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IL-1beta mediates MMP secretion and IL-1beta neosynthesis via upregulation of p22^{phox} and NOX4 activity in human articular chondrocytes

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SUMMARY

Objectives: Osteoarthritis (OA) is characterized by a progressive alteration of the biochemical properties of the articular cartilage. Inflammation plays a major role in OA, particularly through the cytokine Interleukine-1β, promoting reactive oxygen species (ROS) generation and matrix metalloproteinases (MMP) synthesis by the chondrocytes, orchestrating matrix proteolysis. NADPH oxidases (NOX) are membrane enzymes dedicated to the production of ROS. Role of oxidative stress is well established in OA; however, contribution of NOX in this process is still poorly documented. In this study, we addressed the role of NOX in primary human articular chondrocytes (HAC) upon inflammatory conditions – namely IL-1β and OA.

Design: HAC were collected from patients undergoing hip surgery. Chondrocytes were treated with IL-1β and NOX inhibitors Diphenylene Iodonium, GKT136901, Tiron and Heme oxygenase-1 before MMP expression and NOX activity assessment. Finally, NOX4 expression was compared between OA and non OA parts of hip cartilage ($n = 14$).

Results: This study establishes for the first time in human that NOX4 is the main NOX isoform expressed in chondrocytes. We found a significant upregulation of NOX4 mRNA in OA chondrocytes. Expression of NOX4/p22^{phox} as well as ROS production is enhanced by IL-1β. On the other hand, the use of NOX4 inhibitors decreased IL-1β-induced collagenase synthesis by chondrocytes. Moreover, our study support the existence of a redox dependant loop sustaining pro-catabolic pathways induced by IL-1β.

Conclusions: This study points out NOX4 as a new putative target in OA and suggests that NOX-targeted therapies could be of interest for the causal treatment of the pathology.

Introduction

ROS are small reactive molecules derived from molecular oxygen involved in physiological processes such as innate immunity and cell signalling pathways controlling differentiation, adhesion, migration or apoptosis[1]. Impairment of ROS homeostasis has been associated with pathological states such as cell tumoral transformation, fibrosis, osteoporosis or diabetes due to inappropriate cell signalling. ROS production has been suggested to be part on the ageing process[2]. Indeed, most of the common ageing related degenerative pathologies such as Parkinson's disease, Alzheimer, cancer, osteoporosis or osteoarthritis are linked to oxidative stress[1,3].

NOX family are transmembrane proteins composed of seven members sharing specific structural homology regions[4,5]. NOX catalyses the transfer of electron from NADPH across biological membranes via two heme molecules to generate superoxide anion, giving rise to ROS[6]. NOX4 represents the major source of ROS in kidney and is widely expressed in many others tissues including the

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skeletal system[7–9]. Its activity is dependent on $p22^{phox}$ level[10] through a mutual stabilization of the two partners at the protein level[11]. NOX4 activity has recently been shown to be upregulated by quinone compounds *in vitro*[12]. On the other hand, we recently showed that NOX4 activity is negatively regulated by Heme Oxygenase-1 (HO-1), the rate limiting enzyme in heme catabolism[13].

Osteoarthritis (OA) is a common ageing feature related to degenerative disease in which chondrocytes play a central role[14]. In OA, chondrocytes exhibit reticular and oxidative stress markers, lack of proteasome recycling and a decreased mitochondria dependent ATP generation that are common features of degenerative diseases[15,16]. In OA, chondrocytes fail to maintain homeostasis between anabolic and catabolic pathways. IL-1β is one of the main catabolic factors involved in osteoarthritis and many of its biological effects are mediated through a generation of ROS[17,18]. For example, in bovine chondrocytes, both IL-1β and hydrogen peroxide led to an activation of the redox dependant transcription factors NF-KB and AP-1[19] triggering a dramatic decrease in type II collagen transcription associated with a significant increase of MMP synthesis.

Only few studies have addressed the role of NOX enzymes in chondrocytes[18,20,21]. To our knowledge, all published studies were performed on cell lines or in chondrocytes explants from animal models; however, no data concerning the role of NOX in human articular chondrocytes and in OA are presently available. In the human C-20/A4 chondrocyte cell line NOX4 is the sole active isoform of NOX and its activity has been shown to mediate MMP-1 synthesis and chondrocyte apoptosis under IL-1β cytokinic stimulation[13,18]. Interestingly, NOX4 inhibition by HO-1, prevent IL-1β-induced MMP-1 secretion and chondrocyte apoptosis. Given the well-known role of oxidative stress in osteoarthritis and our previous data from the chondrocyte cell line C-20/A4[13], we decided to investigate the role of NOX in human articular chondrocytes and in OA condition.

In this study, we show that NOX4 expression is the highest among other NOX isoforms in HAC and exhibits a constitutive oxidase activity. Similar to chondrocytes cell line, IL-1β drives collagenase synthesis by HAC through an increase of NOX4 and $p22^{phox}$ expression as well as ROS production. Moreover, our results point out the existence of an autocrine/paracrine loop in which ROS mediate IL-1β and $p22^{phox}$ neosynthesis, sustaining catabolic pathways. Interestingly, IL-1β-induced ROS production is prevented by the NADPH oxidase inhibitors Diphenylene Iodonium (DPI) as well as by the use of GKT136901, a specific inhibitor of NOX4, and by the induction of HO-1 expression in HAC. These results suggest that targeting ROS generators and in particular the NOX4 pathway in HAC may have a promising potential for therapeutic strategy in the pathology of OA.

Material and methods

Material

Chemical reagents used in this study and their sources were the following: Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) (life technologies, Saint Aubin, France); AMV Reverse transcriptase (QBiogene, Illkirch, France); TRIzol® reagent, Taq polymerase, Alexa Fluor 546 or 633 labelled goat anti-mouse IgG, Hoechst 33258, (Invitrogen, Cergy Pontoise, France); ECL Western Blotting detection reagents, Goat anti-Mouse IgG-HRP antibody and IL-1β ELISA detection kit (GE healthcare, Buckingamshire, UK); $Na_4P_2O_7$, Na_3VO_4, PMSF, luminol, Horseradish Peroxidase (HRPO), Triton X-100, protoporphyrin-IX cobalt chloride (CoPP-IX), Diphenylene iodonium (DPI), Collagenase type IA and Hyaluronidase (SIGMA, Saint Quentin Fallavier, France); GKT136901 (GenKyoTex, Plan les Ouates, Switzerland); Okadaic acid, leupeptin, pepstatin, trypsin inhibitor, TLCK, human interleukin-1β, complete mini EDTA-free protease inhibitor EASYpack (Roche diagnostics, Meylan, France); diisopropylfluorophosphate (DFP, Acros Organics, Halluin, France); polyclonal IgG from goat against HMOX1 (sc-7695) or against actin (sc-1615) and control Ig (Santa Cruz Biotechnologies, Heidelberg, Germany); Lab-Tek chambered coverglass (Thermo scientific, Courtaboeuf, France); 100 μm nylon cell strainer (BD Falcon, Pont de Claix, France); Decalcifier solution for histology SAKURA TDE 30 (Bayer Healthcare, Newbury, UK), second antibody anti mouse conjugated to peroxidase (DAKO, Courtaboeuf, France); anti-MMP-1 (mAb 901) and anti-MMP-13 monoclonal antibody (R&D Systems, Lille, France); and Rabbit monoclonal Ig against NOX4 (UOTR1B492; Abcam, Paris, France); NOX4 monoclonal antibody (8E9)[22], $p22^{phox}$ monoclonal antibody[23].

Chondrocyte isolation and culture

Cartilage specimens were obtained from 14 patients aged from 46 to 90, undergoing femoral hip replacement. Samples were obtained under patient's consent according to the declaration of Helsinki. Cartilage slices were removed from the femoral head and cut into small pieces. The distinction between the "OA chondrocyte" group and the control chondrocytes was determined by pathologists, according to the OARSI guidelines[24]. Chondrocytes were then isolated by sequential enzymatic digestion: 1 h with 0.1 mg/ml hyaluronidase in sterile PBS at 37°C, followed by 16 h with 1 mg/ml collagenase (type IA) at 37°C in DMEM containing 10% FBS in presence of penicillin (100 U/ml) and streptomycin (100 g/ml) at 37°C in 5% CO_2 atmosphere. The digested tissue was filtered through a 100 μm nylon mesh, washed and centrifuged. The isolated chondrocytes were finally seeded in 6-well plates until they reach confluence and cultured in DMEM containing 4.5 g/L glucose and 0.11 g/L sodium pyruvate, supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine at 37°C, 5% CO_2. For experiments with IL-1β, cell culture medium was replaced by serum free DMEM for 24 h. Chondrocytes were then stimulated by IL-1β, supplemented or not with antioxidants or inhibitors during 24 h. To avoid chondrocyte dedifferentiation, MMP-1 and MMP-13 Western Blot (WB) and real-time quantitative (RT-q-)PCR were performed before the first passage; other experiments were performed after a single passage (75 cm^2 flask). Collagen type II expression, a marker of chondrocyte differentiation was checked after the first passage before launching chemical treatments.

RNA extraction and RT-q-PCR

Total RNA extraction was performed with NucleoSpin® RNA (Macherey Nagel, Hoerd, France) following recommendations. cDNA was reverse transcribed from 1 μg of total RNA with the SuperScriptIII First-Strand Synthesis (Life Technologies, Carlsbad, CA). As recommended an RNase H treatment was added. Real time RT-qPCR was conducted using the QuantiTect SYBR Green RT-PCR kit (Qiagen) and a Stratagene Mx3005P (La Jolla, CA).

Briefly, the transcript expression levels of human IL-1β, CYBA, NOX4, HMOX1, MMP-1, MMP-13, ADAMTS4 and housekeeping genes GAPDH, RPL27 and RPL32 were determined using specific primers chosen to include intron spanning (Supplementary Table I). Gene expression was quantified using the comparative threshold cycle (Ct) method. The amount of target gene, normalized to three endogenous reference genes (RPL27, RPL32 and GAPDH) was expressed relative to the control cells. The specificity of the PCR

products was confirmed by gel electrophoresis migration and a by melting curve analysis.

Evaluation of ROS production by Amplex Red assay

After the first passage chondrocytes were grown in a 75 cm^2 flask at 90% confluence. After 24 h serum starvation, cells were stimulated with 2 ng/ml IL-1β in presence or not of inhibitors (CoPP or DPI 10 μM). Cells were then detached with 0.25% (w/v) trypsin, washed twice with PBS and collected after 8 min centrifugation at 400 *g* at room temperature. The viability of the suspended cells was over 95%, as determined by the trypan blue exclusion method. In a 96-well plate, 5×10^5 living cells resuspended in 20 μl PBS were added per well. Before the start of the assay, 100 μl of a PBS solution containing 5 μM Amplex Red and 10 mUnits/ml horseradish peroxidase was added in each well. Results are expressed as the sum of Relative Fluorescent Units (RFU) recorded every two minutes during 60 min on a fluostar omega spectrofluorimeter (BMG labtech).

Cell extracts preparation

Cells were treated with 3 mM DFP and lysed in Triton X-100 lysis buffer containing 20 mM Tris–HCl pH 7.4, 1% (v/v) Triton X-100, 150 mM NaCl, 1 mM EDTA, 10 mM $Na_4P_2O_7$, 10 nM okadaic acid, 2 mM Na_3VO_4, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 10 μg/ml trypsin inhibitor, 44 μg/ml PMSF, 10 μM TLCK and complete mini EDTA-free protease inhibitor (Triton X-100 cell extract). After 10 min incubation on ice, the mixture was centrifuged at 1000 *g*, 10 min, 4°C. The supernatant was then used for SDS-PAGE and Western Blot.

SDS/PAGE and WB

Triton X-100 cell extract 10× concentrated cell culture supernatant were denatured at 60°C for 30 min or 4°C overnight and loaded on 10% (p/v) SDS-PAGE for migration and then electrotransfer to nitrocellulose. Immunodetection was performed using antibodies raised against NOX4 (mAb 8E9, 1:500 or UOTR 1:2000)[22], $p22^{phox}$ (mAb) MMP-1 (1:500), MMP-13 (1:1000); HO-1 (1:500), or Actin (1:1000). Secondary antibody was conjugated to peroxidase (1:5000). Peroxidase activity was detected using ECL reagents (GE Healthcare, Freiburg).

IL-1β assay

C-20/A4 chondrocytes were treated with IL-1β in a 75 cm^2 flask containing serum free DMEM medium in presence or not of 10 μM CoPP to induce HO-1 expression or with 5 mM Tiron. 24 h later, 3 washes were performed with 1X PBS. Last wash was kept to assess residual IL-1β remaining in the flask. Medium was then replaced by serum free DMEM medium for additional 24 h and was up taken for IL-1β neosynthesis measurement with a commercial ELISA assay. Results are representative of three independent experiments.

Confocal microscopy

Chondrocytes were seeded on coverslips at 60% confluency and fixed with 4% (w/v) paraformaldehyde (PFA) for 10 min, PFA fluorescence was quenched by 50 mM NH_4Cl for 10 additional min at RT. Cells were then washed twice with PBS containing 1% (w/v) BSA (PBS/BSA buffer) and permeabilized with 0.1% (v/v) Triton X-100 during 10 min at RT. NOX4 subcellular localization was assessed by 1 h incubation at room temperature with 5 μg of mouse monoclonal Ig (irrelevant Ig or 8E9 mAb) in 200 μl of PBS/BSA buffer. After two washes with PBS/BSA buffer, 1 h incubation with anti-mouse secondary antibodies conjugated to Alexa 546 (1/200) was performed at RT. Cell nuclei were stained with Hoechst 33258 (0.5 μg/ml) and mounted in DABCO solution; they were sealed, and stored at 4°C in the dark. Confocal microscopy was carried out by using the Zeiss LSM510 NLO META. The pinhole was adjusted to 1 Airy unit resulting in 0.7 μm thick slices. Hoechst fluorescence was visualized using a 2photon excitation.

Immunohistochemistry

Human cartilage was decalcified (Decalcifier SAKURA TDE 30, Bayer-Tissue teck), rinsed in distilled water and embedded in paraffin. 4 μm sections were then layered on polylysine coated slides and deparaffined with xylene treatment. Slides where then incubated for 40 min at 98°C in EDTA solution pH8 to unmask antigen, treated with hydrogen peroxide 3% for 10 min to inhibit endogenous peroxidases and were incubated over night with NOX4 8E9 mAb (1/1250), 4°C. After 3 rinses in PBS 1×, slides were incubated for 45 min with second antibody conjugated to peroxidase before revelation. Nucleus was stained by hematoxilin and samples were mounted on coverslips.

Statistical data

All quantitative data were generated using biological replicates in triplicate unless stated otherwise, and are expressed as the mean plus 95% confidence interval. Data normality was tested by a Shapiro–Wilk test. For each experiment, *P*-values were determined using a paired Student test. All analyses were conducted using StatView (SAS institute, US).

Results

NOX4 is highly expressed in human articular cartilage

IL-1β is one of the main catabolic factors involved in OA and lead to generation of ROS by chondrocytes[17,18]. NOX family dedicated to the ROS production may play a major role in response to the cytokine. To confirm this hypothesis, human articular chondrocytes (HAC) were isolated and cultured from human femoral head according to the Material and Method section. The mRNA expression of NADPH oxidases (NOX1 to NOX5) and their partner $p22^{phox}$ was evaluated by RT-PCR [Fig. 1(A)]. Consistent to our previous data from the human chondrocyte cell line C-20/A4[18], a robust mRNA expression of NOX4 and $p22^{phox}$ was shown in human primary chondrocyte. In contrast, the other NOXs mRNA was below detection thresholds pointing NOX4 as the sole expressed NOX in HAC. The absence of NOX2 was confirmed by real time RT-PCR experiments (data not shown).

To confirm NOX4 expression at the protein level *in vivo*, immunohistochemical experiments were performed on human hip cartilage sections [Fig. 1(B)]. Results showed a gradual staining of NOX4 from the subchondral bone to the articular surface of the cartilage [Fig. 1(B) left panels] compared to the absence of staining when using the irrelevant antibody [Fig. 1(B) right panels]. Consistently, NOX4 proteins were also detected in *ex vivo* cultured HAC by Immunoblot [Fig. 1(C)]. The high expression of NOX4 was confirmed by confocal microscopy, as showed by the staining of NOX4 in the perinuclear area and next to the plasma membrane of HAC [Fig. 1(D)].

NOX4 activity is enhanced upon IL-1β stimulation in human articular chondrocytes

We next addressed the functional impact of NOX by assessing the production of ROS-induced by IL-1β in HAC. After 24 h of stimulation by IL-1β, we observed a significant increase of the

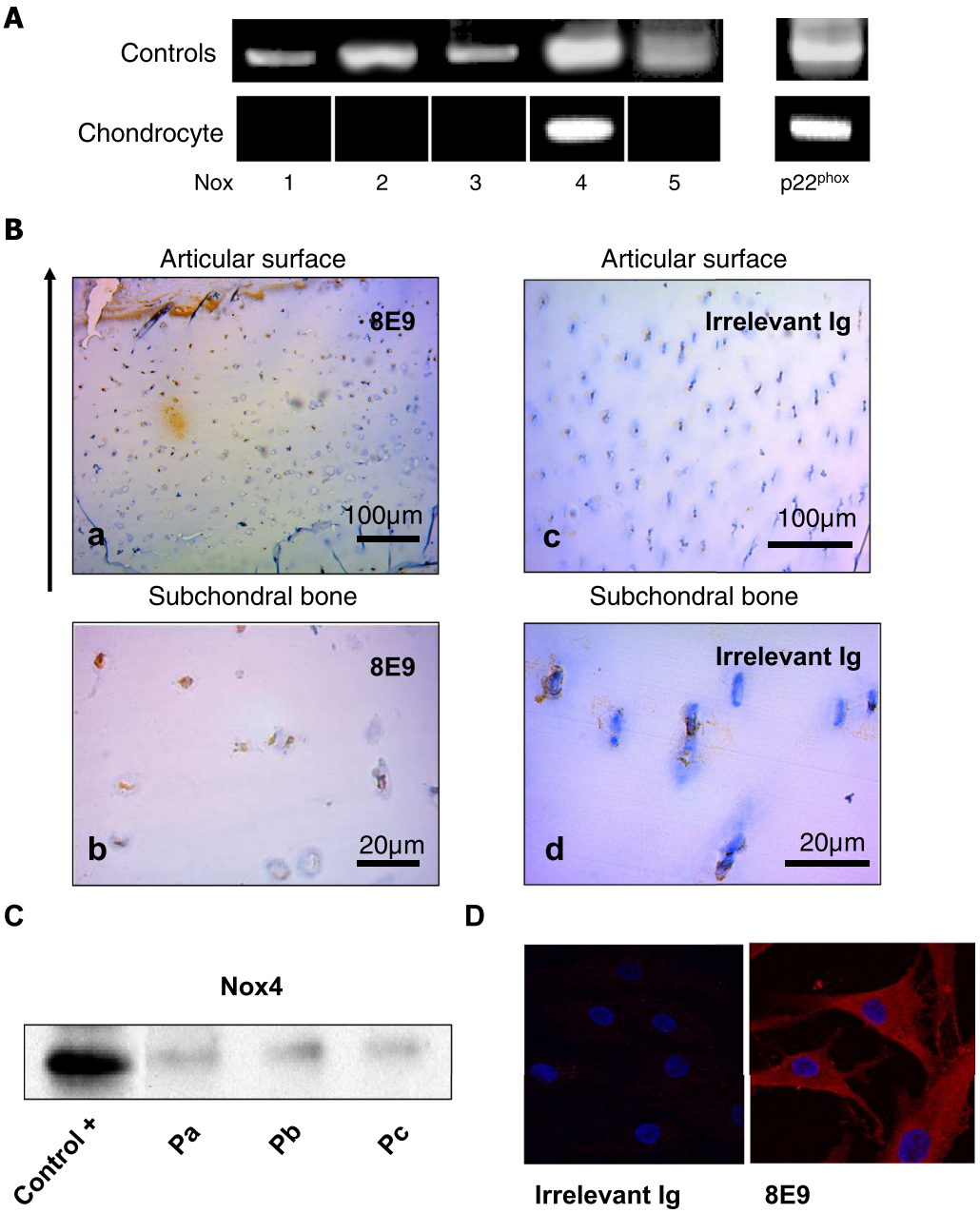

Fig. 1. NOX4 is the chondrocyte NADPH oxidase. (A) RNA was extracted from HAC ($n = 3$) and transcribed to cDNA as described in the Material and Methods section. Specific primers were used to amplify cDNA encoding for NOX1 to NOX5 and $p22^{phox}$ (Table I Supplemental Data). Plasmids encoding NOX1 to NOX5 and $P22^{phox}$ were used as positive controls. (B) Immunohistochemical detection of NOX4 on human cartilage sections by purified mAb8E9 (a and b) or irrelevant Ig (c and d). Photographed and printed to a final magnification of ×500 for the upper pictures, and ×1000 for the lowers. The arrow show NOX4 gradually expressed in human cartilage from the bone side to the articular surface. This result was representative of $n = 3$ human cartilage section. (C) NOX4 expression was assessed by immunoblot upon SDS-PAGE on 150 µg of protein from 1% (v/v) Triton X-100 extract obtained from HAC ($n = 3$ patients). Positive control, was obtained from C-20/A4 NOX4 cells. (D) HAC cultured *ex vivo* (passage 1) were fixed with PFA, permeabilized and were respectively stained with irrelevant IgM or the NOX4 specific 8E9 mAb antibody (red). The nucleus was coloured with Hoechst 33256 (blue). This result was representative of $n = 3$ patients.

hydrogen peroxide (H_2O_2) produced by HAC [Fig. 2(A)] with an optimum dose/response value at 2 ng/ml IL-1β (Supp. Fig. 1). Under these conditions, the addition of the DPI, an inhibitor of NADPH oxidase, led to a significant inhibition of the IL-1β-stimulated ROS production by HAC [Fig. 2(A)]. Moreover the use of GKT136901, a pharmacological inhibitor of NOX4, had a similar effect. Furthermore, treatment with inhibitors of the NO synthase (L-NAME) and of the mitochondrial respiratory chain (Azide and Rotenone), other putative sources of ROS in HAC, did not affect the measured ROS production detected by Amplex Red oxidation rate method [Supp. Fig. 1(B)]. Together, these data suggest that NOX4 is the source of ROS in HAC upon IL-1β stimulation. To confirm this data, we assessed the impact of IL-1β on NOX4 and $p22^{phox}$ expression. Interestingly, results showed a significant increase of both NOX4 and $p22^{phox}$ as shown by immunoblot [Fig. 2(B)]. Hence, given the constitutively active state of NOX4 in complex with $p22^{phox}$, these results suggest that the mechanism leading to the increase of ROS production by IL-1β in HAC cells is mediated by an upregulation of the amount of NOX4/$p22^{phox}$ complex.

NOX4 activity mediates MMP synthesis by chondrocytes

We have previously shown that ROS generated by NOX4 regulate the MMP-1 synthesis pathway in the IL-1β-stimulated C-20/A4

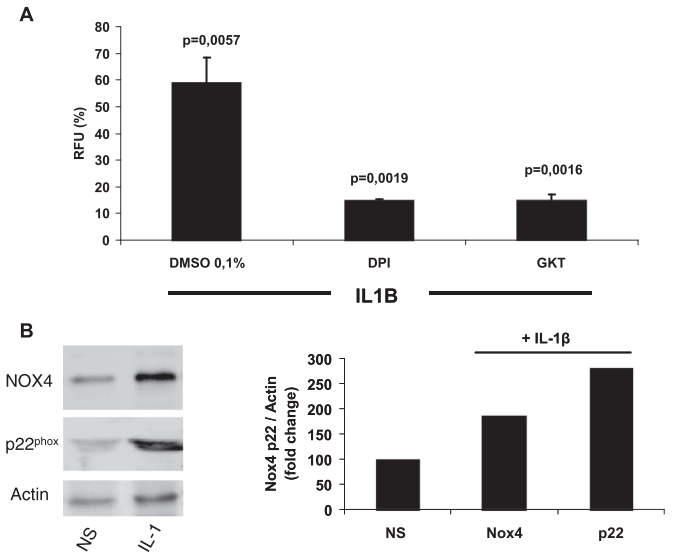

Fig. 2. NOX4 is a major source of ROS upon IL-1β stimulation in human articular chondrocytes. (A) HAC were cultured in absence of FBS during 24 h and stimulated with IL-1β for additional 24 h. Total H_2O_2 production was assessed by the Amplex Red method by using 5×10^5 cells per well. NOX4 inhibitors (10 μM): DPI, GKT136901 or DMSO (0.1%) were added in wells, 20 min before starting the experiment. Results are expressed as the percentage increase vs non stimulated cells (NS) (+DMSO 0.1%) in Relative Fluorescence Units (RFU) and were acquired every minute during 60 min. Values represent the mean and 95% confidence interval of three determinations ($n = 3$ patients). (B) NOX4 and $p22^{phox}$ expression was assessed by immunoblot upon SDS-PAGE on 150 μg of protein from 1% (v/v) Triton X-100 extract obtained from HAC (representative of $n = 3$ patients), stimulated or not (NS) with IL-1β for 24 h. Immunodetection was performed by antibodies raised against NOX4 and $p22^{phox}$ or Actin as loading control.

chondrocyte cell line[13]. In this context, the induction of Heme Oxygenase-1 was potent to inhibit NOX4 activity and the subsequent MMP-1 release. We therefore assessed the impact of NOX inhibition on IL-1β-induced MMP synthesis and release by primary human chondrocytes. The results showed a strong increase of the transcript level and of the quantity of MMP-1 and MMP-13 proteins released in the HAC culture supernatant of after 24 h stimulation with IL-1β [Fig. 3(D)]. Similarly, a strong induction of the transcriptional level of MMP-1, MMP-13 and ADAMTs4 (respectively 300, 200 and 40 fold) was detected by RT-qPCR [Fig. 3(A), (B) and (C)]. To determine the role of NOX4, we treated HAC cells with CoPP for 24 h to induce the expression of HO-1 mRNA (Supp. Fig. 2) or with DPI or GKT136901 to inhibit NOX4 activity. Interestingly, induction of HO-1 expression dramatically decreased IL-1β-stimulated MMP-1, MMP-13 and ADAMTS4 mRNA expression [Fig. 3(A)–(C)]. In addition, despite non-statistically significant results, we observed a similar tendency of the DPI and GKT136901 treatment to reduce the MMP-1 and MMP-13 transcriptional level. This tendency was confirmed at the protein level on HAC isolated from 3 patients [Fig. 3(D)]. However, while ADAMTS4 transcription was repressed by the other inhibitors, no effect of GKT136901 was observed, suggesting that the aggrecanase is not regulated by NOX4 but rather by direct or indirect target of DPI and HO-1.

Together, our data demonstrate that IL-1β-mediated MMP-1 and MMP-13 synthesis and release in HAC are triggered by a production of ROS. The data also strongly suggest a role of NOX4 in this pro-catabolic pathway.

IL-1β induced ROS production is involved in inflammation persistence

It is well established that inflammatory process plays a significant role in the OA pathology[25]. Especially, IL-1β acts in an autocrine/paracrine manner and induces its own neosynthesis by chondrocyte, sustaining pro-catabolic pathways leading to cartilage degradation[26]. We thus assessed whether the inhibition of NOX4 could impact the autocrine-mediated IL-1β synthesis. As assessed by RT-qPCR, IL-1β treatment strongly induced IL-1β mRNA (about 4000 fold increase) [Fig. 4(A)]. Interestingly, the induction of HO-1 expression significantly decreased IL-1β mRNA neosynthesis in HAC. Despite non-statistically significant results, the same tendency was observed with DPI treatment. These results were confirmed at the protein level as assessed by ELISA assay in the culture supernatant of C-20/A4 chondrocytes treated by CoPP or with the antioxidant tiron [Fig. 4(B)]. Moreover, IL-1β-induced $p22^{phox}$ transcription was also significantly prevented by DPI and by HO-1 induction. Similar tendency was observed with GKT136901 treatment [Fig. 4(C)]. Collectively, the results demonstrate for the first time that the autocrine/paracrine loop mediated by IL-1β in HAC is dependent on a production of ROS. Our data also suggest that NOX4 and $p22^{phox}$ could be critical mediator involved in this loop, sustaining in turn IL-1β neosynthesis and downstream catabolic pathways in chondrocytes.

NOX4 is overexpressed in OA chondrocytes

As a whole, mechanistic data collected from HAC suggest that NOX4 is a mediator of IL-1β-induced catabolic pathways. To further confirm the possible pathological implication of NOX4 in OA, we decided to compare the level of NOX4 mRNA between chondrocytes isolated from parts of the hip cartilage of OA and of non OA in a cohort of 14 patients. OA vs non OA criteria of the parts of cartilages were evaluated by pathologists (data not shown). Interestingly, we found a significant upregulation of NOX4 mRNA in OA chondrocytes (fold increase = 13.98 ± 9.7; $P = 0.021$ vs non OA patients) [Fig. 5(A)]. Moreover, the basal production of ROS was significantly enhanced in OA chondrocytes ($P = 0.048$) [Fig. 5(B)]. These clinical data are consistent with our previous observation on IL-1β treated chondrocytes and support the idea that NOX4 may participate to OA pathogenesis. Hence, we propose a mechanistic model in which IL-1β mediates the expression of pro-catabolic players – namely MMP-1 and MMP-13 – by a NOX4/p22phox depend fashion [Fig. 6].

Discussion

We have previously shown the direct relation between NOX4 activity and MMP synthesis by the C-20/A4 chondrocytes cell line[13]. The aim of the present study was to assess the functional relevance of these *in vitro* observation in human pathological conditions, namely in OA. Given the documented role of oxidative stress in degenerative pathologies and especially in OA[27], it is of importance to note that only two recent studies describe the presence of NOX in mice primary chondrocytes[21,28]. To our knowledge, this study demonstrates for the first time the presence of the NOX4 complex (NOX4 and $p22^{phox}$) in human articular chondrocyte. Moreover, at the functional aspect, the increase of NOX4 expression observed upon IL-1β treatment could mediate and sustain pro-catabolic pathways. The relevance of these data in the pathophysiology of osteoarthritis is supported by preliminary clinical data on 14 patients.

Potential sources of ROS have been identified in chondrocytes[3]. Among them, uncoupled NO synthase, mitochondria respiratory chain or NADPH oxidase could modulate chondrocyte behaviour and extracellular matrix homeostasis. In fact, physiological role of ROS in chondrocytes is not well understood. Indeed, according to Martin *et al.* mitochondria dependent generation of ROS is necessary to maintain glycolytic ATP production by chondrocytes[29]. ROS

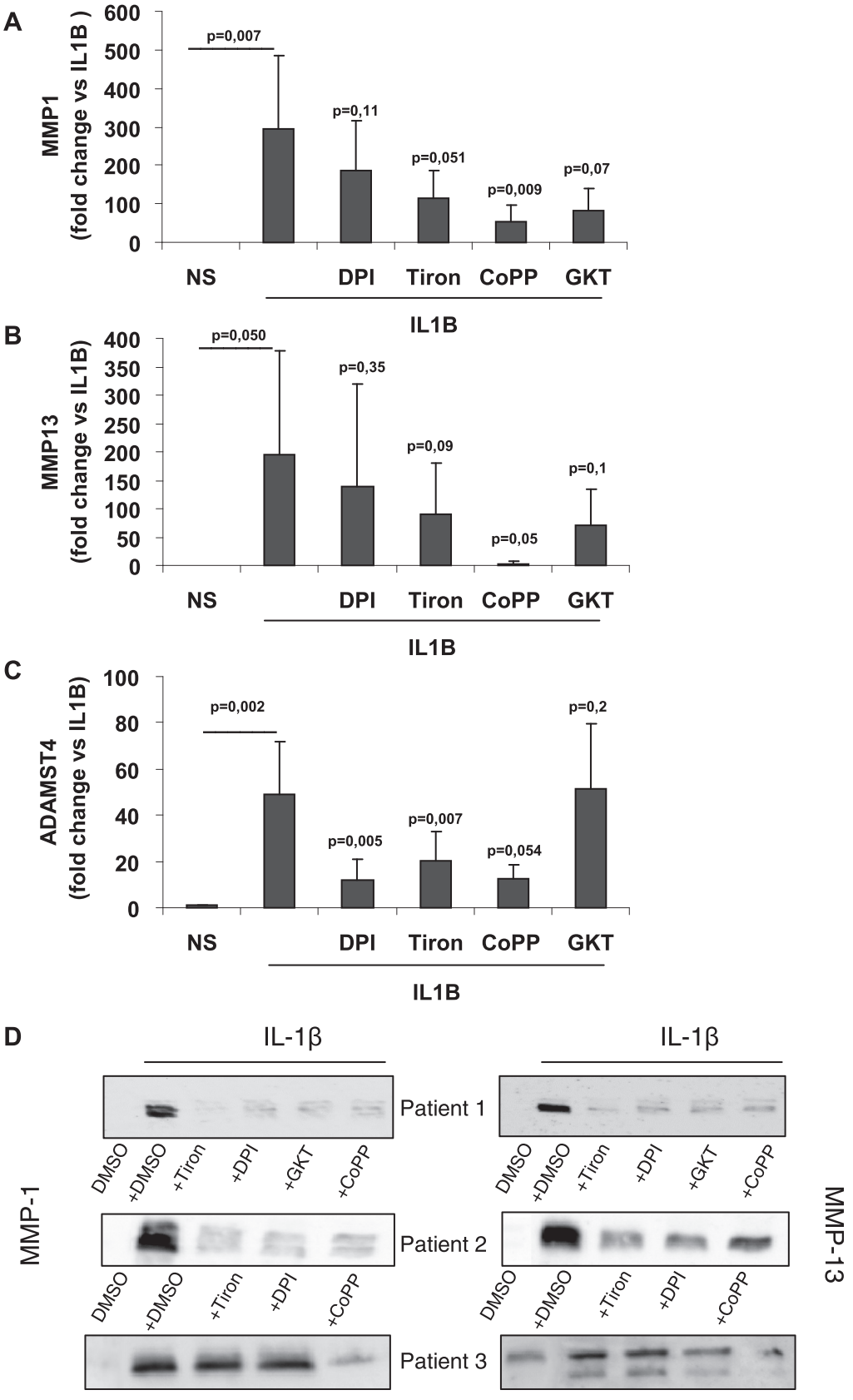

Fig. 3. NOX4 activity regulates MMP synthesis. HAC ($n = 14$ patients) were cultured in absence of FBS and induced or not for HO-1 expression by CoPP. After 24 h treatment with IL-1β and NOX4 inhibitors (Tiron, DPI or GKT136901), total RNA was extracted from HAC for RT-qPCR analysis of MMP-1 (A), MMP-13 (B) and ADAMTs4 (C) (D) In parallel, supernatant was recovered and 10 μg of proteins were loaded for MMP-1 and MMP-13 immunodetection by Western Blot. Experiments were performed on $n = 3$ patients.

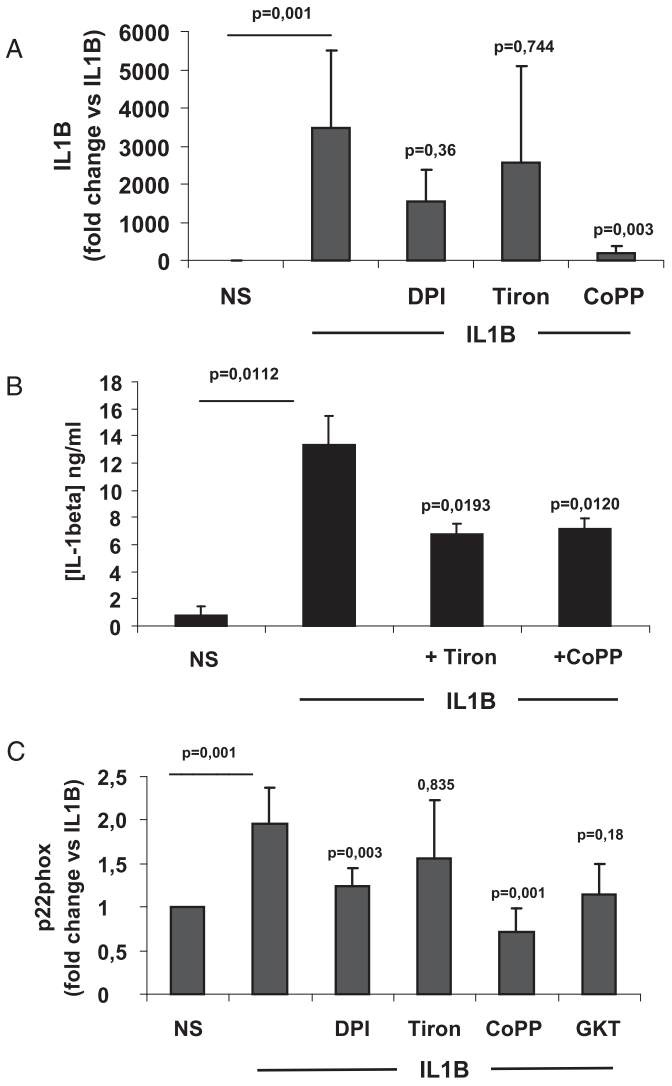

Fig. 4. NOX4 activity sustains IL-1 autocrine/paracrine loop and $p22^{phox}$ upregulation. (A–C) HAC were cultured in absence of FBS and induced or not for HO-1 expression by CoPP. After 24 h treatment with IL-1β and NOX4 inhibitors (DPI and tiron), total RNA was extracted from HAC ($n = 14$ patients) for RT-qPCR analysis of IL-1β (A) and CYBA ($p22^{phox}$) (C). (B) C-20/A4 chondrocytes were treated or not with IL-1β and NOX4 inhibitors (tiron and CoPP). After 24 h, cell were washed 3 times to eliminated exogenous IL-1β and medium was replaced. After additional 24 h neo-synthesized IL-1β was assessed by ELISA method in the cell culture supernatant = 3 patients.

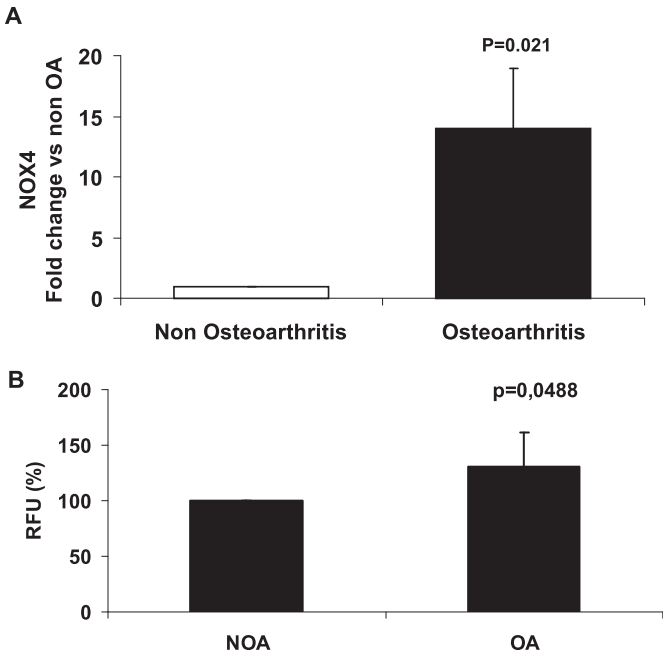

Fig. 5. NOX4 is overexpressed in OA chondrocytes. (A) HAC were extracted and cultured from damaged vs undamaged parts of articular cartilage ($n = 14$ patients). At passage 1, total RNA was extracted from HAC for RT-qPCR analysis of NOX4. Histogram shows the comparison of NOX4 mRNA level between OA and non OA chondrocytes. (B) In parallel, basal production of ROS was assessed by the Amplex Red method on 3 patients.

generated by NOX2 and NOX4 have also been suggested to act as chondrogenesis mediator during the skeletal system development[21]. Moreover, evidences show that ROS are involved in chondrocyte hypertrophic differentiation occurring in endochondral ossification[30,31]. NOX4 immunostaining on human cartilage slides showed a clear gradient of NOX4 expression from the bone side to the articular surface where oxygen is the most abundant. This gradient is particularly relevant given catalytic function of NOX, whose final electron acceptor is molecular oxygen. Hence, it is likely that in physiological conditions, especially during endochondral ossification, NOX4 could play a role in chondrogenesis or in chondrocytes differentiation[21,28].

However, in the inflammatory context, mimicked in our study by the effects of IL-1β or in OA, ROS may play deleterious and pro-catabolic functions in HAC. In the present study and upon IL-1β stimulus, a role of NO synthase and mitochondria respiratory chain was excluded [Supp. Fig. 1(B)]. Indeed, our data showed that ROS generated and NOX4-increased expression are critical mediators of MMP-1, MMP-13 and ADAMTs4 synthesis and release by HAC. Interestingly, the use of NOX inhibitors such as DPI, GKT136901 or HO-1 was able to prevent this release.

It is well documented that IL-1β acts in an autocrine/paracrine manner and induces its own neosynthesis by chondrocytes[26]. Our results show indeed a clear induction of IL-1β mRNA upon IL-1β stimulation of HAC. In addition, we bring in this study the first evidence that this loop is mediated by redox pathways in human chondrocytes. In fact, both $p22^{phox}$ and NOX4 expression increase upon IL-1β treatment probably contributing to sustain the production of ROS and IL-1β neosynthesis. Interestingly, NOX

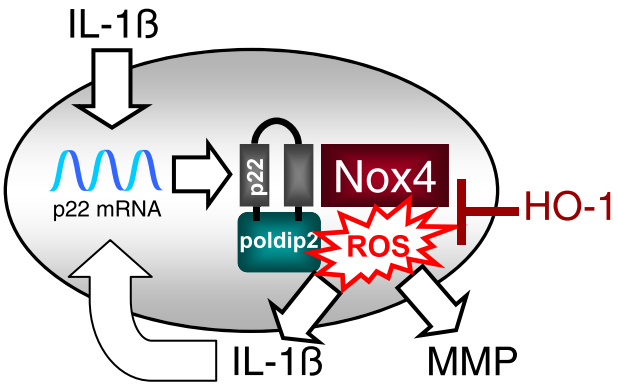

Fig. 6. The NOX4 pathway promote catabolism in human chondrocytes upon inflammatory condition. IL-1β treatment of HAC leads to an upregulation of $p22^{phox}$ mRNA, stabilization of NOX4 at the protein level and an increased production of hydrogen peroxide which acts as a crucial mediator of MMP-1, MMP-13 and ADAMTs4 expressions themselves responsible for chondrocyte extracellular matrix catabolism. This work also point out the redox regulation loop of $p22^{phox}$ and IL-1β suggesting that NOX4 activity sustains catabolic pathways in HAC.

inhibitors (GKT, DPI and HO-1) also abolished the increase in $p22^{phox}$ mRNA, disrupting this autocrine catabolic pathway. Such transcriptional regulation of $p22^{phox}$ was also shown in EaHy926 endothelial cell line[32]. As a general observation, we notice that HO-1 have a slightly better effect than NOX inhibitor on the expression of MMP and on IL-1β neosynthesis. In fact, HO-1 is a multipotent heat shock protein[33]. The multiple effects of HO-1 are all related to the degradation of heme in bioactive compounds, namely bilirubin, iron and carbon monoxide. As HO-1 has been shown to act as a powerful anti-inflammatory agent in the context of OA[34,35], it is likely that the whole effects of HO-1 are not all related to NOX4 inhibition in chondrocytes, by example considering ADAMTS4 transcription.

As a whole, the present study strongly suggests a significant contribution of NOX4/$p22^{phox}$ the IL-1β-mediated expression of MMP and IL-1β autocrine loop in human articular chondrocytes. These results are supported by the significant overexpression of NOX4 mRNA and increased production of ROS observed in OA compared to non OA condition in a cohort of 14 patients.

Nowadays, no available causal treatments are able to slow the degenerative condition of articular cartilage during OA[36]. On the other hand, many dietary supplements also displaying antioxidant capacities have been assessed in OA[37]. Among them, the most widely used compounds include proteoglycans such as chondroitin or glucosamine sulfate and polyphenols such as resveratrol or green tea extracts[38]. Some of these compounds seem to display modest benefits on the disease progression, however, antioxidant therapies are often disappointing *in vivo*, mainly because of the highly instable nature of ROS and their subsequent short half-life[39]. Indeed, it would expect that scavengers are bioavailable in sufficient concentration and in very close proximity of the site of production of the ROS. In this respect, we believe that pharmacological inhibitors of NOX4 as well as HO-1 agonists might be a sound strategy in the causal treatment of the disease. Further work, especially using *in vivo* models, should be done to confirm the potential of this strategy.

Author contributions

Conceived and designed the experiments: FR BL FM. Provision of study materials: BRD LG. Performed the experiments: FR CP AS FHP CD. Analysed the data: FR FHP CP BL. Wrote the paper: FR MVCN FHP BL FM.

Competing interests

The authors have declared that no competing interests exist.

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Supplementary data

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