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Heme oxygenase-1 induction modulates microsomal prostaglandin E synthase-1 expression and prostaglandin E₂ production in osteoarthritic chondrocytes

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ABSTRACT

Pro-inflammatory cytokines such as interleukin-1 β (IL-1 β) may participate in the pathogenesis of cartilage damage in osteoarthritis (OA) through the production of catabolic enzymes and inflammatory mediators. Induction of heme oxygenase-1 (HO-1) has previously been shown to exert anti-inflammatory effects in different cell types. We have investigated whether HO-1 induction may modify chondrocyte viability and the production of relevant mediators such as oxidative stress and prostaglandin E₂ (PGE₂) elicited by IL-1 β in OA chondrocytes. Chondrocytes were isolated from OA cartilage and used in primary culture. Cells were stimulated with IL-1 β in the absence or presence of the HO-1 inducer cobalt protoporphyrin IX (CoPP). Gene expression was assessed by quantitative real-time PCR, protein levels by ELISA and Western blot, apoptosis by laser scanning cytometry using annexin V-FITC and TUNEL assays, and oxidative stress by LSC with dihydrorhodamine 123. HO-1 induction by CoPP enhanced chondrocyte viability and aggrecan content while inhibiting apoptosis and oxidative stress generation. PGE₂ is produced in OA chondrocytes stimulated by IL-1 β by the coordinated induction of cyclooxygenase-2 and microsomal PGE synthase 1 (mPGES-1). The production of PGE₂ was decreased by HO-1 induction as a result of diminished mPGES-1 protein and mRNA expression. Transfection with HO-1 small interfering RNA counteracted CoPP effects. In addition, the activation of nuclear factor- κ B and early growth response-1 was significantly reduced by CoPP providing a basis for its anti-inflammatory effects. These results confirm the protective role of HO-1 induction in OA chondrocytes and suggest the potential interest of this strategy in degenerative joint diseases.

Key words: Heme oxygenase-1, Chondrocyte, Osteoarthritis, Interleukin-1 β , Prostaglandin E₂, microsomal PGE synthase-1.

Running headline: HO-1 in chondrocytes

1. Introduction

The chronic production of inflammatory mediators by articular tissues is an important feature of rheumatoid arthritis and osteoarthritis (OA) contributing to the degenerative process. In these conditions, high levels of pro-inflammatory cytokines such as interleukin(IL)-1 β have been found in synovial fluids and may participate in collagen and proteoglycan degradation [1,2]. Moreover, chondrocyte activation by pro-inflammatory cytokines has been implicated in joint destruction through the production of catabolic enzymes and inflammatory mediators such as reactive oxygen species (ROS) and prostaglandin E₂ (PGE₂) [3,4].

ROS can modulate cell signaling and exert damaging effects on cartilage as a consequence of extracellular matrix degradation or chondrocyte apoptosis [4-7]. Furthermore, studies examining the life span of articular chondrocytes have demonstrated that oxidative stress is related to premature senescence and may be responsible for the development of OA [8,9].

The arachidonic acid metabolite PGE₂, a mediator of inflammation and pain, is produced in OA chondrocytes stimulated by IL-1 β by the coordinated induction of cyclooxygenase-2 (COX-2) and microsomal PGE synthase 1 (mPGES-1) [10]. Previous studies have suggested the interest of this last enzyme as a novel target for OA [10]. Although some studies have reported that mice lacking mPGES-1 do not show significant differences with respect to wild type animals in OA induced by surgical procedures [11], it is interesting to note that mPGES-1 deficiency results in milder arthritis and lower cartilage degradation [12].

Heme oxygenase-1 (HO-1) is induced by oxidative stress (reviewed in [13]) and can inhibit apoptosis in several cell types such as T cells [14,15], endothelial cells [16]

and enterocytes [17]. In human OA chondrocytes, HO-1 induction by low doses of sodium nitroprusside has been shown to protect against apoptosis induced by high doses of this agent [18]. We have reported previously that HO-1 expression in OA chondrocytes is down-regulated by pro-inflammatory cytokines such as IL-1 β , IL-17 and tumor necrosis factor- α but up-regulated by the antiinflammatory cytokine IL-10 [19]. Recently, we have also shown a protective effect of HO-1 induction on cartilage degradation [20]. We hypothesized that HO-1 could control metabolic processes in chondrocytes relevant in the progression of OA. Therefore, we have investigated whether HO-1 induction by cobalt protoporphyrin IX (CoPP) may modify cell viability and the production of oxidative stress and PGE₂ in OA chondrocytes in primary culture, thus avoiding the phenotypic changes associated with cell culture [21].

2. Material and methods

2.1. Reagents

IL-1 β was from Peprotech EC Ltd. (London, UK). COX-2 and mPGES-1 polyclonal antibodies were purchased from Cayman Chemical (Ann Arbor, MI, USA) and the HO-1 antibody was from Stressgen (Victoria, Canada). The peroxidase-conjugated IgGs were purchased from Dako (Copenhagen, Denmark). [5,6,8,11,12,14,15(n)-³H]PGE₂ was from GE Healthcare (Barcelona, Spain). Pre-designed small interfering RNA (siRNA) oligonucleotides and SiPORTTMAmine were purchased from Ambion Inc. (Austin, TX, USA). CoPP was purchased from Frontier Scientific Europe Ltd. (Carnforth, UK). Dihydrorhodamine 123 (DHR) was from Molecular Probes®, (Invitrogen S.A., Barcelona, Spain). The rest of reagents were from Sigma Aldrich (St Louis, MO, USA).

2.2. Chondrocyte culture

Cartilage specimens were obtained from 18 patients with the diagnosis of advanced OA undergoing total knee joint replacement. Samples were obtained under the Institutional Ethical Committee approved protocol. Cartilage slices were removed from the femoral condyles and tibial plateaus and cut into small pieces. Chondrocytes were isolated by sequential enzymatic digestion: 1 h with 0.1 mg/ml hyaluronidase (Sigma) followed by 12 h with 2 mg/ml collagenase (type IA) (Sigma) in DMEM/Ham's F-12 (Sigma) containing penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in 5% CO₂ atmosphere. The digested tissue was filtered through a 70 µm nylon mesh, washed and centrifuged. Cell viability was greater than 95 % according to the Trypan blue exclusion test. The isolated chondrocytes were seeded at 2.5×10^5 cells/well in six-well plates. Cells were cultured in growth medium: DMEM/Ham's F-12 supplemented with 10 % human serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified 5 % CO₂ incubator at 37°. Chondrocytes were allowed to grow until nearly confluence and then incubated with CoPP (10 µM) or vehicle for 1 h before stimulation with IL-1β (100 U/ml). In experiments using HO-1 siRNA (100 nM, sense 5'-GGCAGAGAAUGCUGAGUUCtt-3', antisense 5'-GAACUCAGCAUUCUCUGCCtg-3'), transfection was performed in SiPORT™Amine following the manufacturer's recommendations, 24 h before other experimental procedures. A nonspecific siRNA (Ambion) was used as negative control. Determination of aggrecan was performed by ELISA using a kit from Biosource Europe S.A. (Nivelles, Belgium) with sensitivity of 0.9 ng/ml. For lentivirus transduction of chondrocytes, OA chondrocytes isolated as indicated above were cultured in alginate [22]. The pellet of primary chondrocytes (1.5×10^6 /ml) was resuspended in sterile alginate (2% in 0.15 M NaCl) and slowly added

dropwise into a solution containing 100 mM CaCl_2 (alginate- CaCl_2 , 1/2 (v/v)). The alginate beads polymerized in the presence of CaCl_2 after 10 minutes and they were washed three times with 0.15 M NaCl solution and twice with growth medium. Chondrocytes in alginate beads were cultured for 10 days in growth medium supplemented with 25 $\mu\text{g/ml}$ ascorbic acid.

2.3. Transduction with lentiviral vectors

Human embryonic kidney (HEK) 293T cells were grown in DMEM/Ham's F-12 supplemented with 10% human serum, penicillin (100 U/ml) and streptomycin (100 $\mu\text{g/ml}$) in a humidified 5% CO_2 incubator at 37 °C. For lentiviral production we used the expression vectors psPAX2, pMD2G and pWXL (Dr T. Didier, School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland). Plasmid pWXL (green fluorescence protein (GFP) minus)Flag-hHO-1 was generated in two steps: first, the GFP coding sequence was replaced by an oligonucleotide cloned between the BamHI and EcoRI sites of pWXL (forward: 5'-CGCGTATTCGGTCACCGTAAG-3' and reverse: 5'-AATTCTTACGGTGACCGAATA-3'); second, the amplification product using pFlag-hHO-1 as template and the following primers (forward: 5'-TCGCGGATCCATGGACTACAAAGACGATGA-3' and reverse: 5'-TCCGGAATTCTGGTACCGATATCAGATCTAT-3') was digested with BamHI/EcoRI and subcloned in the same sites of pWXL (GFP)minus). Lentiviral vector stocks were generated in HEK293T cells by calcium phosphate-mediated transient transfection of three plasmids: the transfer vector plasmid (pWXL-Flag-hHO-1), the packaging plasmid psPAX2, and the VSV-G envelope protein-coding plasmid pMD2G. After transfection for 24 and 48 h, the cellular supernatants were removed,

centrifuged at 700 *g* for 10 min at 4°C, passed through 45 µm pore size filters and kept at -80°C. The titers of lentiviral stocks were in the range of 3-5x10⁵ IU/ml as determined by immunocytochemical analysis of HEK293T-infected cells [23]. The three-dimension chondrocyte culture in alginate was infected with 500 µl of each lentiviral stock for 24 h in a humidified 5% CO₂ incubator at 37°C. After infection and culture in growth medium for two days, alginate beads were stimulated with IL-1β (100 U/ml) for 24 h. Finally, HO-1 and mPGES-1 expression was measured by immunocytochemical analysis. The beads were fixed with 4% formaldehyde in PBS for 10 minutes and incubated with two antibodies at the same time: anti-FLAG monoclonal antibody (Sigma, 1/400) and anti-mPGES-1 polyclonal antibody (Cayman Chemical, 1/50) for 1.5 h, followed by incubation with the secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) and R-phycoerythrin goat anti-rabbit IgG (Invitrogen) for 45 minutes at 37° C. Cell nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) solution (1/1000). Slides were examined under a fluorescence microscope (Eclipse E800, Nikon Instruments Europe, Amstelveen, The Netherlands). Each experiment was performed in triplicate.

2.4. Determination of PGE₂

Chondrocytes in primary culture were stimulated with IL-1β (100 U/ml) or IL-1β+CoPP (10 µM) for 24 h. Supernatants were harvested and frozen at -80°C until analysis. PGE₂ was measured by radioimmunoassay [24]. In another set of experiments, chondrocytes in primary culture were stimulated with IL-1β (100 U/ml) for 24 h to induce COX-2 and mPGES-1, and then cells were washed with medium and incubated with arachidonic acid (10 µM) and either CoPP or NS-398 (10 µM). After 3 h, supernatants were harvested and PGE₂ measured as above.

2.5. Western blotting

After 24 h stimulation with IL-1 β (100 U/ml) or IL-1 β +CoPP (10 μ M), chondrocytes in primary culture were lysed in 100 μ l of buffer (1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl and 25 mM Tris, pH 7.4) and centrifuged at 4°C for 10 min at 10,000 x g. Proteins (25 μ g) in cell lysates were separated by 12.5% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 3% bovine serum albumin and incubated with specific antibodies (1:1000) for 2 h at room temperature. Finally, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG and the immunoreactive bands were visualized by enhanced chemiluminescence (GE Healthcare) using the AutoChemi image analyzer (UVP Inc., Upland, CA, USA).

2.6. Oxidative stress and apoptosis

Oxidative stress and apoptosis rate were assessed by laser scanning cytometry (LSC) analysis. Chondrocytes (75,000/well) were spread in 8-well Lab-tek chambers (Nalge Nunc International, Naperville, IL, USA) with DMEM/Ham's F-12, penicillin (100 U/ml), streptomycin (100 μ g/ml) and 10% human serum in a humidified 5 % CO₂ incubator at 37°C. Confluent cells were treated with IL-1 β (100 U/ml) or IL-1 β +CoPP (10 μ M) for 24 h. Analysis of viability and apoptosis was made using LSC Bx50 CompuCyte Olympus, (Cambridge, MA, USA) using the Vybrant Apoptosis Assay Kit (Molecular Probes®, Invitrogen S.A.), according to the manufacturer's protocol. In this assay the externalization of phosphatidylserine, a marker of apoptosis, is detected by annexin V-FITC binding. Apoptotic cell death was also determined by the terminal

deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method using an assay kit (Roche Applied Science, Barcelona, Spain).

Formation of intracellular ROS was detected using DHR which is oxidized to fluorescent rhodamine (excitation at 485 nm and emission at 534 nm). Chondrocytes (75,000/well) were incubated with DMEM without phenol red and DHR (5 μ M) for 15 min at 37°C. After washing, fresh medium was added and cells were incubated with CoPP for 4 h and then with IL-1 β in the presence or absence of CoPP for other 30 min. Cultures were washed twice with fresh medium and analyzed by LSC.

2.7. Real-time PCR

Chondrocytes in primary culture were stimulated with IL-1 β (100 U/ml) or IL-1 β +CoPP (10 μ M) for 12 h. Total RNA was extracted using the TRIzol reagent (Life Technologies Inc. Barcelona, Spain) according to the manufacturer's instructions. Reverse transcription was accomplished on 1 μ g of total RNA using random primers (TaqMan reverse transcription reagents, Applied Biosystems Spain, Madrid). PCR assays were performed in duplicate on an iCycler Real-Time PCR Detection System using SYBR Green PCR Master Mix (Bio-Rad Laboratories, Richmond, CA, USA) as previously described [20]. Primer sequences are shown in table 1. Cycle threshold (CT) values for each gene were corrected using the mean CT value for β -actin. Relative gene expression was calculated using the ΔC_T method and expressed as fold change ($2^{-\Delta\Delta C_T}$) relative to the expression values in nonstimulated cells.

2.8. Activation of transcription factors

Chondrocytes were seeded into 6-well plates and grown to 50-60 % confluence. Transient transfection was performed overnight with 2 μ g of the reporter construct

nuclear factor- κ B(NF- κ B)-luc, activator protein(AP-1)-luc or early growth response(EGF)-1-luc (Stratagene, La Jolla, CA, USA) and 1 μ g of the internal control pRL-TK (Promega Corporation, Madison, WI, USA) by the Magnetofection™ system (OZ Biosciences, Marseille, France) according to the manufacturer's recommendations. The medium was then replaced and after 18 h cells were treated for 24 h with CoPP in the absence or presence of IL-1 β (100 U/ml). After lysis and centrifugation, aliquots of supernatants were used to assay firefly and *Renilla* luciferase activity using the Dual-Luciferase Reporter Assay System kit (Promega Corporation). Luminescence was measured in a Microbeta counter (Wallac, Turku, Finland) and firefly luciferase activity was normalized to *Renilla* luciferase activity.

2.9. Data analysis

Results are presented as mean $\pm$ SEM. Statistical analyses were performed using one-way ANOVA followed by Dunnett's *t*-test for multiple comparisons and unpaired Student's *t*-test for dual comparisons.

3. Results

3.1. HO-1 induction increases chondrocyte viability

According to previous studies [20], we used CoPP (10 μ M) to induce HO-1 in OA chondrocytes. To determine whether cell viability is modified in our experimental conditions, we performed LSC experiments. We observed a significant reduction in cell viability after IL-1 β treatment for 24 h (Fig. 1A), which was counteracted by CoPP. We determined whether HO-1 induction by CoPP was able to control apoptosis, an important factor in the evolution of OA [29]. Fig. 1B shows that IL-1 β weakly induced

apoptosis measured by LSC (annexin V-FITC) and CoPP treatment significantly reduced the rate of apoptosis to basal levels. Our results suggest that CoPP effects on apoptosis were specific, as in cells treated with HO-1 siRNA, CoPP failed to modify this process. Additional experiments using the TUNEL method (Fig. 1C) confirmed the beneficial effects of CoPP treatment on chondrocyte apoptosis in the presence of IL-1 β , although the level of apoptosis induced by this cytokine was a bit higher in this assay, with percentages of 11.4 $\pm$ 2.3 (B), 6.2 $\pm$ 2.2 (CoPP), 21.2 $\pm$ 5.3 (IL-1 β) and 12.0 $\pm$ 2.9 (IL-1 β +CoPP, $n=6$, $p<0.05$ with respect to IL-1 β).

The ability to synthesize extracellular matrix components is a feature of chondrocyte metabolism. Our results indicate that CoPP treatment was also able to counteract the loss of aggrecan induced by IL-1 β . Treatment with this cytokine for 24 h reduced aggrecan content in chondrocytes from 11618 $\pm$ 1664 to 7699 $\pm$ 567 ng proteoglycan/mg protein ($n=6$, $p<0.05$). In contrast, chondrocytes treated with CoPP+IL-1 β showed proteoglycan levels similar to those of non-stimulated cells (12063 $\pm$ 522, ng proteoglycan/mg protein, $n=6$, $p<0.05$ with respect to IL-1 β). Thus, our investigations suggest that overexpression of HO-1 confers to OA chondrocytes an increased resistance to the deleterious effects of IL-1 β .

3.2. HO-1 induction inhibits oxidative stress

Since many of the effects of IL-1 β on chondrocyte metabolism are mediated by the generation of ROS, we wanted to determine whether the production of oxidative stress could be modified by HO-1 induction. Stimulation of chondrocytes by this cytokine resulted in a significant level of ROS generation (Fig. 2). Consistent with an antioxidative role for HO-1 induction, oxidative stress was significantly decreased in cells treated with CoPP. The effect of HO-1 induction was confirmed in experiments

using HO-1 siRNA as this agent prevented the down-regulation of ROS production by CoPP treatment.

3.3. Effects on PGE₂, COX-2 and mPGES-1

As a result of COX-2 and mPGES-1 induction, IL-1 β strongly increased PGE₂ levels after chondrocyte stimulation for 24 h. Production of PGE₂ was significantly reduced in cells treated with CoPP and stimulated with IL-1 β (Fig. 3A). To confirm that CoPP effects were due to the induction of HO-1, we used a siRNA specific for human HO-1. This agent nearly abolished the induction of HO-1 protein by CoPP, whereas a nonspecific siRNA failed to modify HO-1 protein expression (Fig. 3B). Interestingly, the treatment with the first siRNA reverted the inhibitory effects of CoPP on PGE₂ (Fig. 3A). The possible contribution of inhibitory effects on enzymatic activities regulating PGE₂ synthesis was tested by using chondrocytes previously treated with IL-1 β for 24 h to induce COX-2 and mPGES-1 and then washed and incubated in fresh medium with CoPP and arachidonic acid for 3 h. In this experimental setting, the production of PGE₂ was dramatically increased in cells treated with IL-1 β in comparison with non-stimulated cells (119.3 ± 21.6 versus 9.2 ± 1.2 ng/mg protein, $n=8$, $p<0.01$). As expected, the COX-2 inhibitor NS-398 (10 μ M) reduced PGE₂ production to basal levels (5.9 ± 1.2 , $p<0.01$) but CoPP (10 μ M) was devoid of significant effects (113.5 ± 13.7 ng/mg protein). We investigated whether the effects of HO-1 induction on PGE₂ were the consequence of a reduced expression of COX-2 or mPGES-1. As shown in Fig. 3B, IL-1 β induced the expression of COX-2 and mPGES-1 in OA chondrocytes. CoPP treatment down-regulated mPGES-1 protein whereas HO-1 siRNA tended to revert this effect. In contrast, COX-2 expression was not significantly modified by CoPP. The ability of CoPP to reduce mPGES-1 expression was also observed at the mRNA level

(Table 2). To confirm that HO-1 overexpression results in reduced mPGES-1 expression, a three-dimension culture of OA chondrocytes was transduced with a lentiviral HO-1 vector (LV-HO-1). Fig. 4 shows that mPGES-1 was strongly induced by IL-1 β in cells transduced with the empty vector LV-. In contrast, in cells transduced with LV-HO-1, mPGES-1 was hardly detected after IL-1 β stimulation.

3.4. Effects on the activation of transcription factors

Because NF- κ B is a main regulator of pro-inflammatory genes, we analyzed the contribution of this transcription factor to the effects of CoPP. As shown in Fig. 5A, IL-1 β activated NF- κ B in OA chondrocytes. Although CoPP treatment of cells did not modify basal luciferase activity, it significantly decreased the activation of this transcription factor in the presence of IL-1 β . In contrast, the activation of AP-1, another transcription factor involved in the expression of pro-inflammatory genes [30], was not influenced by CoPP (Fig. 5B). As EGR-1 is a key transcription factor in regulating the inducible expression of mPGES-1 [31], we investigated the effects of CoPP treatment on EGR-1 activation. When IL-1 β was incubated with chondrocytes, EGR-1 activity was significantly enhanced. It is interesting that the treatment of chondrocytes with CoPP led to a significant reduction of EGR-1-luc promoter activation in cells stimulated with IL-1 β as well as in non-stimulated cells (Fig. 5C).

4. Discussion

Pro-inflammatory cytokines such as IL-1 β induce the formation of intracellular oxidants which may function as second messengers leading to augmentation of gene expression of degradative enzymes [32]. In addition, ROS have been implicated in cartilage degeneration in OA through the initiation of chondrocyte apoptosis [33], decreased

replicative potential, catabolic changes in matrix cartilage, telomere instability and senescence [8]. It is known that HO-1 induction can decrease the levels of the pro-oxidant heme and increase the generation of antioxidant molecules (biliverdin and bilirubin) and the anti-apoptotic and anti-inflammatory agent carbon monoxide (reviewed in [13]). In the present study we provide evidence of a protective role for HO-1 induction against oxidative stress in human OA chondrocytes. Our results also suggest that inhibition of ROS generation may contribute to the beneficial effects of CoPP on viability, apoptosis and extracellular matrix metabolism in primary OA chondrocytes. Therefore, the antioxidant properties of HO-1 may be relevant for its chondroprotective effect.

Previous studies have demonstrated an increased expression of COX-2 and mPGES-1 in OA cartilage [28] and chondrocytes [34]. Experimental evidence indicate that IL-1 β induces in chondrocytes large elevations of COX-2 and mPGES-1 leading to increased levels of PGE₂. Besides its pro-inflammatory effects, this mediator can participate in the catabolic-anabolic imbalance in OA by inhibiting tissue inhibitor of metalloproteinases-1 synthesis or enhancing matrix metalloproteinase production [35,36]-[37]. In contrast, low concentrations of PGE₂ may downregulate collagenases and collagen 2A1 cleavage [38]. PGE₂ may enhance chondrocyte death [39] and induce apoptosis in bovine articular chondrocytes through the cAMP pathway [40]. In this study, we investigated the effects of HO-1 induction on IL-1 β -stimulated PGE₂ production in primary OA chondrocytes. We have shown that these effects were not dependent on the inhibition of COX-2/mPGES-1 enzyme activity. Although the reduction in heme availability may result in COX-2 protein possessing a low activity, our recent data [41] suggest that a product of HO-1 activity such as CO may be responsible for the reductions in PGE₂ production after HO-1 induction. Our results

have demonstrated the down-regulation of mPGES-1 gene expression by HO-1 that translates into accompanying declines in PGE₂ production. This may result in the control of PGE₂ overproduction during inflammatory responses without inhibition of other metabolites derived from COX-2 activity, with possible protective effects [42]. Recently, we have reported that HO-1 protects against cartilage degradation through the inhibition of the expression of several matrix metalloproteinases [20]. Here, we show for the first time a regulatory role of HO-1 in PGE₂ production due to mPGES-1 modulation, in primary OA chondrocytes stimulated with IL-1 β , which may participate in the observed protective effects on cartilage.

NF- κ B and AP-1 are involved in the transcription of a number of inflammatory genes and MMPs [30,43]. Our data suggest that the inhibitory effects of HO-1 induction on the first transcription factor could play a role in the recently reported inhibition of MMP expression [20]. Although the COX-2 promoter contains multiple regulatory sites including those able to interact with both transcription factors, it has been reported that COX-2 induction by IL-1 β in human chondrocytes may be dependent on other transcription factors [44], which would be consistent with the lack of effect of CoPP on COX-2 expression.

mPGES-1 is induced by pro-inflammatory cytokines in many cell types and has been proposed as a target in inflammatory diseases [45] and OA [10,46]. Recent studies have revealed that EGR-1 could be a key regulator of mPGES-1 transcription in response to cytokine stimulation [47]. Therefore, we sought to establish whether HO-1 induction could regulate this transcription factor. Our data have shown that HO-1 induction results in a significant decrease of EGR-1 activation in primary OA chondrocytes stimulated with IL-1 β , thus providing a mechanism for the inhibition of mPGES-1 expression. Previous studies from our lab have shown the inhibitory effects

of HO-1 induction on extracellular signal related kinase-1/2 phosphorylation [20]. This mechanism may also participate in the down-regulation of mPGES-1 by HO-1 induction, as the activity of mitogen activated protein kinases could be critical for the expression of mPGES-1 [10].

Therefore, HO-1 induction has shown consistent inhibitory effects toward a number of processes involved in the pathology and progression of OA. The present data revealed new facets of HO-1 actions in OA chondrocytes that could be relevant to cell survival in cartilage degeneration. These studies open the potential for therapy for a range of inflammatory and degenerative conditions and provide an opportunity to clarify HO-1 effects in human articular cells.

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FIGURE LEGENDS

Fig. 1. Effect of CoPP on viability and apoptosis of OA chondrocytes. (A) Viability by the LSC assay. (B) Apoptosis by the LSC assay. Cells in primary culture were stimulated with IL-1 β (100 U/ml) in the presence or absence of CoPP (10 μ M) and siRNA specific for HO-1 or siRNA control (100 nM). Data are expressed as mean $\pm$ SEM ($n=6-8$). ** $p<0.01$ with respect to IL-1 β ; ### $p<0.01$ with respect to nonstimulated cells. (C) TUNEL assay: B (nonstimulated cells). The picture is representative of results from three separate experiments. Magnification x200.

Fig. 2. Effect of CoPP on oxidative stress in human OA chondrocytes. Cells were stimulated with IL-1 β (100 U/ml) in the presence or absence of CoPP (10 μ M) and a siRNA specific for HO-1 or siRNA control (100 nM). Oxidative stress were measured by LSC, as indicated in Materials and methods. Data are expressed as mean $\pm$ SEM ($n=8$). ** $p<0.01$ with respect to IL-1 β ; ## $p<0.01$ with respect to nonstimulated cells. Rho: rhodamine.

Fig. 3. Effect of CoPP on (A) PGE₂ levels and (B) COX-2, mPGES-1 and HO-1 protein expression in human OA chondrocytes. Cells were stimulated with IL-1 β (100 U/ml) for 24 h in the presence or absence of CoPP (10 μ M). PGE₂ was measured in supernatants by radioimmunoassay and protein expression was determined in cell lysates by Western blotting. Data are expressed as mean $\pm$ S.E.M. ($n=10-15$). ** $p<0.01$ with respect to IL-1 β ; ## $p<0.01$ with respect to nonstimulated cells. The immunoblot is representative of three independent experiments.

Fig. 4. Effect of HO-1 (after LV-HO-1 transduction) on mPGES-1 protein in OA chondrocytes stimulated by IL-1 β . Three-dimension cultures of OA chondrocytes in alginate were transduced with LV-HO-1 or the empty vector LV-. mPGES-1 expression was induced by IL-1 β stimulation for 24 h and HO-1 and mPGES-1 expression was characterized by immunofluorescence. Cell nuclei were counterstained with DAPI. Fluorescence micrographs representative of three separate experiments. Magnification x200.

Fig. 5. Effect of CoPP on the activation of (A) NF- κ B, (B) AP-1 and (C) EGR-1. Transient transfection was performed overnight with the reporter construct (NF- κ B-luc, AP-1-luc or EGR-1-luc) and the internal control pRL-TK, as indicated in Materials and methods. Cells were treated for 24 h with CoPP in the absence or presence of IL-1 β (100 U/ml). Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are expressed as mean $\pm$ SEM ($n=6$). ** $p<0.01$ with respect to IL-1 β ; # $p<0.05$; ## $p<0.01$ with respect to nonstimulated cells.

Table 1. Sequences of primers used in real-time PCR experiments

	Forward	Reverse
β -actin [25]	5'-AGGCTACGAGCTGCCTGACG-3'	5'-GTAGTTTCGTGGATGCCACAGGACT-3'
HO-1 [26]	5'-CAGGCAGAGAATGCTGAGTTC-3'	5'-GCTTCACATAGCGCTGCA-3'
COX-2 [27]	5'-AAATTGCTGGCAGGGTTGC-3'	5'-TTTCTGTACTGCGGGTGGAAC-3'
mPGES-1 [28]	5'-GAAGAAGGCCTTTGCCAA-3'	5'-GGAAGACCAGGAAGTGCATC-3'

Table 2. Relative gene expression of OA chondrocytes stimulated with IL-1 β .

Treatment	HO-1	COX-2	mPGES-1
IL-1 β	-1.1 $\pm$ 1.5	20.9 $\pm$ 2.7	21.8 $\pm$ 0.6
IL-1 β +CoPP	4.9 $\pm$ 0.3*	21.7 $\pm$ 1.7	15.7 $\pm$ 0.7*

Mean fold change ($\pm$ SEM, $n=5$) in gene expression relative to control 12 h post stimulation. IL-1 β : 100 U/ml; CoPP: 10 μ M. * $p<0.05$, with respect to IL-1 β .

C

1C

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1C