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ABSTRACT

Epidemiological findings support the hypothesis that type 2 diabetes mellitus (T2DM) is a risk factor for osteoarthritis (OA). Moreover, OA cartilage from patients with T2DM exhibits a greater response to inflammatory stress, but the molecular mechanism is unclear. To investigate whether the antioxidant defense system participates in this response, we examined here the expression of nuclear factor-erythroid 2-related factor (Nrf-2), a master antioxidant transcription factor, and of heme oxygenase-1 (HO-1), one of its main target genes, in OA cartilage from T2DM and non-T2DM patients, as well as in murine chondrocytes exposed to high glucose (HG). *Ex vivo* experiments indicated that Nrf-2 and HO-1 expression is reduced in T2DM vs. non-T2DM OA cartilage (0.57-fold [Nrf-2] and 0.34-fold [HO-1]), and prostaglandin E_2 (PGE_2) release was increased in samples with low HO-1 expression. HG-exposed, IL-1β-stimulated chondrocytes had lower Nrf-2 levels *in vitro*, particularly in the nuclear fraction, than chondrocytes exposed to normal glucose (NG). Accordingly, HO-1 levels were also decreased (0.49-fold) in these cells. The HO-1 inducer cobalt protoporphyrin-IX more efficiently attenuated PGE_2 and IL-6 release in HG+IL-1β-treated cells than in NG+IL-1β-treated cells. A greater reduction in HO-1 expression and increase in PGE_2/IL-6 production were observed in HG+IL-1ß-stimulated chondrocytes from Nrf-$2^{-/-}$ mice than in chondrocytes from wild type mice. We conclude that the Nrf-2/HO-1 axis is a critical pathway in the hyperglucidic-mediated dysregulation of chondrocytes. Impairments in this antioxidant system may explain the greater inflammatory responsiveness of OA cartilage from T2DM patients and may inform treatments of such patients.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder associated with many adverse complications. Accumulating epidemiological and experimental findings support the hypothesis that T2DM is an independent risk factor for osteoarthritis (OA), the most frequent joint disease (1), or for its severity (2-4). However, the mechanisms underlying the connection between both diseases remain unclear.

Together, oxidative stress and pro-inflammatory mediators, particularly interleukin 1 beta (IL-1β), actively induce the changes in articular cartilage that predispose this tissue to the development of OA (5,6), as well as to T2DM and T2DM complications (7-10). Elevated intracellular glucose levels primarily generate oxidative damage as a consequence of glycolytic pathway saturation in the cell and the subsequent production of advanced glycation end products (AGEs) (11-13). This oxidative stress mediated by excess glucose-induced impairments in the antioxidant defense system may also contribute to diabetic complications. Moreover, similar oxidative stress disturbances could occur in OA (14,15).

Nuclear factor-erythroid 2-related factor-2 (Nrf-2), a master transcription factor involved in antioxidant signaling and the cell survival response, regulates a wide battery of cytoprotective responses and helps attenuate metabolic, neurodegenerative and other age-related diseases (16-19). Likewise, evidence for altered Nrf-2 signaling in aging and metabolic disorders has been reported (19,20).

As shown in recent studies, Nrf-2 is a pivotal target for the prevention and attenuation of diabetes mellitus (17,21) and for controlling bone and cartilage destruction induced by oxidative stress (22,23). Under physiological conditions, Nrf-2

is generally located in the cytoplasm and binds to its inhibitor, Kelch-like ECH-associated protein 1 (Keap1), leading to its degradation. However, in response to oxidative or electrophilic stress, Nrf-2 dissociates from Keap1 and translocates to the nucleus to bind antioxidant-responsive elements (ARE) in the promoter regions of its downstream antioxidant genes, including heme oxygenase-1 (HO-1) (24).

HO-1 is a crucial antioxidant enzyme that catalyzes the degradation of heme into iron, carbon monoxide, and biliverdin (25). This enzyme regulates catabolic and anabolic processes in OA chondrocytes (26). HO-1 overexpression in cartilage prevents the pro-inflammatory mediator-induced activation of catabolic, apoptotic or senescence pathways (27-29). Thus, HO-1 represents an important part of the cellular response to inflammatory and oxidative stress in joints (30). Interestingly, the expression and activity of this enzyme are downregulated in the vasculature and retinal tissue from patients with diabetes (31-33), as well as in *in vitro* and *in vivo* cellular models of high glucose exposure (34,35). However, the expression and involvement of the Nrf-2/HO-1 antioxidant axis in type 2 diabetes-related OA has not yet been studied.

For a better understanding of the link between OA and T2DM, we recently reported the higher responsiveness of OA cartilage from patients with T2DM to interleukin-1β (IL-1β)-induced inflammatory stress (36). Similarly, the exposure of IL-1β-stimulated chondrocytes to high glucose exacerbates the activation of pathological pathways, which is blocked by ROS scavengers (36). These findings further support the critical role of oxidative stress generated under hyperglucidic conditions in the activation of catabolic responses in cartilage. However, the pathological mechanisms triggered by a high glucose environment that ultimately

establish the redox imbalance in cartilage remain evasive. We studied the Nrf-2/HO-1 axis in human OA cartilage from patients with or without diabetes and in chondrocytes in a high-glucose environment to determine whether the antioxidant defense system is impaired in this excess glucose- and low-grade inflammation-induced phenomenon in cartilage.

RESULTS

Nrf-2/HO-1 expression is decreased in OA cartilage from patients with diabetes and is inversely correlated with the production of inflammatory mediators.

We have recently observed a more pronounced inflammatory phenotype of OA cartilage from patients with diabetes, based on IL-6 and prostaglandin E_2 (PGE_2) release (36). We evaluated the expression of the Nrf-2 and HO-1 proteins in 8 cartilage explants from patients with T2DM and 8 explants from non-diabetic patients matched for age and body mass index (BMI) to elucidate whether impairments to the antioxidant system underlie this altered phenotype (age: 64.8±11.1 vs. 68.0±8.7; BMI: 30.1±4.9 vs. 29.9±4.7; gender: 6 vs. 3 females for T2DM and non-T2DM patients respectively). Additionally, the other clinical characteristics of all patients were similar.

The Nrf-2 and HO-1 expression levels were reduced in OA cartilage from patients with T2DM compared with the expression levels in non-diabetic patients (0.57-fold and 0.34-fold for Nrf-2 and HO-1, respectively, $p<0.05$) (**Figure 1A and Figure 1B**). Likewise, the HO-1 and Nrf-2 expression levels varied similarly: Nrf-2 expression was higher in patients with HO-1 expression levels above the median (**Figure 1C**). Conversely, PGE_2 release and HO-1 expression varied in opposite ways: PGE_2 release was higher in patients with HO-1 expression levels below the median (**Figure 1D**).

A high-glucose environment reduces Nrf-2/HO-1 expression in IL-1β-stimulated murine chondrocytes.

Glucose has been shown to downregulate HO-1 promoter activity and HO-1 levels (37). Consequently, a high glucose environment in T2DM may be responsible for the

reduced Nrf-2/HO-1 expression, as observed in OA cartilage from patients with T2DM. Murine chondrocytes were stimulated with or without IL-1β (5 ng/mL) for the indicated times in the presence of normal glucose (5.5 mM) or high glucose (25 mM) to explore this possibility. As shown in **Figure 2 and Figure 3**, reduced HO-1 and Nrf-2 mRNA expression levels were observed at 72 h of incubation with high glucose compared with cells treated with normal glucose; however, these differences in expression were not significant at the protein level. The IL-1β treatment further enhanced the impact of high glucose on the Nrf-2 and HO-1 expression levels at both the mRNA (**Figure 2A and Figure 3A**) and protein levels (**Figure 2D and Figure 3B**). This effect was significant at 48 h and particularly at 72 h (0.81-fold [Nrf-2] and 0.48-fold [HO-1] reductions at the protein level, $p<0.05$). Additionally, we did not observe a simultaneous regulation of Keap-1 expression (Nrf-2 cytoplasmic inhibitor) that counteracted the variations in the Nrf-2 levels (**Figure 2B**). Subsequently, the Nrf-2/Keap-1 ratio confirmed the modulation of Nrf-2 expression by high glucose (**Figure 2C**).

We assayed the variations in the nuclear levels of this transcription factor 30 min after treatment to determine whether Nrf-2 nuclear translocation was impaired (**Figure 2E**). Nuclear levels of Nrf-2 was reduced in chondrocytes grown under high-glucose conditions in both the presence and absence of IL-1β compared with that of cells grown under normal glucose conditions (0.73-fold and 0.61-fold, respectively). Consistent with these findings and similar to our observations with human OA cartilage, we observed a significant dependence between the Nrf-2 and HO-1 expression levels in cultured murine chondrocytes, as the levels of these proteins varied in the same way (**Figure 3C**).

Reduced HO-1 expression favors the increased responsiveness of chondrocytes to IL-1β in a high-glucose environment.

Consistent with our observations in OA cartilage, HO-1 expression was inversely correlated with PGE_2 production in IL-1β-stimulated murine chondrocytes (**Figure 3D).** Based on these findings, HO-1 expression may regulate chondrocyte activation by pro-inflammatory cytokines. Chondrocytes were co-incubated with cobalt protoporphyrin IX (CoPP), an inducer of HO-1 activity, for 72 h and IL-6 and PGE_2 release were measured to further confirm this hypothesis (**Figure 4A and Figure 4B**). CoPP attenuated both IL-1β-induced IL-6 and PGE_2 production. Interestingly, this reduction was significantly stronger in cells grown in high glucose, with a 49% decrease in IL-6 release and a 98% decrease in PGE_2 release, compared with a 25% and 91% release, respectively, from cells grown in normal glucose. Moreover, since HO-1 is an antioxidant enzyme, we also determined whether CoPP reduced the previously observed high glucose-stimulated increase in IL-1β-induced ROS production (36). As expected, CoPP, through HO-1 strongly reduced the ROS levels, returning them to the control values (**Figure 4C**).

Nrf-2 knockout chondrocytes show reduced HO-1 expression and higher responsiveness to IL-1β in a high glucose environment

Nrf-2 is recognized as the main inducer of HO-1 gene expression (38). Here, Nrf-2 and HO-1 expression were shown to vary in the same way in OA cartilage and in murine chondrocytes exposed to a high glucose environment. Additionally, sulforaphane, a known natural inducer of Nrf-2, increased HO-1 expression, reduced

ROS accumulation and attenuated the production of IL-6 in chondrocytes exposed to IL-1β (**Figure 5**), suggesting a role for Nrf-2 in controlling the catabolic response. Therefore, we conducted studies in chondrocytes from Nrf-2 knockout (Nrf-$2^{-/-}$) mice using the same experimental approach to further address the dependence of IL-1β-induced HO-1 expression in response to high glucose on Nrf-2 (**Figure 6**). As expected, HO-1 expression was drastically reduced in Nrf-$2^{-/-}$ chondrocytes at 72 h compared with that in the wild type cells (**Figure 6A**). IL-6 and PGE_2 production were measured to determine whether the Nrf-2 knockout also had an impact on the pro-inflammatory phenotype of chondrocytes. We observed a strengthening of PGE_2 production induced by IL-1β in chondrocytes from Nrf-$2^{-/-}$ mice compared to wild type mice, achieving significant differences in those cells incubated in high glucose (**Figure 6B**); moreover, similar results were obtained for IL-6 levels.

DISCUSSION

T2DM is currently considered an additional risk factor for OA occurrence and OA severity, delineating the T2DM-associated OA phenotype (2,3,39). In addition to insulin resistance, chronic hyperglycemia is one of the main biological features involves in diabetic complications (10,13). Moreover, we have recently reported a more pronounced inflammatory phenotype in OA cartilage from patients with diabetes and enhanced IL-1β-induced inflammation in cultured chondrocytes exposed to excess glucose (36). To further elucidate these findings, we shown in this study the expression of Nrf-2, the master transcriptional regulator of antioxidant responses, and one of its main target genes, HO-1, is reduced in OA cartilage from patients with T2DM. These observations are mimicked *in vitro* by exposing IL-1β-stimulated chondrocytes to high glucose. Linking our findings, impaired Nrf-2/HO-1 signaling is responsible for the increased responsiveness to IL-1β, as shown by the increased IL-6 and PGE_2 release and ROS production by chondrocytes.

The Nrf-2/HO-1 axis is a crucial cell survival mechanism that counteracts oxidative stress and inflammation (24,40). Deficiencies in this axis have been identified in some systemic diabetic complications, such as retinopathy or cardiopathy (33-35,41). In the present study, we investigated whether the Nrf-2/HO-1 pathway was also impaired in OA joints from patients with T2DM and whether it participates in the pathological mechanisms predisposing cartilage to diabetes-associated OA. For this purpose, we selected osteoarthritic cartilage explants from patients with and without diabetes, and matched them based on age, BMI, gender and other metabolic co-morbidities, similar to our previous report (36). Both Nrf-2 and HO-1 expression were decreased in diabetic OA cartilage. The HO-1 promoter (HMOX1)

contains binding sites for several transcription factors, notably the ARE site for Nrf-2; however, activator protein-1 (AP-1), cAMP response element binding protein (CREB), and nuclear factor- κB (NF-κB) can also activate its expression (38). In our study, HO-1 expression in OA cartilage at least partially depended on the Nrf-2 levels. Nrf-2/HO-1 signaling protects the joint against the activation of pathological pathways (42-44). Here, we revealed a negative correlation between PGE_2 production and the HO-1 levels, suggesting a deleterious effect of a reduction in Nrf-2/HO-1 expression on the inflammatory profile of cartilage.

A decrease in Nrf-2/HO-1 signaling and a subsequent increase in ROS release are associated with exposure to a high glucose environment in different cell types. Retinal endothelial cells incubated with high glucose exhibit reduced Nrf-2 transcriptional activity (45). Similar results were observed in human microvessel endothelial cells (46). Interestingly, decreased HO-1 levels were also detected in these *in vitro* models (46), as well as in animal models of diabetes mellitus (31,35). Therefore, we used an *in vitro* approach to evaluate whether a diabetes-related high glucose environment may participate in reducing Nrf-2/HO-1 signaling in T2DM OA cartilage. Murine chondrocytes were stimulated with IL-1β, a cytokine known to be involved in the pathophysiology of OA and T2DM, and were exposed to high glucose. The hyperglucidic environment reduced the early nuclear translocation of Nrf-2 as well as its total protein level. As expected, HO-1 expression was also reduced and was positively correlated with the Nrf-2 levels. This correlation between the expression levels of both proteins was further confirmed in chondrocytes from Nrf-$2^{-/-}$ mice. The genetic invalidation of Nrf-2 expression drastically reduced HO-1 expression.

The transcriptional activity of Nrf-2 is mainly regulated by its cytoplasmic repressor, Keap-1, although other inhibitors also block its activity in the nucleus (24). We failed to detect any modulation of Keap-1 expression under high glucose conditions and/or IL-1β stimulation, suggesting that repressors other than Keap-1 participate in the alterations in the Nrf-2 pathway observed in our model. For instance, the glycogen synthase kinase 3β (GSK3β)/Fyn pathway and BTB domain and CNC homolog 1 (bach1) (47) impair Nrf-2 signaling in diabetic complications and have been involved in pathological processes in joints (48-52). Based on these findings, hyperglycemia/diabetes modulates the Nrf-2/HO-1 axis through different repressors; however, additional studies are required to elucidate the specific pathways activated in chondrocytes.

The beneficial effect of activation of the Nrf-2/HO-1 axis on diabetic conditions is widely accepted (17,35). Moreover, accumulating evidence has revealed a protective role for the Nrf-2/HO-1 axis in joint diseases (22,23,26,43,53). As shown in the present study, the reduction of the Nrf-2/HO-1 levels contributes to a pro-inflammatory imbalance in IL-1β-stimulated chondrocytes exposed to a high glucose environment. HO-1 expression was negatively correlated with the production of inflammatory mediators. Likewise, treatment with sulforaphane, a known natural inductor of Nrf-2, and subsequent HO-1 up-regulation, protected chondrocytes against ROS accumulation and inflammatory production. Accordingly, the chemical induction of HO-1 activity reduced IL-6 and PGE_2 release in a more significant manner in those in chondrocytes exposed to high glucose. Finally, HO-1 expression was reduced in chondrocytes from Nrf-2 knockout mice incubated with high glucose and the inflammatory response to IL-1β was further exacerbated. Our results are

corroborated by Cai et al. (2015), who observed that an Nrf-2 deletion results in increased disease severity in different animal models of OA (43). Additionally, the recovery of Nrf-2 activity inducing HO-1 expression decreased OA pathogenesis (43). Accordingly, deficiency of bach-1 favoring Nrf-2 transcriptional activity protects against development of two different types of OA: aging- and post-traumatic associated OA pathogenesis (51). Thereby, these findings suggest anti-catabolic roles of Nrf-2 in different OA subsets. Here, we provide for the first time strong evidences that Nrf2 is also pivotal to counteract the pathological pathways activated by high glucose stress in a diabetes-related OA context. However, HO-1 activation independent of Nrf-2 may also protect against the development of this disease (51,54). In this sense, Nrf-2 knockout mice failed to completely block the effect of high glucose on HO-1 expression, suggesting that pathways other than Nrf-2 signaling may also be involved in regulating HO-1 expression.

In conclusion, the Nrf-2/HO-1 axis is dysfunctional in diabetic osteoarthritic cartilage and represents a critical pathway involved in the hyperglucidic-mediated dysregulation of articular chondrocytes (**Figure 7**). The impairment of this antioxidant system may explain the greater inflammatory responsiveness of cartilage from patients with T2DM and may provide new targeted therapeutic avenues for treating patients with the diabetes-related OA phenotype.

EXPERIMENTAL PROCEDURES

Collection of OA human cartilage

Human knee explants were obtained from patients with OA who were undergoing total joint replacement at Saint-Antoine Hospital (Paris, France). The diagnosis of OA of the knee was based on criteria from the American College of Rheumatology (55). Patients were screened for diabetes using their medical files, drug prescriptions, and patient interviews. For each patient with diabetes who was included in the study, we matched a non-diabetic patient undergoing total knee joint replacement due to OA by age and BMI to avoid confounding factors. The explants from each patient were manually dissected from all remaining cartilage zones (*i.e.*, tibial plateaus and femoral condyles), mixed to obtain homogeneous isolated cartilage samples and managed using previously described methods (36). Briefly, the cartilage explants were cut into small pieces (~5 mm^3), washed several times with PBS and incubated in DMEM (25 mM) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 4 mM glutamine for 24 h at 37°C. After incubation, the explants were frozen and protein extracts were obtained using previously established methods (56). Briefly, the tissues were ground in liquid nitrogen using a mortar and pestle, after which the proteins were extracted with lysis buffer and used for the western blot experiments. In parallel, conditioned media (CM) were also collected, centrifuged (1,600 g for 6 min) and stored at -20°C. Each volume of medium was normalized to the wet weight of the explants (6 mL/g tissue) (36). Informed consent was obtained from each patient for the use of their tissues and clinical data. All experiments using human samples were approved by a French Institutional Review Board (Comité de Protection des Personnes, Paris Ile de France 5, April 2012).

Primary culture and treatment of murine articular chondrocytes

Mouse primary chondrocytes were isolated from the articular cartilage of 5- to 6-day-old newborn C57BL/6 mice from Janvier (St. Berthevin, France) and were seeded at a density of 8×10^3 cells per cm, as previously described (57). Articular chondrocytes obtained from newborn mice using this protocol were validated as cells presenting characteristics similar to fully mature murine chondrocytes (58). Chondrocytes were cultured in complete DMEM for a week and were then incubated in serum-free DMEM containing 0.1% bovine serum albumin (BSA) for 24 h before treatment (basal medium). Subsequently, the murine chondrocytes were incubated with normal glucose (5.5 mM) or high glucose (25 mM) in the presence or absence of IL-1β (5 ng/mL) (PeproTech, Rocky Hill, NJ, USA) for the indicated times. Cell lysates were collected for mRNA or protein extraction, and supernatants were collected for the assays.

For mechanistic studies, chondrocytes that had been cultured with normal or high glucose in the presence or absence of IL-1β (5 ng/mL) for 72 h were co-treated with CoPP, an inducer of HO-1 activity (10 µM) (Enzo Life Sciences, Villeurbanne, France) whose non-toxic effect at this concentration has been checked using LDH assay and its specificity described in previous publications (29,59,60). In additional experiments, cells were pre-treated for 30 min with the Nrf2 activator compound sulforaphane (SFN; 5µM) (Sigma). Moreover, experiments were also performed using Nrf-2 knockout (Nrf-$2^{-/-}$) (61) mice generated from inbred Nrf-2 heterozygous mice on a C57BL/6J background, as described by El Ali et al (62). The mice were

housed in a pathogen-free facility and were handled in accordance with the principles and procedures outlined in Council Directive 86/609/EEC.

The efficiency of the IL-1β dose employed was assayed previously, and cytotoxic effects of the treatments and an osmotic effect of high glucose were excluded (36). The CoPP dose was chosen based on dose-effect experiments (data not shown) and literature data. All experiments with murine articular chondrocytes were performed using protocols approved by the French and European ethics committees (Comité Régional d'Ethique en Expérimentation Animale N°3 de la région Ile de France).

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

Total RNA was extracted from the chondrocytes using the ReliaPrep RNA Cell Miniprep System kit (Promega, Madison, WI, USA), and the concentrations were determined by spectrophotometry (Eppendorf, Le Pecq, France). Reverse transcription utilized 500 ng of total RNA and the Omniscript RT kit (Qiagen). The levels of the IL-6, HO-1, Nrf-2 and Keap-1 mRNAs were quantified using a Light Cycler LC480 (Roche Diagnostics, Indianapolis, IN, USA). The PCR amplification conditions were: initial denaturation for 5 min at 95°C, followed by 40 cycles consisting of 10 s at 95°C, 15 s at 60°C and 10 s at 72°C. Product formation was detected at 72°C in the fluorescein isothiocyanate channel. The relative mRNA expression levels were calculated and normalized to the levels of the murine hypoxanthine guanine phosphoribosyltransferase (HPRT) mRNA using the 2 ΔΔCT method (specific mouse primer sequences are shown in **Table 1**). All measurements were performed in duplicate.

IL-6 and PGE_2 assessments

The IL-6 concentrations in the murine cell supernatants were measured using the Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Lille, France). The concentrations of human IL-6 were measured in human CM using the Pelikine compact kit (Sanquin, Amsterdam, Netherlands). The concentrations of the murine and human pro-inflammatory bioactive lipid PGE_2 in the cell supernatants and CM were measured using 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 for the murine/human IL-6 and PGE_2 assessments, respectively. Duplicate measurements were performed.

Protein extraction and western blotting

Protein extracts from human cartilage explants were prepared using previously described methods (56). Murine chondrocytes were cultured in 12-well plates (4×10^4 cells/well) in duplicate and were treated as indicated. Subsequently, the total intracellular proteins were obtained as previously described (63). In some experiments, cytosolic and nuclear fractions were obtained from the murine chondrocytes using commercially available NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. Protein extracts from human cartilage (30 μg) and total proteins (30 μg) or nuclear fractions (20 μg) from murine chondrocytes were resolved by SDS-PAGE and immunoblotted with the indicated antibodies: Nrf-2 antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-722) (80-100 kDa)(64), HO-1 antibody (1:1,000; Enzo Life Sciences, Lausen, Switzerland; SPA-895)

(32kDa), or actin antibody (1:5,000; Sigma-Aldrich, Lyon, France; A5316). Signals were detected using enhanced chemiluminescence (ECL), and the blots were exposed to Fujifilm LAS-300 (Fujifilm Medical Systems, Stamford, CT, USA). The relative levels of protein expression were calculated using densitometry and were normalized to the actin levels. We used Image-Gauge software (Science Lab 2004; Fujifilm) for the densitometry analysis.

Cellular ROS production

Chondrocytes were seeded and cultured in 96-well plates at a density of 1×10^4 cells per well, as described above. After 72-h treatment, ROS production was measured using a fluorometric assay with dichlorodihydrofluorescein diacetate (DCFDA) (Molecular Probes, Life Technologies, Saint Aubin, France). Briefly, chondrocytes were incubated with 17 µM DCFDA diluted in the fasting medium for 60 min at 37°C in the dark. Subsequently, the chondrocytes were washed with PBS, and fluorescence was measured using the Fluostar Galaxy reader (BMG Labtech, Ortenberg, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm and then analyzed using the Biolise system (Labsystems, Helsinki, Finland). The intracellular proteins were collected with NaOH (0.5 M), and the concentrations were measured using a spectrophotometer and a protein assay kit (Bio-Rad) to normalize the results. The ROS production is represented as fold induction from that of the control and by micrograms of protein. All measurements were performed in triplicate.

Statistical analysis

All data are reported as points representing one single experiment from one litter of mice or one patient with standard deviation to represent error. All tests were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) with the Wilcoxon test for paired variables and the Mann-Whitney test for unpaired variables. Additionally, analysis of data from Nrf-$2^{-/-}$ experiments was performed by paired t test. $P \leq 0.05$ was considered statistically significant.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest regarding the contents of this article.

AUTHORS' CONTRIBUTIONS

CV-G, AC, M-CL, XH, SK-R, RS, FB and JS were responsible for the study design, manuscript preparation, and data interpretation. AS organized and collected the human tissue samples and participated in designing the experiments with human tissue and in data interpretation. CV-G, AC and AP performed the experiments. SK-R was responsible for generating the Nrf-$2^{-/-}$ mice and was involved in data interpretation. All authors reviewed and approved the final manuscript.

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FOOTNOTES

CoPP, cobalt protoporphyrin-IX; DCFDA, 2',7' dichlorofluorescein diacetate; HG, high glucose; HO-1, heme oxygenase-1; HPRT, hypoxanthine guanine phosphoribosyltransferase; IL-6, interleukin-6; IL-1β, interleukin-1β; KEAP-1, Kelch-like ECH-associated protein 1; NG, normal glucose; Nrf-2, nuclear factor erythroid 2-related factor 2; OA, osteoarthritis; PGE_2, prostaglandin E_2; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction T2DM, type 2 diabetes mellitus.

TABLE 1

Gene	*foward*	*reverse*
Nrf-2	5’-CATGATGGACTTGGAGTTGC-3’	5’-CCTCCAAAGGATGTCAATCAA-3’
Keap-1	5’-CACAGCAGCGTGGAGAGA-3’	5’-CAACATTGGCGCGACTAGA-3’
HO-1	5’-AGGCTAAGACCGCCTTCCT-3’	5’-TGTGTTCCTCTGTCAGCATCA-3’
HPRT	5’-AGGACCTCTCGAAGTGT-3’	5’-ATTCAAATCCCTGAAGTACTCAT-3’

FIGURE LEGENDS

Figure 1: Reduced nuclear factor-erythroid 2-related factor-2/heme oxygenase-1 (Nrf-2/HO-1) expression in OA cartilage from patients with T2DM is associated with increased production of pro-inflammatory mediators. The HO-1 (**A**) and Nrf-2 (**B**) levels in explants of OA cartilage from patients with or without T2DM were evaluated by western blotting. The values were normalized to the β-actin levels. Each symbol represents an OA patient without (OA; □) or with T2DM (OA-T2DM; ●) (n=8 per condition). The dependence between Nrf-2 levels (**C**) or PGE_2 production (**D**) and HO-1 expression in all groups of patients (△) was assayed by dichotomizing the Nrf-2/PGE_2 values as a function of the median HO-1 levels. The bars represent the means ± SD for each condition. * p≤0.05. OA, osteoarthritis; HO-1, heme oxygenase-1; Nrf-2, nuclear factor erythroid 2-related factor 2; PGE_2, prostaglandin E_2; T2DM, type 2 diabetes mellitus.

Figure 2: Nrf-2 expression is reduced in murine chondrocytes incubated with high glucose. Chondrocytes were incubated with normal glucose (5 mM; NG) or high glucose (25 mM; HG) in the presence or absence of interleukin-1β (IL-1β; 5 ng/ml) for the indicated times. The expression levels of the Nrf-2 (**A**) and Kelch-like ECH-associated protein 1 (Keap-1) (**B**) genes were evaluated by quantitative RT-PCR (qRT-PCR). The values were normalized to hypoxanthine guanine phosphoribosyltransferase (HPRT) expression (n=6). **C.** The Nrf-2/Keap-1 expression ratio was calculated. The total (**D**) and nuclear (**E**) Nrf-2 protein expression levels were measured by western blotting. The values were normalized to β-actin expression (n=6). Each symbol represents an experiment from one litter of mice in

normal glucose (○) and high glucose (●) conditions. The bars represent the means ± SD for each condition. * p≤0.05. Nrf-2, nuclear factor erythroid 2-related factor 2; RT-PCR, reverse transcription-polymerase chain reaction.

Figure 3: HO-1 expression is reduced in murine chondrocytes incubated with high glucose and is associated with Nrf-2 levels and negatively associated with the production of inflammatory mediators. Chondrocytes were incubated with NG or HG in the presence or absence of IL-1β for the indicated times. Expression of the HO-1 gene (**A**) or protein (**B**) was evaluated by qRT-PCR or western blotting, respectively. The values were normalized to the HPRT levels for gene expression or to the β-actin levels for protein quantification (n=6 per condition). Each symbol represents an experiment from one litter of mice in normal glucose (○) and high glucose (●) conditions. The bars represent the means ± SD for each condition. * p≤0.05. Dependence analysis between Nrf-2 expression (**C**) or IL-1β-induced PGE_2 production (**D**) and HO-1 expression was assayed by dichotomizing Nrf-2/PGE_2 values as a function of the median HO-1 levels. Each symbol (△) represents the Nrf-2/PGE_2 value obtained from chondrocytes incubated in both normal and high glucose, and whose HO-1 expression is lower or higher than the median level (n=72 for the Nrf-2/HO-1 analysis; n=10 for the PGE2/HO-1 analysis). The bars represent the means ± SD for each condition. * p≤0.05; **p≤0.01. HO-1, heme oxygenase-1; Nrf-2, nuclear factor erythroid 2-related factor 2; NG, normal glucose; HG, high glucose; IL-1β, interleukin-1β; qRT-PCR, quantitative reverse transcription polymerase chain reaction; HPRT, hypoxanthine guanine phosphoribosyltransferase; PGE_2, prostaglandin E_2.

Figure 4: HO-1 protects chondrocytes against increased responsiveness to IL-1β in a high glucose environment. Chondrocytes were co-incubated with an inducer of HO-1, CoPP (10 µM), for 72 h. Release of the IL-6 (**A**) and PGE_2 (**B**) was assayed. **C.** ROS production was evaluated using a fluorimetric assay with DCFDA. Represented data are fold induction compared to the control condition without IL-1β, and normalized to intracellular protein quantity. Each symbol represents an experiment from one litter of mice in normal glucose (○) and high glucose (●) conditions. The bars represent the means ± SD for each condition (n=6). * p≤0.05. HO-1, heme oxygenase-1; IL-1β, interleukin-1β; CoPP, cobalt protoporphyrin-IX; IL-6, interleukin-6; PGE_2, prostaglandin E_2; ROS, reactive oxygen species; DCFDA, 2,7 dichlorofluorescein diacetate.

Figure 5: Sulforaphane increases HO-1 expression and attenuates ROS and inflammatory production. Chondrocytes were pre-incubated with Sulforaphane (SFN; 5µM) for 30min before stimulation in normal glucose (5mM; NG) or high glucose (25mM; HG) with/without interleukin-1β (IL-1β; 5ng/ml). HO-1 expression (**A.**) and ROS (**B.**) and IL-6 production (**B.**) were assayed after 72 h treatment. Each symbol represents an experiment from one litter of mice in normal glucose (○) and high glucose (●) conditions. The bars represent the means ± SD for each condition. * p≤0.05. HO-1, heme oxygenase-1; ROS, reactive oxygen species; IL-6, interleukin-6.

Figure 6: Nrf-2 knockout (Nrf-$2^{-/-}$) chondrocytes exposed to high glucose exhibit

lower HO-1 expression and increased responsiveness to IL-1β. A. The expression of the HO-1 protein was evaluated in chondrocytes from wild type (WT) or Nrf-$2^{-/-}$ mice that were stimulated as described above for 72 h. The values were normalized to β-actin expression. Represented data are fold induction compared to the control condition without IL-1β. **B.** PGE_2 production was also measured. Each symbol represents an experiment from one litter of wild type (○) and Nrf-$2^{-/-}$ (●) mice. The bars represent the means ± SD for each condition. * p≤0.05.(n=4). Nrf-2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; IL-1β, interleukin-1β; PGE_2, prostaglandin E_2.

<u>Figure 7:</u> Hypothetical mechanism by which impairments in the Nrf-2/HO-1 axis favor catabolic responsiveness to IL-1β in chondrocytes exposed to a high glucose environment. The Nrf-2/HO-1 axis is one of the most important anti-inflammatory and antioxidant protective systems. Under stress conditions, Nrf-2 is translocated to the nucleus to activate antioxidant gene expression, including one of its main target HO-1. Next, HO-1 counteracts the catabolic pathways induced by inflammatory stimuli such as IL-1β. However, Nrf-2 signaling is impaired in a high glucose environment, which is manifested as dysfunction in Nrf-2 synthesis and translocation and a subsequent reduction in the HO-1 levels. Thus, chondrocytes lost the capacity to control ROS production and inflammation (*i.e.*, PGE_2 and IL-6 release). This event further favors the activation of pathological pathways, leading to an increase in the susceptibility to OA and disease severity. SFN, a Nrf-2 activator, and CoPP, an inducer of HO-1, attenuates this phenomenon. HO-1, heme oxygenase-1; Nrf-2, nuclear factor erythroid 2-related factor 2; IL-1β, interleukin-1β; PGE_2, prostaglandin E_2; IL-6,

interleukin-6; OA, osteoarthritis; SFN, sulforaphane; CoPP, cobalt protoporphyrin-IX.

Table 1: Primer sequences used for real-time PCR. HO-1, heme oxygenase-1; Nrf-2, nuclear factor erythroid 2-related factor 2; KEAP-1, Kelch-like ECH-associated protein 1; HPRT, hypoxanthine guanine phosphoribosyltransferase.