelletier JP, Lascau-Coman V, Jovanovic D, Fernandes JC, Manning P, Connor JR, et al. Selective inhibition of inducible nitric oxide synthase in experimental osteoarthritis is associated with reduction in tissue levels of catabolic factors. J Rheumatol 1999;26(9):2002-14.

24. Rosa SC, Goncalves J, Judas F, Mobasheri A, Lopes C, Mendes AF. Impaired glucose transporter-1 degradation and increased glucose transport and oxidative stress in response to high glucose in chondrocytes from osteoarthritic versus normal human cartilage. Arthritis Res Ther 2009;11(3):R80.

25. Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, et al. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. Arthritis Rheum 1986;29(8):1039-49.

26. Laiguillon MC, Houard X, Bougault C, Gosset M, Nourissat G, Sautet A, et al. Expression and function of visfatin (Nampt), an adipokine-enzyme involved in inflammatory pathways of osteoarthritis. Arthritis Res Ther 2014;16(1):R38.

27. Chauffier K, Laiguillon MC, Bougault C, Gosset M, Priam S, Salvat C, et al. Induction of the chemokine IL-8/Kc by the articular cartilage: possible influence on osteoarthritis. Joint Bone Spine 2012;79(6):604-9.

28. Gosset M, Berenbaum F, Thirion S, Jacques C. Primary culture and phenotyping of murine chondrocytes. Nat Protoc 2008;3(8):1253-60.

29. Salvat C, Pigenet A, Humbert L, Berenbaum F, Thirion S. Immature murine articular chondrocytes in primary culture: a new tool for investigating cartilage. Osteoarthritis Cartilage 2005;13(3):243-9.

30. Shikhman AR, Brinson DC, Valbracht J, Lotz MK. Cytokine regulation of facilitated glucose transport in human articular chondrocytes. J Immunol 2001;167(12):7001-8.

31. Windhaber RA, Wilkins RJ, Meredith D. Functional characterisation of glucose transport in bovine articular chondrocytes. Pflugers Arch 2003;446(5):572-7.

32. Gabbay KH. The sorbitol pathway and the complications of diabetes. N Engl J Med 1973;288(16):831-6.

33. Terashima H, Hama K, Yamamoto R, Tsuboshima M, Kikkawa R, Hatanaka I, et al. Effects of a new aldose reductase inhibitor on various tissues in vitro. J Pharmacol Exp Ther 1984;229(1):226-30.

34. Hotta N. Is there a place for inhibition of transforming growth factor-beta and the polyol pathway in therapy for diabetic retinopathy? J Diabetes Investig 2010;1(4):134-6.

35. Yagihashi S, Mizukami H, Sugimoto K. Mechanism of diabetic neuropathy: Where are we now and where to go? J Diabetes Investig 2011;2(1):18-32.

36. Hotta N, Sakamoto N, Shigeta Y, Kikkawa R, Goto Y. Clinical investigation of epalrestat, an aldose reductase inhibitor, on diabetic neuropathy in Japan: multicenter study. Diabetic Neuropathy Study Group in Japan. J Diabetes Complications 1996;10(3):168-72.

37. Schemmel KE, Padiyara RS, D'Souza JJ. Aldose reductase inhibitors in the treatment of diabetic peripheral neuropathy: a review. J Diabetes Complications 2010;24(5):354-60.

38. Hotta N, Kawamori R, Fukuda M, Shigeta Y. Long-term clinical effects of epalrestat, an aldose reductase inhibitor, on progression of diabetic neuropathy and other microvascular complications: multivariate epidemiological analysis based on patient background factors and severity of diabetic neuropathy. Diabet Med 2012;29(12):1529-33.

39. Blanco FJ, Rego I, Ruiz-Romero C. The role of mitochondria in osteoarthritis. Nat Rev Rheumatol 2011;7(3):161-9.

40. Halliwell B, Chirico S. Lipid peroxidation: its mechanism, measurement, and significance. Am J Clin Nutr 1993;57(5 Suppl):715S-724S; discussion 724S-725S.

41. Beckman JS, Chen J, Ischiropoulos H, Crow JP. Oxidative chemistry of peroxynitrite. Methods Enzymol 1994;233:229-40.

42. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 2007;87(1):315-424.

43. Abramson SB. Nitric oxide in inflammation and pain associated with osteoarthritis. Arthritis Res Ther 2008;10 Suppl 2(2):S2.

44. Cheng X, Ni B, Zhang Z, Liu Q, Wang L, Ding Y, et al. Polyol pathway mediates enhanced degradation of extracellular matrix via p38 MAPK activation in intervertebral disc of diabetic rats. Connect Tissue Res 2013;54(2):118-22.

45. Capurso C, Capurso A. From excess adiposity to insulin resistance: the role of free fatty acids. Vascul Pharmacol 2012;57(2-4):91-7.

46. Alvarez-Garcia O, Rogers NH, Smith RG, Lotz MK. Palmitate has proapoptotic and proinflammatory effects on articular cartilage and synergizes with interleukin-1. Arthritis Rheumatol 2014;66(7):1779-88.

47. Kellner K, Schulz MB, Gopferich A, Blunk T. Insulin in tissue engineering of cartilage: a potential model system for growth factor application. J Drug Target 2001;9(6):439-48.

48. Claassen H, Schluter M, Schunke M, Kurz B. Influence of 17beta-estradiol and insulin on type II collagen and protein synthesis of articular chondrocytes. Bone 2006;39(2):310-7.

49. Claassen H, Schicht M, Paulsen F. Impact of sex hormones, insulin, growth factors and peptides on cartilage health and disease. Prog Histochem Cytochem 2011;45(4):239-93.

50. Rosa SC, Rufino AT, Judas F, Tenreiro C, Lopes MC, Mendes AF. Expression and function of the insulin receptor in normal and osteoarthritic human chondrocytes: modulation of anabolic gene expression, glucose transport and GLUT-1 content by insulin. Osteoarthritis Cartilage 2011;19(6):719-27.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

TABLE

Table 1: Clinical details and pathological characteristics of patients with osteoarthritis 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/m^2)	30.6 ±5.95	29.4 ± 5.46	0.90
Obesity (BMI ≥30 kg/m^2)	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

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

FIGURE LEGENDS

Figure 1: Responsiveness of cartilage explants from osteoarthritic (OA) diabetic and non-diabetic patients to interleukin 1β (IL-1β) stimulation. IL-6 and prostaglandin E_2 (PGE_2) release by cartilage explants with or without IL-1β stimulation (5 ng/mL). Each symbol represents a patient, non-diabetic (ND) n=5 (○) or diabetic (D) n=5 (●). The bar represents the mean for each condition and 95% confidence interval is represented. † *P*=0.016, * *P*=0.03, ‡ *P*=0.048.

Figure 2: Potentiating effect of high glucose on pro-inflammatory induction of murine chondrocytes stimulated with IL-1β. Induction of pro-inflammatory mediators with normal glucose (5 mM) and high glucose (25 mM) in murine chondrocytes treated with IL-1β (5 ng/mL) at 24 and 72 h. **A**, Quantitative RT-PCR (qRT-PCR) analysis of mRNA levels of IL-6 and cyclooxygenase 2 (COX2) relative to that of hypoxanthine guanine phosphoribosyltransferase (HPRT), n=5 for each condition. **B**, IL-6 and PGE_2 release, n=5 for each condition. Each symbol represents an experiment from one litter of mice in normal glucose (○) and high glucose (●) conditions. The bar represents the mean for each condition and 95% confidence interval is represented. * *P*=0.031.

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

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

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

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

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

Figure 7: Hypothetical scheme of high-glucose conditions potentiating the pro-inflammatory effect of IL-1β on articular chondrocytes. Under high glucose, glucose uptake is increased when cells are in a pro-inflammatory condition (i.e., IL-1β stimulation) induced by increased expression of glucose transporters GLUT-1 and -9. Intracellular glucose increase is responsible for the potentiation of the pro-inflammatory effect of IL-1β by activating the alternative polyol pathway, the production of mitochondrial ROS and inducible nitric oxide synthase (iNOS) for the production of NO. This activation leads to IL-6 and PGE_2 release.

ACCEPTED MANUSCRIPT

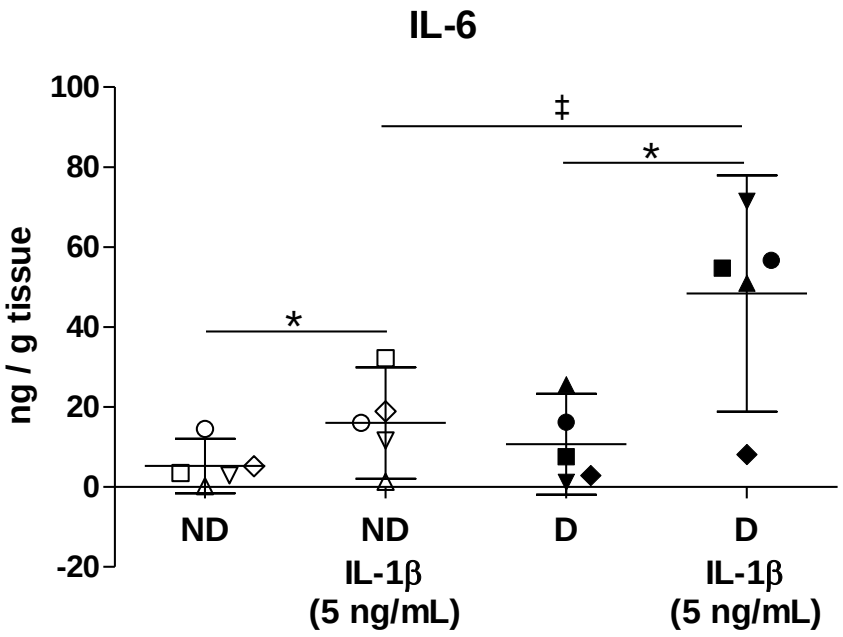
Supplementary file

Figure S1: Polyol pathway involvement in murine chondrocytes stimulated with IL-1β. Cells were treated with epalrestat (Epal, 10 µM). Protein release of PGE_2, n=5 for each condition. Each symbol represents an experiment from one litter of mice in normal glucose (○) and high glucose (●) conditions. The bar represents the mean for each condition and 95% ci is represented.* *P*=0.031.

Figure S2: Involvement of nitric oxide in the pro-inflammatory profile induction of high-glucose and IL-1β-stimulated chondrocytes. Cells were treated with an inhibitor of NO-synthase, L-NAME (D). Protein release of PGE_2, n=5 for each condition. Each symbol represents an experiment from one litter of mice in normal glucose (○) and high glucose (●) conditions. The bar represents the mean for each condition and 95% ci is represented.* *P*=0.031.

ACCEPTED MANUSCRIPT

Figure 1


IL-6
ng / g tissue
100
80
60
40
20
0
-20
‡
*
*
ND
ND
IL-1β
(5 ng/mL)
D
D
IL-1β
(5 ng/mL)

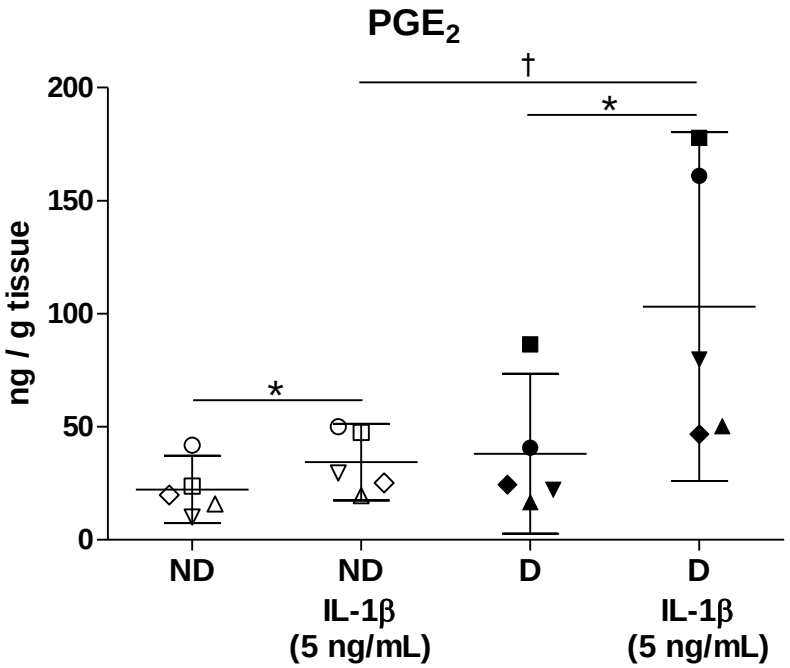

PGE2
ng / g tissue
200
150
100
50
0
†
*
*
ND
ND
IL-1β
(5 ng/mL)
D
D
IL-1β
(5 ng/mL)

Figure 2

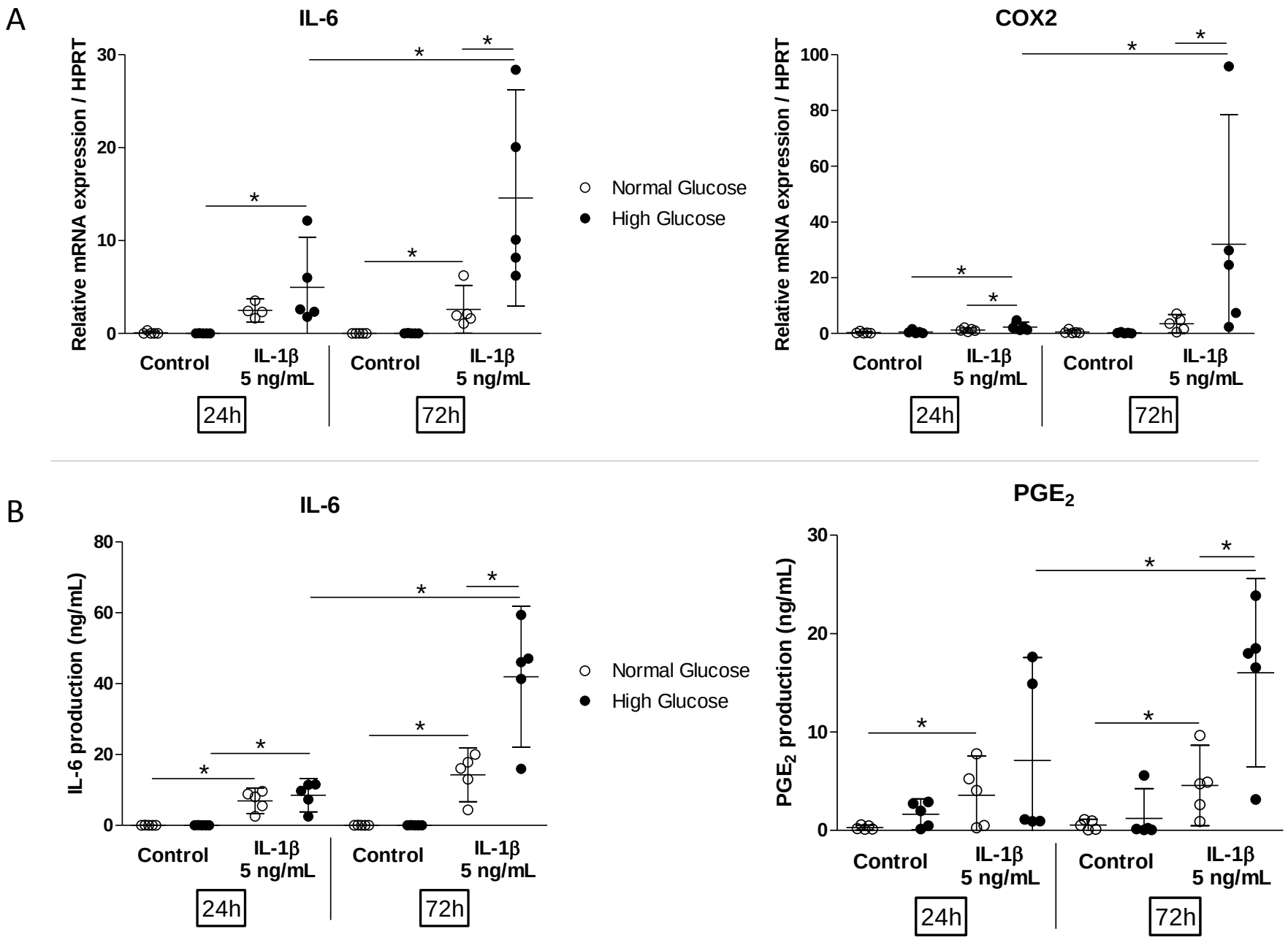
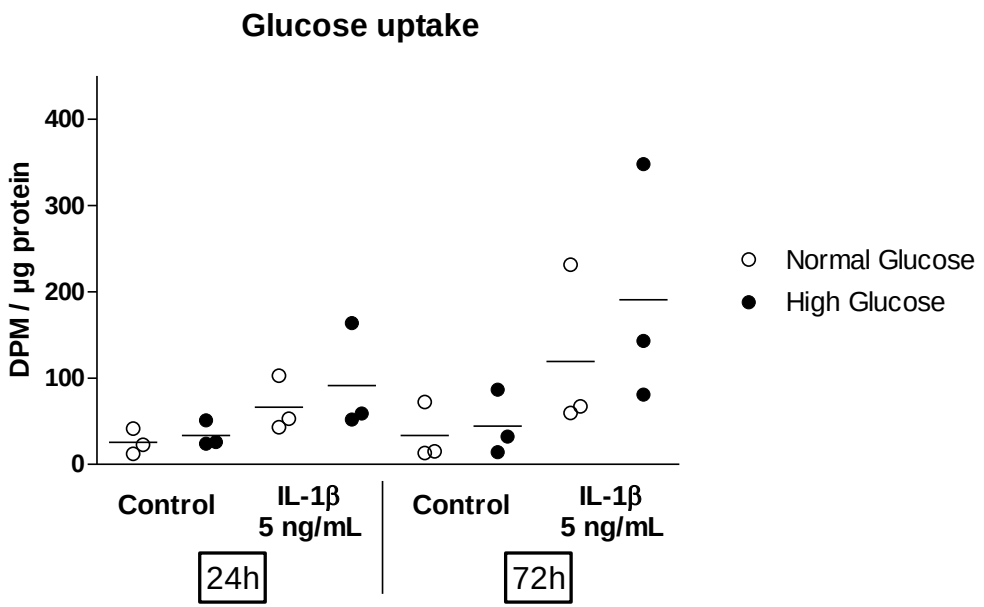

A
IL-6
Relative mRNA expression / HPRT
30
20
10
0
Control
IL-1β 5 ng/mL
Control
IL-1β 5 ng/mL
24h
72h
Normal Glucose
High Glucose
COX2
Relative mRNA expression / HPRT
100
80
60
40
20
0
Control
IL-1β 5 ng/mL
Control
IL-1β 5 ng/mL
24h
72h
B
IL-6
IL-6 production (ng/mL)
80
60
40
20
0
Control
IL-1β 5 ng/mL
Control
IL-1β 5 ng/mL
24h
72h
Normal Glucose
High Glucose
PGE2
PGE2 production (ng/mL)
30
20
10
0
Control
IL-1β 5 ng/mL
Control
IL-1β 5 ng/mL
24h
72h

Figure 3

A


Glucose uptake
DPM / µg protein
400
300
200
100
0
Control
IL-1β
5 ng/mL
Control
IL-1β
5 ng/mL
24h
72h
Normal Glucose
High Glucose

B

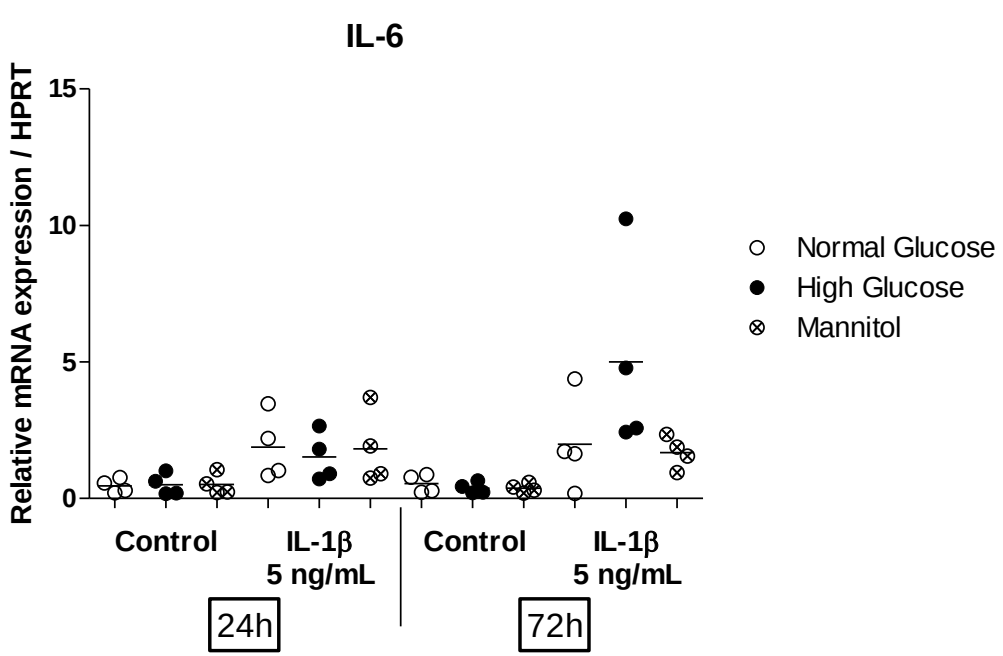

IL-6
Relative mRNA expression / HPRT
15
10
5
0
Control
IL-1β
5 ng/mL
Control
IL-1β
5 ng/mL
24h
72h
Normal Glucose
High Glucose
Mannitol

Figure 4

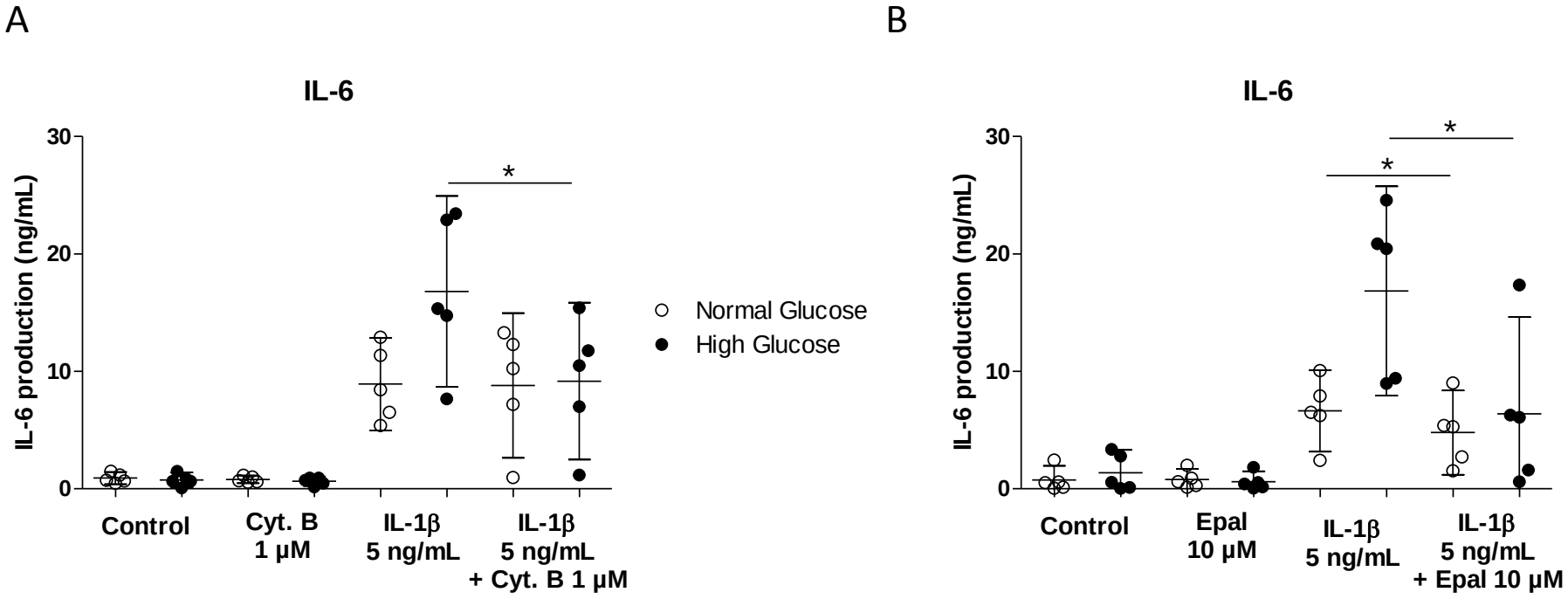

A
IL-6
IL-6 production (ng/mL)
30
20
10
0
*
Control
Cyt. B
1 μM
IL-1β
5 ng/mL
IL-1β
5 ng/mL
+ Cyt. B 1 μM
Normal Glucose
High Glucose
B
IL-6
IL-6 production (ng/mL)
30
20
10
0
*
*
Control
Epal
10 μM
IL-1β
5 ng/mL
IL-1β
5 ng/mL
+ Epal 10 μM

Figure 5

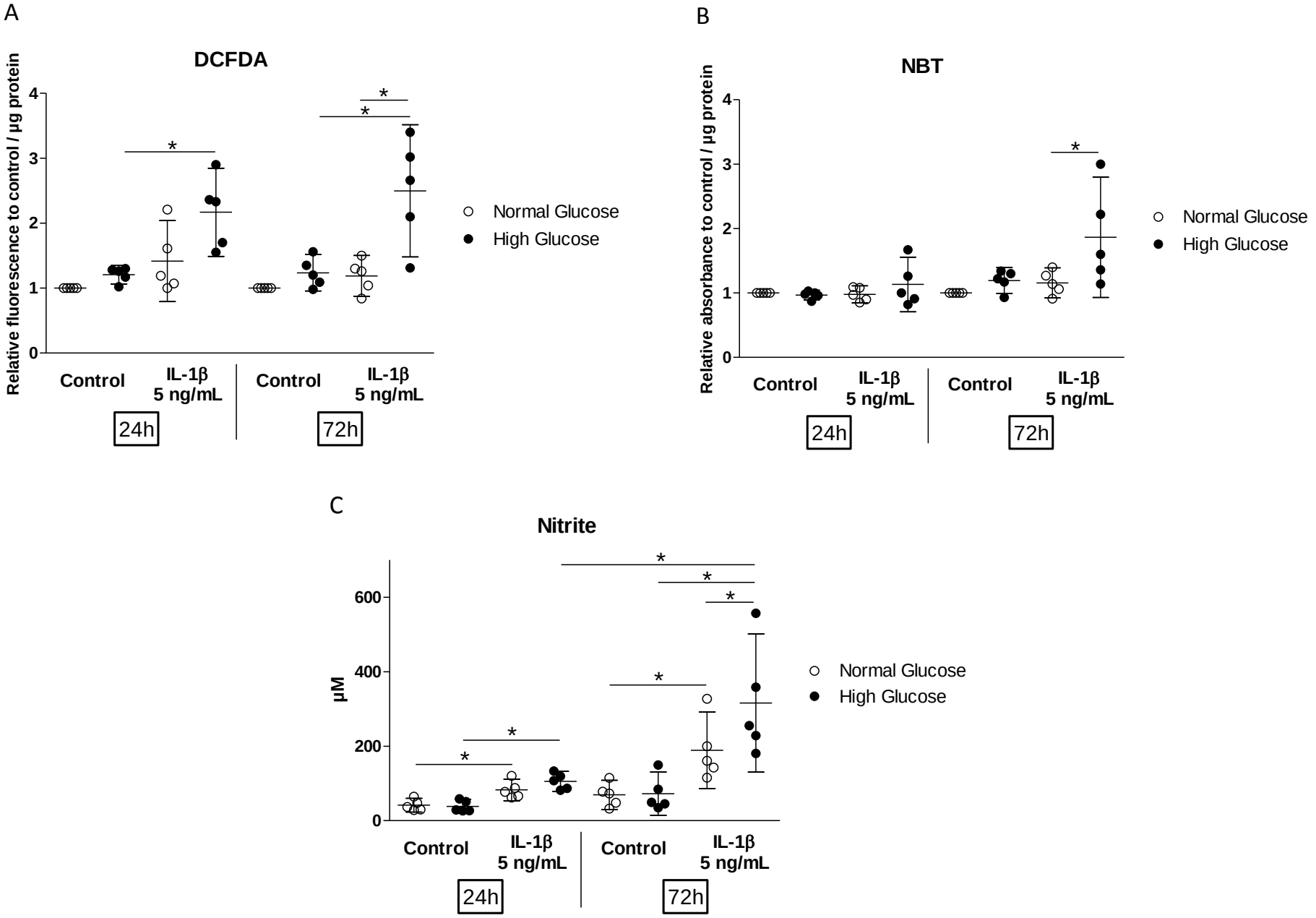
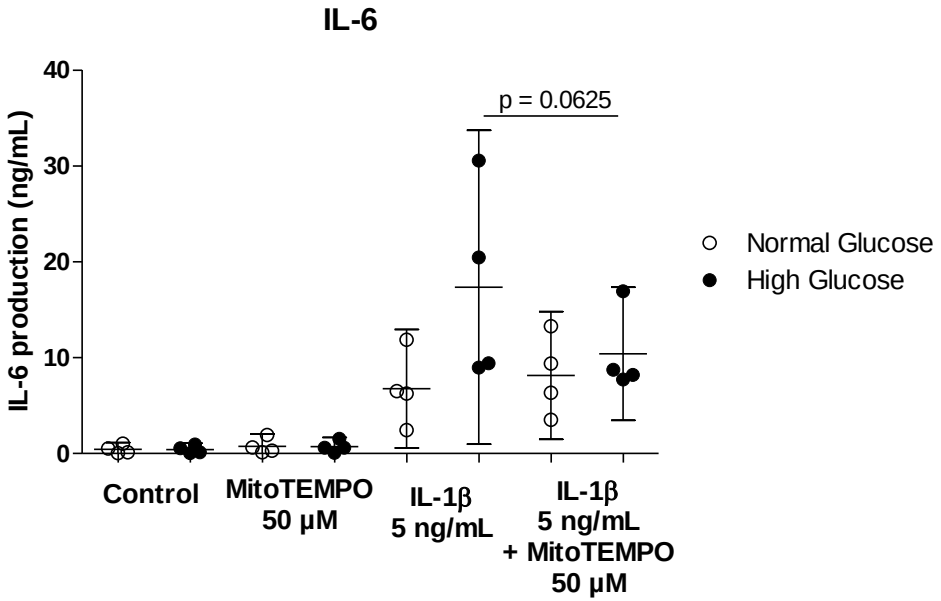

A
DCFDA
Relative fluorescence to control / µg protein
0
1
2
3
4
*
*
*
Control
IL-1β
5 ng/mL
Control
IL-1β
5 ng/mL
24h
72h
Normal Glucose
High Glucose
B
NBT
Relative absorbance to control / µg protein
0
1
2
3
4
*
Control
IL-1β
5 ng/mL
Control
IL-1β
5 ng/mL
24h
72h
Normal Glucose
High Glucose
C
Nitrite
µM
0
200
400
600
*
*
*
*
*
*
Control
IL-1β
5 ng/mL
Control
IL-1β
5 ng/mL
24h
72h
Normal Glucose
High Glucose

Figure 6

A


IL-6
IL-6 production (ng/mL)
40
30
20
10
0
p = 0.0625
Control
MitoTEMPO 50 µM
IL-1β 5 ng/mL
IL-1β 5 ng/mL + MitoTEMPO 50 µM
Normal Glucose
High Glucose

B

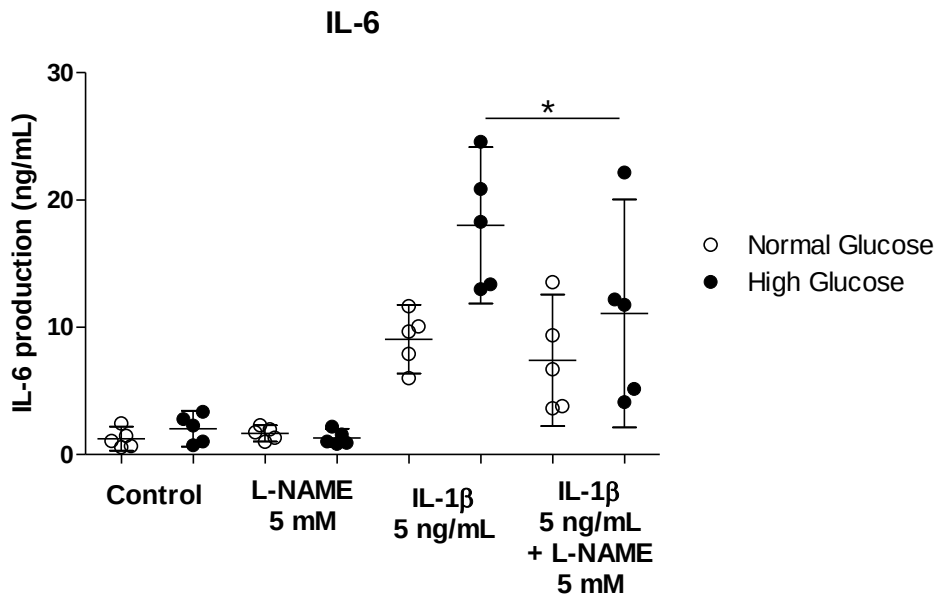
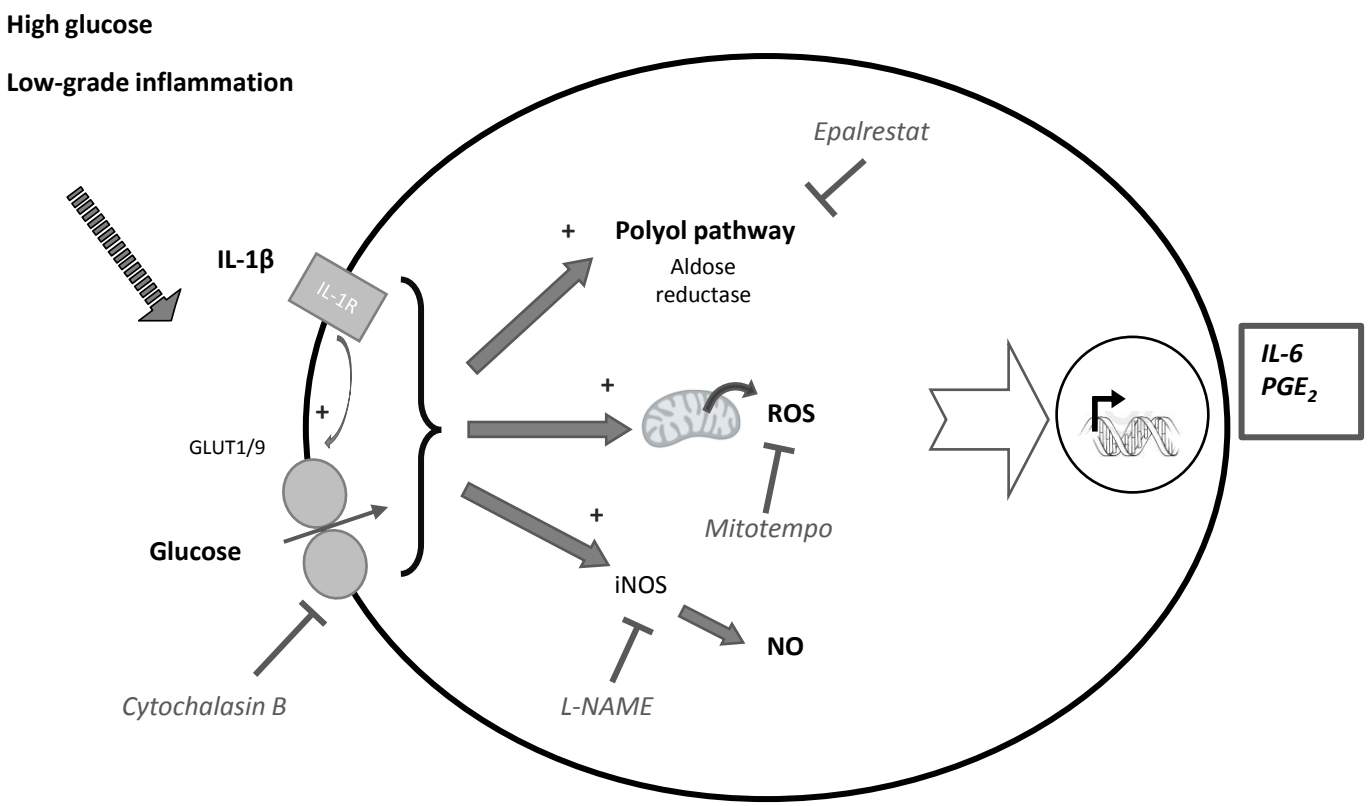
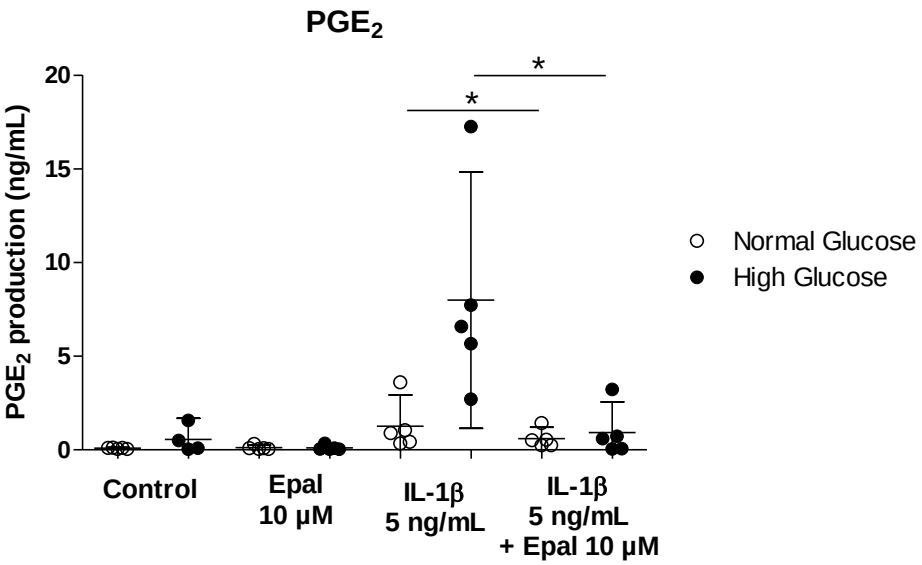

IL-6
IL-6 production (ng/mL)
30
20
10
0
*
Control
L-NAME 5 mM
IL-1β 5 ng/mL
IL-1β 5 ng/mL + L-NAME 5 mM
Normal Glucose
High Glucose

Figure 7


High glucose
Low-grade inflammation
IL-1β
IL-1R
+
GLUT1/9
Glucose
Cytochalasin B
Epalrestat
+
Polyol pathway
Aldose reductase
+
ROS
Mitotempo
+
iNOS
NO
L-NAME
IL-6
PGE2

Supplementary file

Figure S1



Supplementary file

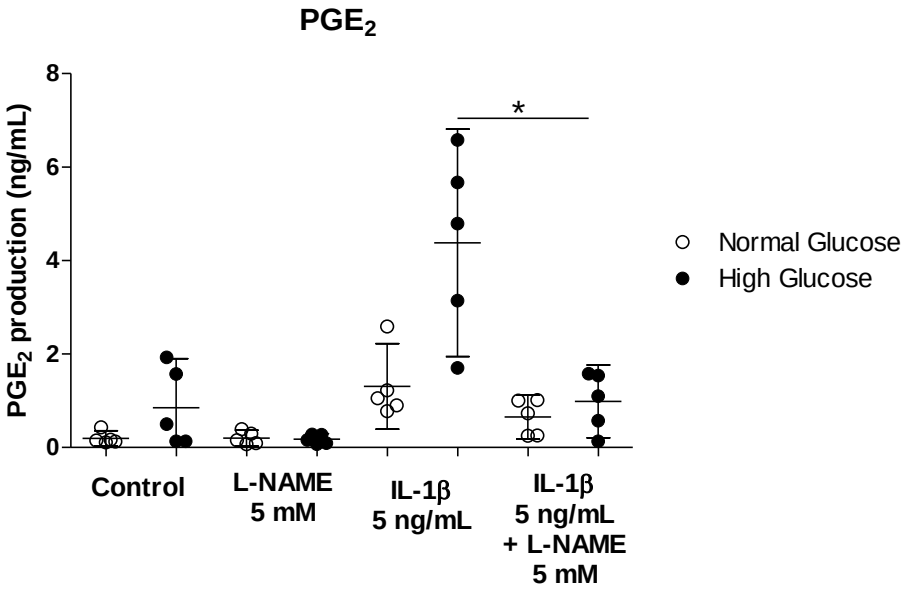



Figure S2