Haem oxygenase-1 down-regulates high mobility group box 1 and matrix metalloproteinases in osteoarthritic synoviocytes

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Abstract

Objectives. Activation of osteoarthritic synoviocytes by pro-inflammatory cytokines results in the release of biochemical mediators such as MMPs and high mobility group box 1 (HMGB1). Extracellular HMGB1 can play an important role in joint diseases as a mediator of synovitis. We have shown previously that haem oxygenase-1 (HO-1) exerts protective effects during inflammatory responses. In this study, we have examined whether HO-1 induction would be an effective strategy to control MMP and HMGB1 production in osteoarthritic synoviocytes.

Methods. Osteoarthritic synoviocytes were obtained by digestion with collagenase and cultured until third passage. HO-1 was induced by cobalt protoporphyrin IX (CoPP). Lentiviral HO-1 vector (LV-HO-1) was also used for HO-1 overexpression. HO-1 gene silencing was achieved by using a specific small interfering RNA. Gene expression was analysed by quantitative PCR and protein expression by western blot, ELISA and IF. MMP activity was studied by fluorometric procedures.

Results. Induction of HO-1 by CoPP in the presence of IL-1β decreased the expression of MMP-1 and -3, and MMP activity. IL-1β stimulation of synoviocytes increased HMGB1 expression, its translocation into the cytoplasm and secretion. HO-1 induction exerted inhibitory effects on these processes. The consequences of HO-1 induction were counteracted by HO-1 gene silencing, whereas transfection with LV-HO-1 confirmed the effects of pharmacological HO-1 induction.

Conclusions. We have provided direct evidence that HO-1 down-regulates MMP-1, -3 and HMGB1 in osteoarthritic synoviocytes. HO-1 may be a potential strategy to control inflammatory and degradative processes in the progression of OA.

Key words: Haem oxygenase-1, High mobility group box 1, Osteoarthritis, Synoviocyte, Matrix metalloproteinases.

Introduction

OA is a degenerative disease characterized by the degradation of cartilage and subchondral bone [1].

The progression of this chronic disease is related to multiple factors, including an inflammatory response to cartilage components [2]. Several studies have shown that OA synovial cells play a role in sustaining joint lesion by producing inflammatory mediators and MMPs [3, 4]. Therefore, the up-regulation of IL-1β and other pro-inflammatory cytokines in OA synovial cells could contribute to synovitis and increased matrix degradation in articular cartilage [5, 6].

The nuclear DNA-binding protein, high mobility group box 1 (HMGB1), can be released passively by necrotic cells and actively secreted by myeloid cells after stimulation with lipopolysaccharide or pro-inflammatory cytokines.
cytokines (reviewed in [7]). In addition to the role of this protein in the nucleus, several lines of evidence indicate that extracellular HMGB1 acts as a pro-inflammatory cytokine in synovial tissues [8–10]. This is further indicated by the overexpression and extracellular localization of HMGB1 in synovial tissue of RA patients and its relation to the progression of arthritis in experimental models [11].

Up-regulation of the inducible isofrom of haem oxygenase-1 (HO-1), serves as an adaptive response to protect cells from stress [12]. The protective effects of HO-1 could be related to the generation of the anti-oxidant molecules, biliverdin and bilirubin [13] and carbon monoxide [14], and are of particular interest in the context of inflammatory responses (for review see [15]). Recent findings revealed that HO-1 induction in animals protects them against the development of arthritis [16] and may control HMGB1 levels in acute lung injury [17] and sepsis [18, 19].

We have shown that HO-1 induction in OA chondrocytes results in decreased levels of MMPs and could exert protective effects against cartilage degradation [20]. Considering the importance of synovial cells as regulators of inflammation and cartilage degradation in OA [21], in the present study we have investigated whether the mode of action of HO-1 involves the regulation of degradative and inflammatory pathways in OA synoviocytes, focusing on MMP and HMGB1 production.

Materials and methods

Reagents

IL-1β was from PeproTech EC (London, UK). HMGB1 polyclonal antibody was purchased from Upstate and anti-collagen I from Chemicon (Millipore Iberica, Madrid, Spain). The HO-1 antibody was from Stressgen (Victoria, Canada). The peroxidase-conjugated IgGs were purchased from Dako (Copenhagen, Denmark). Pre-designed small interfering RNA (siRNA) oligonucleotides and SiPORT Amine were purchased from Ambion (Austin, TX, USA). Cobalt protoporphyrin IX (CoPP) was purchased from Frontier Scientific Europe (Camforuth, UK). The rest of the reagents were from Sigma Aldrich (St Louis, MO, USA).

Cell culture and treatments

Knee SMs were obtained from patients with OA [seven females, five males, aged 70 (2) years, mean (S.E.M.)] undergoing joint replacement surgery. All studies were performed under the University of Valencia and University Hospital Ethical Committees’ approved protocol and patients’ consent according to the Declaration of Helsinki. Synovial specimens were finely minced and isolated by enzymatic digestion with collagenase type 1A (Sigma Aldrich) in DMEM/HAM F12 (Sigma Aldrich) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in 5% CO2 atmosphere for 16 h. The digested tissue was filtered through a 70-μm nylon mesh, washed and centrifuged. Cell viability was >95% according to the Trypan blue exclusion test. Collected cells were resuspended in DMEM/HAM F12 (Sigma Aldrich) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) and supplemented with 10% fetal bovine serum (Sigma Aldrich) and cultured at 37°C in 5% CO2 atmosphere until third passage (95% fibroblasts, detected by immunocytochemistry with anti-collagen I antibody), where we performed all our experiments. Synoviocytes were allowed to grow nearly up to confluence and then they were incubated with CoPP or vehicle for 30 min before stimulation with IL-1β (10 ng/ml) for 24 h. In preliminary experiments, we selected this cytokine and concentration due to its inhibitory effects on HO-1 expression (supplementary figure 1, available as supplementary data at Rheumatology Online). In experiments using siRNA (100 nM), transfection was performed in SiPORT Amine following the manufacturer’s recommendations, 24 h before other experimental procedures. A non-specific siRNA (Ambion) was used as negative control. Viability studies were performed for all the experimental conditions of this study. None of the treatments significantly affected cell viability, which was >90% as tested by Trypan blue exclusion (data not shown).

Western blot analysis

After 24 h stimulation with IL-1β (10 ng/ml) or IL-1β+CoPP, synoviocytes were lysed in 100 μl 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 g. In another set of experiments, for separation of nuclear and cytoplasmic extracts, cells were washed twice with ice-cold phosphate buffered saline, and then treated with 0.2 ml of buffer A (20 mM Tris–HCl, pH 7.8, 10 mM KCl, 1 mM EGTA, 1 mM diethiothreitol, 1 mM p- nylmethylsulphonyl fluoride and 10 μM leupeptin) for 15 min followed by addition of Nonidet P-40 (0.5%). The tubes were vortexed for 15 s, and nuclei were sedimented by centrifugation at 8000 g for 30 s. Aliquots of the supernatant were stored at −80°C (cytoplasmic extract) and the nuclei pellet was resuspended in 50 μl of buffer A supplemented with 0.4 M KCl. After centrifugation at 13,000 g for 15 min, aliquots of the supernatant (nuclear extract) were stored at −80°C. Protein was determined by the DC Bio-Rad protein reagent (Richmond, CA, USA). Proteins (25 μg) in cell lysates were separated by 12.5% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 3% BSA and incubated with specific antibodies (anti-HO-1, 1:1000; anti-HMGB1, 1:500) 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, Barcelona, Spain) using the AutoChem image analyzer (UVP, Upland, CA, USA).

Determination of MMP activity

Cells were stimulated with IL-1β (10 ng/ml) or IL-1β+CoPP (10 μM) for 24 h and supernatants were harvested, centrifuged and incubated with ρ-aminohippuricuric acid for 6 h at 37°C to activate MMPs. Aliquots of supernatants were then transferred to a 96-well plate and after addition
of the 5-FAM peptide substrate (AnaSpec, San Jose, CA, USA), fluorescence was measured for different times at 490 nm (excitation)/520 nm (emission) in a Victor3 microplate reader (PerkinElmer España, Madrid, Spain).

ELISA
Synoviocytes were stimulated with IL-1β (10 ng/ml) for 24 h, in the presence or absence of CoPP (10 μM). Supernatants were harvested, centrifuged and frozen at −80°C until analysis. HMGB1 protein was quantified in supernatants by using the ELISA kit from IBL-Hamburg (Hamburg, Germany), with sensitivity of 0.2 ng/ml. MMP-1 protein was quantified using the ELISA kit from AnaSpec (sensitivity 8 pg/ml) and MMP-3 protein with the ELISA kit from Raybiotech (Norcross, GA, USA), with sensitivity of 0.3 ng/ml.

Transduction with lentiviral vectors
For lentiviral production, we used the expression vectors psPAX2, pMD2G and pWXL (Dr T. Didier, School of Life Sciences, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland). 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 titres of lentiviral stocks were in the range of 3–5×10^5 IU/ml as determined by immunocytochemical analysis of HEK293T-infected cells [22]. The synoviocyte culture was infected with 500 μl of each lentiviral stock for 24 h in a humidified 5% CO₂ incubator at 37°C. After infection of lentivector HO-1 (LV-HO-1) or empty vector (LV–), cells were cultured in growth medium for 2 days and then stimulated with IL-1β (10 ng/ml).

Real-time PCR
Total RNA was extracted using the TRizol reagent (Life Technologies, Barcelona, Spain) according to the manufacturer’s instructions. Reverse transcription was accomplished on 1 μg 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) as previously described [20, 23]. For each sample, differences in cycle threshold (ΔCt) values were calculated by correcting the Ct of the gene of interest to the Ct of the reference gene β-actin. Relative gene expression was expressed as ΔΔCt with respect to non-stimulated cells (experiments using CoPP) or non-stimulated cells transduced with the empty vector (LV–) (experiments using LV).

Immunocytochemical analysis
Cells were fixed with 4% formaldehyde in phosphate buffered saline for 10 min and incubated with primary antibody for 1.5 h, followed by incubation with the secondary antibody R-phycocerythin goat anti-rabbit IgG (Invitrogen, Barcelona, Spain) for 45 min 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 done in triplicate.

Statistical analysis
Results are presented as mean (±SEM). Statistical analyses were performed using one-way ANOVA followed by Dunnett’s t-test for multiple comparisons and two-tailed unpaired Student’s t-test for dual comparisons.

Results
Induction of HO-1 by CoPP
Cell stimulation with IL-1β down-regulated HO-1 protein, but treatment with CoPP induced HO-1 protein in a concentration-dependent manner (Fig. 1A). The concentration of 10 μM CoPP was selected due to its strong HO-1 induction and lack of toxicity. Figure 1B shows that HO-1 induction by CoPP (10 μM) was prevented by HO-1 siRNA, but not for the siRNA control, indicating a specific effect of this metalloporphyrin. Similar results were obtained at mRNA level (Fig. 1C).

Effect of HO-1 induction on MMPs
Induction of HO-1 by CoPP (10 μM) resulted in a significant reduction in MMP activity, released into the medium, in cells stimulated with IL-1β for 24 h (Fig. 2A). This effect of CoPP was reverted by incubation with HO-1 siRNA. Quantitative RT–PCR analysis revealed that CoPP significantly decreased the expression of mRNA for MMP-1 and -3 in OA synoviocytes stimulated with IL-1β (Fig. 2B). In addition, protein levels of MMP-1 and -3 measured in the medium by ELISA were significantly reduced by CoPP (Fig. 2C and D). Treatment with HO-1 siRNA significantly counteracted the effects of CoPP.

Effect of HO-1 induction on HMGB1
IL-1β stimulation of synoviocytes augmented the expression of total intra-cellular HMGB1 protein (Fig. 3A) and mRNA (Fig. 3B). In cells treated with CoPP, we observed a reduction in the expression of HMGB1 protein and mRNA induced by IL-1β. The effects of CoPP were counteracted by HO-1 siRNA. Immunocytochemical analyses indicated that HMGB1 protein is present mainly in nuclear localization in non-stimulated cells, whereas stimulation with IL-1β augmented the presence of HMGB1 protein in the cytoplasm (Fig. 4A). CoPP treatment decreased HMGB1 translocation from the nucleus into the cytoplasm, the necessary step for secretion. These results were confirmed by western blot analyses of nuclear and cytoplasmic extracts (Fig. 4B). In addition, Fig. 4C shows that IL-1β increased HMGB1 protein secretion into the medium, whereas CoPP significantly reduced
this effect. Treatment of cells with HO-1 siRNA prevented this reduction in HMGB1 levels induced by CoPP.

**Effects of the LV-HO-1 vector**

Additional experiments were conducted to confirm that the observed effects of CoPP were due to HO-1 overexpression. For this purpose, synoviocyte cultures were transduced with a LV-HO-1. Figure 5A shows the increased expression of HO-1 protein in cells transduced with LV-HO-1, with respect to those transduced with the LV-, either in the absence or presence of IL-1β. We also observed that HMGB1 translocation into the cytoplasm induced by IL-1β was decreased in cells transduced with LV-HO-1. In addition, an increase in MMP-1 and -3 protein and mRNA was induced by IL-1β in cells transduced with the LV- (Fig. 5B and C). In contrast, in cells transduced with LV-HO-1, IL-1β stimulation resulted in a lower level of protein and mRNA expression for both MMPs.

**Discussion**

Inflammatory mechanisms are involved in OA progression and chronicity [2]. In this context, the production of biochemical factors by synoviocytes contributes to the degradation of essential cartilage components [4]. We have recently reported the protective effects of HO-1 induction in OA chondrocytes, which were due in part to the inhibition of MMP gene expression [20]. In the present work, we have provided additional insight into the control of MMP function by HO-1 by demonstrating that induction of HO-1 in human OA synoviocytes decreases the gene expression of MMP-1 and -3 induced by IL-1β. These findings are of interest in the context of a possible role of HO-1 against cartilage degradation, as MMP-1 plays an important role in the degradation of collagen II in the extracellular matrix [24–26] and MMP-3 (stromelysin 1) participates in the activation of collagenases [27]. Furthermore, we have examined the consequences of HO-1 overexpression after lentiviral transduction and confirmed the down-regulation of MMPs by HO-1. Our results thus suggest that the protective effects of HO-1 overexpression in joint tissues are related to the control of MMP production by chondrocytes and synoviocytes [16].

Several proteins of the HMGB family may have a role in the physiopathology of human articular cartilage, as HMGB1 released from differentiating chondrocytes may participate in endochondral ossification during osteogenesis [28], and HMGB2 has been related to ageing and OA [29]. Particularly for synovial tissues, there is increasing evidence that extracellular HMGB1 could play a pathogenetic role in synovitis [8]. Recent studies indicate that HMGB1 alone has a low pro-inflammatory activity, which is potentiated through binding to bacterial products, IL-1β and other inflammatory mediators to create complexes inducing innate immune responses and the production of inflammation [30]. Therefore, HMGB1 induces the release of pro-inflammatory cytokines [31] and might regulate nitric oxide production in mononuclear cells [32]. HMGB1 stimulates the motility of a wide range of cells and thus elicits the migration to the site of tissue damage [33]. Moreover, in fibroblast-like synoviocytes from RA patients, HMGB1 has been shown to increase cellular invasiveness [34], whereas the inhibition of HMGB1 translocation in activated macrophages may be a possible mechanism for the anti-rheumatic effects of gold salts [35]. Additionally, the interaction of HMGB1
**FIG. 2** Effect of CoPP on (A) MMP activity, (B) MMP mRNA and (C and D) MMP protein expression in human OA synoviocytes.

**FIG. 3** Effect of CoPP on (A) HMGB1 protein and (B) HMGB1 mRNA expression in OA synoviocytes.

Cells were stimulated with IL-1β (10 ng/ml) for 24 h (A, C and D) or 16 h (B) in the presence or absence of CoPP (10 μM) and a siRNA specific for HO-1 or siRNA control (100 nM). MMP activity was measured in supernatants by a fluorometric procedure, as indicated in ‘Materials and methods’ section and expressed as fluorescence units (FUs) × 10^3/mg of protein. mRNA levels were determined by real-time PCR. Protein levels were quantified in cell supernatants by ELISA. Data are expressed as mean (s.e.m.) (n = 6–8). Open bars: non-stimulated cells; solid bars: cells stimulated with IL-1β.

**P < 0.05, **P < 0.01 with respect to non-stimulated cells.

#P < 0.05, ##P < 0.01 with respect to IL-1β.

Cells were stimulated with IL-1β (10 ng/ml) for 24 h (A) and 16 h (B) in the presence of CoPP (10 μM) and a siRNA specific for HO-1 or siRNA control (100 nM). HMGB1 protein expression was determined in cell lysates by western blotting. Relative expression of HMGB1 and β-actin protein bands was calculated after densitometric analysis. mRNA levels were determined by real-time PCR. Data are expressed as mean (s.e.m.) (n = 6–8). Open bars: non-stimulated cells; solid bars: cells stimulated with IL-1β.

**P < 0.05, **P < 0.01 with respect to non-stimulated cells.

#P < 0.05, ##P < 0.01 with respect to IL-1β.

The immunoblot is representative of three independent experiments.
with phosphatidylserine on the cell surface inhibits the phagocytosis of apoptotic neutrophils by macrophages, which may retard the resolution of the inflammatory response [36].

Our results indicate that IL-1β increases the expression of HMGB1 at the protein and mRNA levels, whereas induction of HO-1 by CoPP treatment is able to decrease HMGB1 expression. Stimulation of OA synoviocytes with IL-1β leads to the translocation of HMGB1 from the nucleus into the cytoplasm followed by its secretion into the medium. We have shown that HO-1 induction by CoPP is able to control HMGB1 translocation and secretion, thus reducing the levels of extracellular HMGB1. These observations were confirmed by cell transduction with LV-HO-1. The inhibitory effects of HO-1 overexpression on HMGB1 expression, translocation and secretion represent a novel finding, indicating a role of HO-1 in the regulation of the inflammatory response in OA synoviocytes.

In conclusion, our data show that HO-1 can down-regulate degradative and inflammatory mediators in OA synoviocytes. These results suggest that

![Fig. 4 Effect of CoPP on HMGB1 translocation into the cytoplasm and secretion, in OA synoviocytes.](https://academic.oup.com/rheumatology/article-abstract/49/5/854/1786321/)

(A) Immunocytochemical analysis. (B) Western blot analysis. (C) Protein release into the medium. Cells were stimulated with IL-1β (10 ng/ml) for 24 h in the presence or absence of CoPP (10 μM) and a siRNA specific for HO-1 or siRNA control (100 nM). (A) Translocation was followed by immunocytochemical analysis of HMGB1. Cell nuclei were counterstained with DAPI. Fluorescence micrographs representative of three separate experiments. Magnification ×400. (B) HMGB1 expression was determined by western blotting in nuclear and cytoplasmic extracts. (C) HMGB1 was measured in the medium by ELISA. Data are expressed as mean (S.E.M.) (n = 6–8). Open bars: non-stimulated cells; solid bars: cells stimulated with IL-1β. *P < 0.05, with respect to non-stimulated cells; †P < 0.05 with respect to IL-1β; ‡P < 0.05 with respect to IL-1β + CoPP. The immunoblot is representative of three independent experiments.
therapeutic targeting of HO-1 may be a potential strategy to control the progression of OA.

**Rheumatology key message**

- Overexpression of haem oxygenase-1 down-regulates degradative and inflammatory processes in osteoarthritic synoviocytes.

**Acknowledgements**

I.G.-A. would like to thank Generalitat Valenciana for a fellowship. Moreover, the authors are grateful to Dr Antonio Cuadrado (Department of Biochemistry and Alberto Sols Institute of Biomedical Investigations, UAM-CSIC, Madrid, Spain) for his collaboration in the preparation of LV-HO-1.

**Funding**: This work was supported by grants SAF2007-61769 and RETICEF RD06/0013/2001 (Ministerio de Ciencia e Innovación-FEDER).

**Disclosure statement**: The authors have declared no conflicts of interest.

**Supplementary data**

Supplementary data are available at *Rheumatology* Online.

**References**


