



Heme oxygenase-1 mediates protective effects on inflammatory, catabolic and senescence responses induced by interleukin-1 β in osteoarthritic osteoblasts

Victoria Clérigues^a, Maria Isabel Guillén^{a,b}, Miguel Angel Castejón^c,
Francisco Gomar^d, Vicente Mirabet^e, Maria José Alcaraz^{a,*}

^a Department of Pharmacology, University of Valencia, Burjasot, 46100 Valencia, Spain

^b Department of Chemistry, Biochemistry and Molecular Biology, Cardenal Herrera-CEU University, Moncada, 46113 Valencia, Spain

^c Department of Orthopaedic Surgery and Traumatology, De la Ribera University Hospital, Alzira, 46600 Valencia, Spain

^d Department of Surgery, Faculty of Medicine, University of Valencia, 46010 Valencia, Spain

^e Valencia Transfusion Center, Generalitat Valenciana, 46014 Valencia, Spain

ARTICLE INFO

Article history:

Received 20 September 2011

Accepted 23 November 2011

Available online 2 December 2011

Keywords:

Osteoblasts

Heme oxygenase-1

Osteoarthritis

Cytokines

Senescence

ABSTRACT

Osteoarthritis (OA) is a chronic degenerative joint disease showing altered bone metabolism. Osteoblasts contribute to the regulation of cartilage metabolism and bone remodeling. We have shown previously that induction of heme oxygenase-1 (HO-1) protects OA cartilage against inflammatory and degradative responses. In this study, we investigated the effects of HO-1 induction on OA osteoblast metabolism. HO-1 was induced with cobalt protoporphyrin IX (CoPP) and by transduction with LV-HO-1. In osteoblasts stimulated with interleukin (IL)-1 β , CoPP enhanced mineralization, the expression of a number of markers of osteoblast differentiation such as Runx2, bone morphogenetic protein-2, osteocalcin, and collagen 1A1 and 1A2, as well as the ratio osteoprotegerin/receptor activator of nuclear factor- κ B ligand. HO-1 induction significantly reduced the expression of matrix metalloproteinase (MMP)-1, MMP-2 and MMP-3, and the production of pro-inflammatory cytokines such as tumor necrosis factor- α and IL-6 whereas IL-10 levels increased. HO-1 also exerted inhibitory effects on prostaglandin (PG)E₂ production which could be dependent on cyclooxygenase-2 and microsomal PGE synthase-1 down-regulation. The activity of senescence-associated β -galactosidase and the expression of the senescence marker caveolin-1 were significantly decreased after HO-1 induction. The inhibition of nuclear factor- κ B activation induced by IL-1 β in OA osteoblasts may contribute to some HO-1 effects. Our results have shown that HO-1 decreases the production of relevant inflammatory and catabolic mediators that participate in OA pathophysiology thus eliciting protective effects in OA osteoblasts.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

Osteoarthritis (OA) is a degenerative disease of the joints leading to progressive cartilage damage. OA is characterized by changes to all components of the joints, with cartilage degradation, joint space narrowing, synovitis, and sclerosis of subchondral bone (reviewed in [1]). Pharmacological treatment is mainly centered on symptomatic agents although in recent years new approaches are being explored. There is an urgent need to develop new treatment strategies as there is no therapy able to stop the progression of disease.

Several studies have demonstrated that OA is associated with altered bone metabolism and turnover in subchondral areas where bone is sclerotic but mechanically weaker because of

under mineralization and increased collagen metabolism [2,3]. In addition, sclerotic OA subchondral osteoblasts could contribute to cartilage degradation by stimulating chondrocytes to produce matrix metalloproteinases (MMPs) and by inhibiting aggrecan synthesis [4]. There is growing evidence for a generalized involvement of bone in OA. Hence, sites more distal to the joint articular surface show a decrease in mineralization and a more rigid structure of the trabecular bone. Interestingly, a number of differentially expressed genes with a potential role in the pathology of OA have been identified in femoral trabecular bone associated with altered bone remodeling and trabecular microarchitecture [5–7]. Therapeutic strategies aiming at modifying the abnormal metabolism of bone cells have been proposed for OA [8].

Pro-inflammatory cytokines such as interleukin (IL)-1 β have been shown to play an important role in OA. This cytokine is involved in cartilage degradation [9] and bone remodeling in both physiological and pathological conditions [10]. It is known that IL-1 β activates osteoblasts leading to the synthesis of mediators

* Corresponding author at: Department of Pharmacology, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjasot, Valencia, Spain.
Tel.: +34 963544292; fax: +34 963544499.

E-mail address: maria.j.alcaraz@uv.es (M.J. Alcaraz).

involved in OA [8,11]. A major cytokine-induced signaling pathway involves the activation of nuclear factor- κ B (NF- κ B) which is responsible for the transcription of key genes in inflammation and OA [12].

Heme oxygenase (HO) activity catalyses the degradation of heme into free iron, carbon monoxide (CO) and biliverdin which is converted to bilirubin by biliverdin reductase. HO-1 is induced by a wide range of stimuli as an adaptive response against stress (reviewed in [13]). We have reported previously that induction of HO-1 results in protective effects against inflammatory and degradative responses induced by IL-1 β in OA chondrocytes and synoviocytes [14,15]. Nevertheless, the role of HO-1 in bone pathophysiology is not completely understood [16]. Both the constitutive isoform HO-2 and the inducible enzyme HO-1 have been detected in bone tissue and mechanical loading generating physiological levels of strain sufficient to initiate an osteogenic response induces HO-1 in rats. HO-1 up-regulation by CO or nitric oxide protects MC3T3E1 osteoblasts against tumor necrosis factor- α (TNF- α)-induced apoptosis [17]. In addition, induction of HO-1 in human bone marrow-derived mesenchymal stem cells augmented osteoblast differentiation [18], and activation of Nrf2 and HO-1 has been linked to the up-regulation of osteogenic differentiation in human periodontal ligament cells [19]. Interestingly, induction of HO-1 has been shown to inhibit osteoclast differentiation and inflammatory bone loss *in vivo* [20]. In contrast, it has also been reported that HO-1 overexpression inhibits maturation of osteoblasts from rat calvaria precursor cells and osteoclastogenesis in rat bone marrow cells [21].

Several lines of evidence indicate that osteoblasts contribute to the regulation of cartilage metabolism and bone remodeling in OA [22]. However, the role of HO-1 in the regulation of OA osteoblast metabolism has not been reported yet. Given the relevance of osteoblast function in OA and our demonstration of the protective effects of HO-1 induction in OA cartilage [7], we sought to study the consequences of HO-1 overexpression in OA osteoblasts.

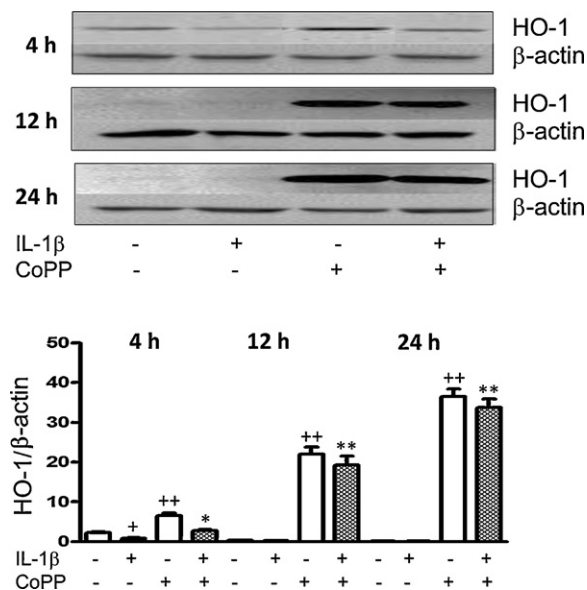


Fig. 1. Time-course of HO-1 protein induction by CoPP in OA osteoblasts. Cells were incubated with CoPP for different times in the presence or absence of IL-1 β . HO-1 protein expression was analyzed by Western blotting. Relative expression of HO-1 and β -actin protein bands was calculated after densitometric analysis. Data are expressed as mean \pm S.E.M. Samples from 3 patients were used. $^+p < 0.05$, $^{++}p < 0.01$ with respect to nonstimulated cells; $^*p < 0.05$, $^{**}p < 0.01$ with respect to IL-1 β .

2. Materials and methods

2.1. Cells and culture media

The knee specimens were obtained from patients with the diagnosis of advanced OA (33 women and 13 men, aged 71.5 ± 7.1 years mean \pm S.E.M.) undergoing total knee joint replacement. Diagnosis was based on clinical, laboratory and radiological evaluation. The design of the work was approved by the Institutional Ethical Committees (University of Valencia and University Clinical Hospital, Valencia, Spain). Samples were obtained under patient's consent according to the declaration of Helsinki. The pieces of trabecular bone were obtained from the femoral condyles and tibial plateaus, cut into small pieces and subjected to enzymatic digestion with 2 mg/ml of collagenase type IA (Sigma–Aldrich St. Louis, MO, USA) at 37 °C in DMEM/Ham's F-12

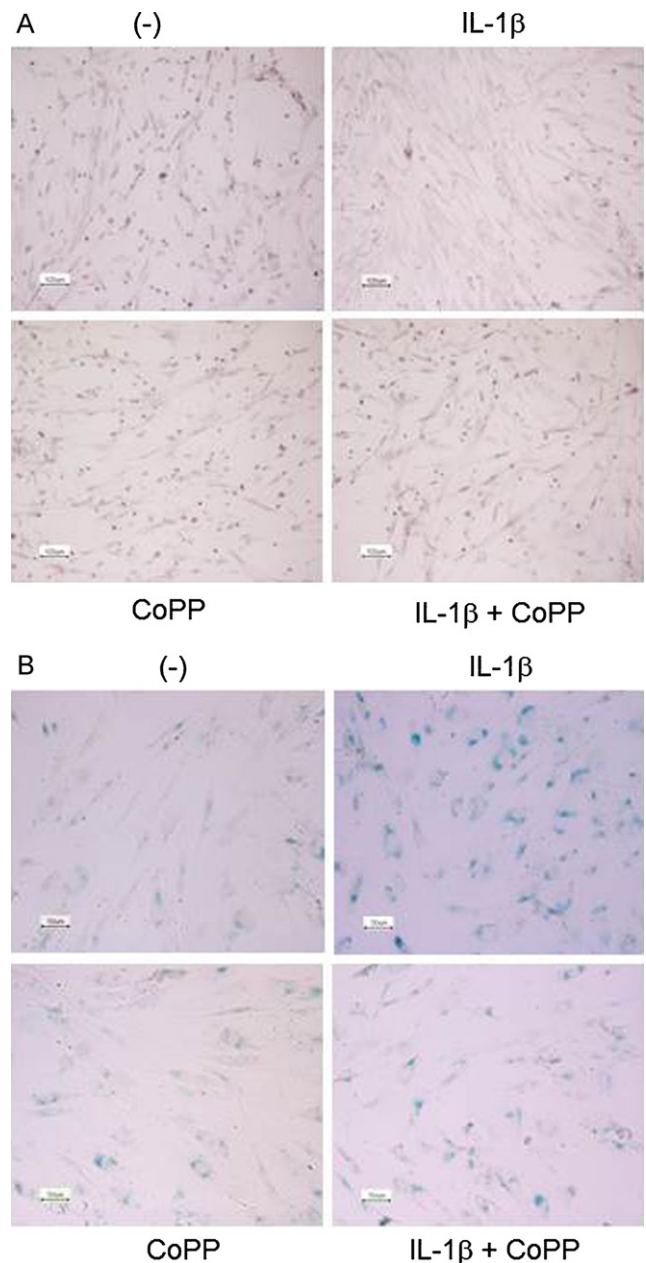


Fig. 2. Effects of CoPP on mineralization and senescence-associated β -galactosidase activity in OA osteoblasts. (A) Mineralization was measured by the Alizarin red method. (B) Senescence-associated β -galactosidase activity was determined by using the cellular senescence assay kit, as indicated in Section 2. Figure representative of three different experiments.

(Sigma–Aldrich) supplemented with penicillin (100 U/ml) and streptomycin (100 U/ml) (Sigma–Aldrich) for 2 h in agitation. The digest was cultured in osteogenic medium (Promocell, Labclinics S.A., Barcelona, Spain) in a humidified incubator with 5% CO₂ at 37 °C. This medium was replaced twice a week. Cell phenotype was characterized by flow cytometric analysis using a Becton Dickinson FACSCanto II cytometer (BD, Franklin Lakes, NJ, USA) and antibodies anti-human-CD90(1/50), -CD105(1/20), -CD34(1/20) and -CD45(1/10). When cells were at 70% of confluence bone fragments were removed and cells were cultured until third passage where there is a high number of cells with osteoblast-like differentiation [23]. Cells were incubated with the HO-1 inducer cobalt protoporphyrin IX (CoPP, 10 μM) (Frontier Scientific Europe Ltd., Carnforth, UK) or vehicle for 30 min before stimulation with IL-1β (10 ng/ml) (Peprotech EC Ltd., London, UK), or transduced with lentiviral vectors and then stimulated with IL-1β (10 ng/ml), as indicated. The concentrations used were selected from previous studies [14,24] and preliminary experiments. 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 test (data not shown).

2.2. Mineralization assay

The ability to form a mineralized matrix was assessed by the Alizarin red staining method using the osteogenesis quantitation kit,

(Chemicon/Millipore, Schwalbach, Germany), according to manufacturer's instructions. Cells (5×10^5) were seeded in Petri dishes and incubated in growth medium for 10 days [25] in the presence or absence of the HO-1 inducer CoPP (10 μM) and IL-1β (10 ng/ml). Cells were washed with phosphate-buffered saline, fixed with 10% formaldehyde, rinsed with deionized water and then stained with Alizarin red solution for 1 h at room temperature. After washing with deionized water, mineral deposition was examined under light microscopy (Eclipse E800, Nikon Instruments Europe, Amstelveen, The Netherlands) and photographed with a Nikon Digital Camera DXM1200 using the software Nikon ACT-1. Quantitative analysis of Alizarin red staining was performed by extracting the dye with 10% acetic acid from the stained monolayer and determining OD₄₀₅ in comparison with a standard curve of Alizarin red. Each experiment was done in triplicate.

2.3. Senescence-associated β-galactosidase activity assay

Cells (5×10^5) were seeded in Petri dishes and incubated in growth medium for 7 days in the presence or absence of IL-1β (10 ng/ml) and CoPP (10 μM). Senescence-associated β-galactosidase activity was measured using the cellular senescence assay kit from Cell Biolabs Inc. (San Diego, CA, USA). Cells were counterstained with hematoxylin to count the total cell numbers. Osteoblasts were analyzed using a Nikon Eclipse E800 microscope and photographed.

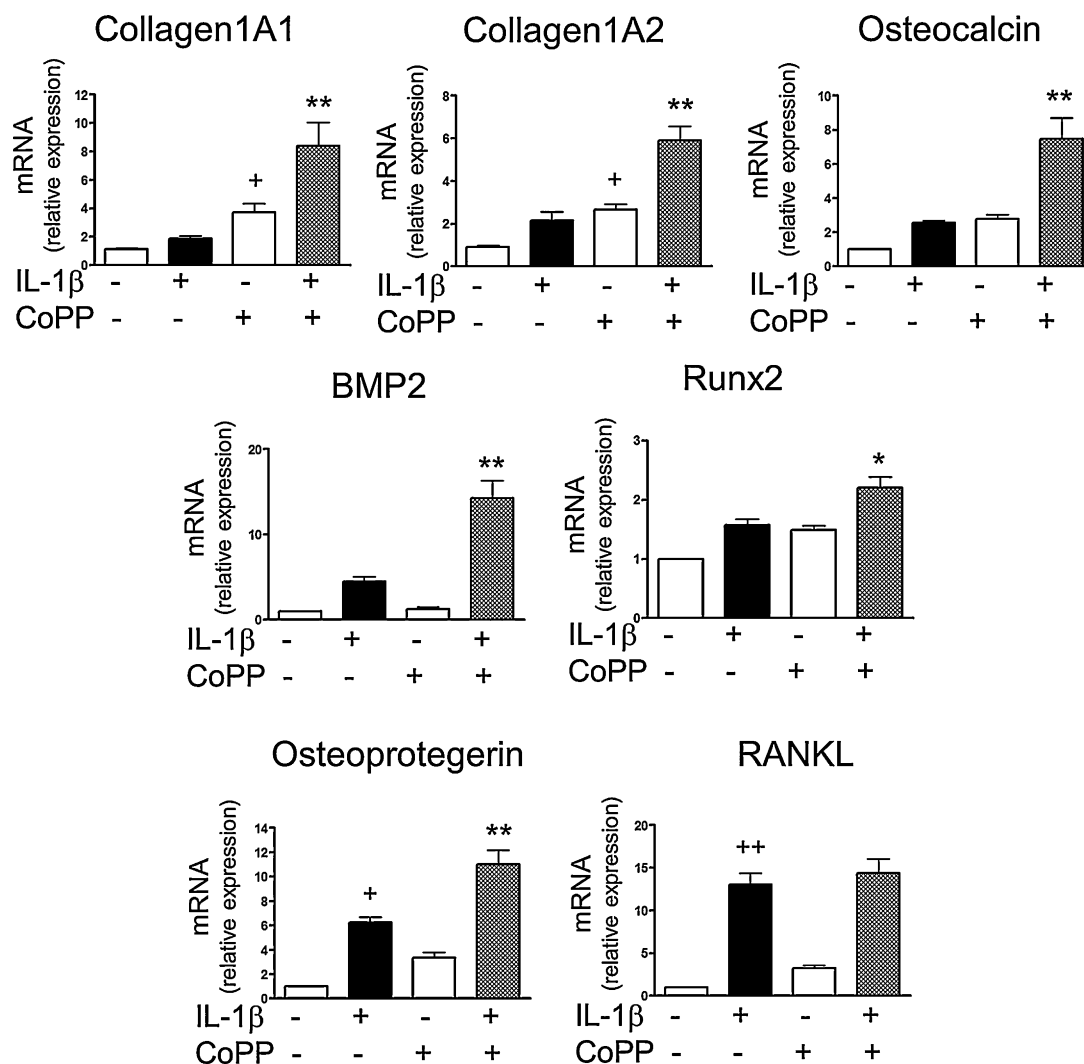


Fig. 3. Effects of CoPP on mRNA expression of osteogenesis-related genes. Real-time PCR analysis was performed in OA osteoblasts incubated with CoPP and IL-1β for 16 h. Data represent mean ± S.E.M., $n = 6$. + $p < 0.05$, ++ $p < 0.01$ with respect to nonstimulated cells; * $p < 0.05$, ** $p < 0.01$ with respect to IL-1β.

2.4. Transduction with lentiviral vectors

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). 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 $700 \times g$ for 10 min at 4°C , passed through $45\text{-}\mu\text{m}$ pore size filters and kept at -80°C . The titres of lentiviral stocks were in the range of $3\text{--}5 \times 10^5$ IU/ml as determined by immunocytochemical analysis of HEK293T-infected cells [26]. The osteoblasts culture was infected with 2.5 ml of each lentiviral stock for 24 h in a humidified 5% CO_2 incubator at 37°C . After infection of lentiviral vector HO-1 (LV-HO-1) or empty vector (LV(-)), cells were cultured in growth medium for 2 days and then stimulated with IL-1 β (10 ng/ml) for different times.

2.5. Immunocytochemical analysis

Cells were fixed with 4% formaldehyde in phosphate-buffered saline for 10 min and incubated with primary antibody anti-Flag (Sigma–Aldrich) (1/400) for 1.5 h, followed by incubation with the secondary antibody Alexa Fluor 488 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) (Sigma–Aldrich). Cells were examined under fluorescence microscopy (Nikon Eclipse E800) and photographed.

2.6. Real-time PCR

Total RNA was extracted using the TriPure reagent (Roche Applied Science, Barcelona, Spain) according to the manufacturer's instructions. Reverse transcription was accomplished on $1\ \mu\text{g}$ 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). Sequences of primers used have been reported previously [3,27–32] and were synthesized by Eurofins MWG Operon (Ebersberg, Germany). Caveolin-1 and hTERT primer sets were from SA Biosciences Corporation (Tebu-Bio, Barcelona, Spain). For each sample, differences in threshold cycle (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 $2^{-\Delta\Delta\text{Ct}}$ with respect to nonstimulated cells.

2.7. Western blot analysis

Cells were lysed in $100\ \mu\text{l}$ 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 g. Protein content was determined by the DC Bio-Rad protein reagent (Richmond, CA, USA). Proteins (20 μg) in supernatants 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 polyclonal antibodies against cyclooxygenase-2 (COX-2, Cayman Chemical, Ann Arbor, MI, USA), HO-1 (Stressgen, Victoria, British Columbia, Canada) and β -actin

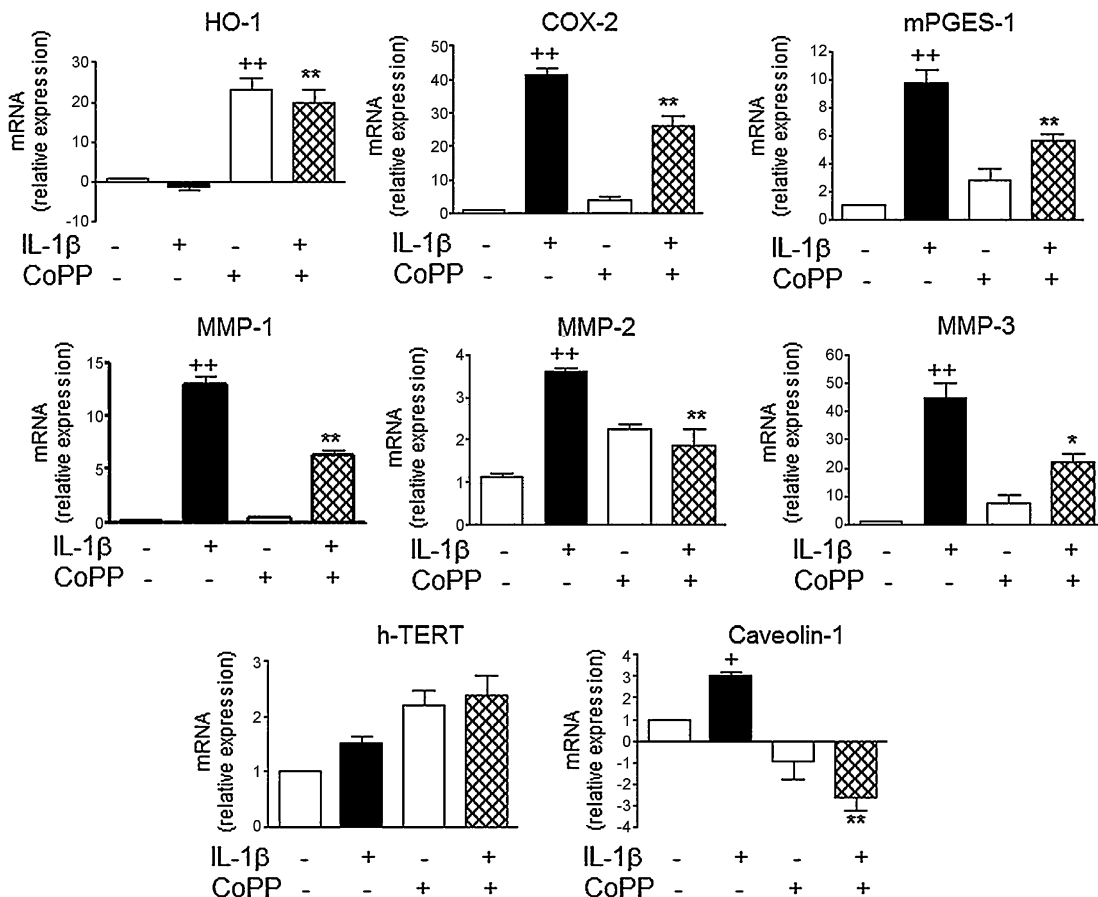


Fig. 4. Effects of CoPP on mRNA expression of genes related with inflammation or senescence. Real-time PCR analysis was performed in OA osteoblasts incubated with CoPP and IL-1 β for 3 h. Data represent mean \pm S.E.M., $n = 6$. $+p < 0.05$, $++p < 0.01$ with respect to nonstimulated cells; $*p < 0.05$, $**p < 0.01$ with respect to IL-1 β .

(Sigma–Aldrich) for 2 h at room temperature. Finally, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (Dako) and the immunoreactive bands were visualized by enhanced chemiluminescence (GE Healthcare, Barcelona, Spain) using the AutoChemi image analyzer (UVP Inc., Upland, CA, USA).

2.8. Determination of MMP activity

Cells were stimulated with IL-1 β (10 ng/ml) for 24 h and supernatants were harvested, centrifuged and incubated with p-aminophenylmercuric acetate 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 Inc., San Jose, CA, USA), fluorescence was measured for different times at 490 nm (excitation)/520 nm (emission) in a Victor3 microplate reader (PerkinElmer, Madrid, Spain).

2.9. Enzyme-linked immunosorbent assay

Osteoblasts were stimulated with IL-1 β (10 ng/ml) or IL-1 β + CoPP (10 μ M) for 4 or 24 h. Supernatants were harvested, centrifuged and frozen at –80 °C until analysis. TNF- α , IL-6 and IL-10 were measured by ELISA kits from eBioscience (San Diego, CA, USA) with sensitivity of 4 pg/ml for TNF- α and 2 pg/ml for IL-6 and IL-10 kits. Osteoprotegerin was measured by an ELISA kit from Cusabio Biotech (Newark, DE, USA) with sensitivity of 0.078 ng/ml and receptor activator of NF- κ B ligand (RANKL) was assayed by an

ELISA kit from Wuhan EIAab Science Co. Ltd. (Wuhan, China) with sensitivity of 0.78 pg/ml. NF- κ B inhibitory protein- α (I κ B α) phosphorylation was measured in cytosolic extracts of cells stimulated with IL-1 β (10 ng/ml) in the presence or absence of LV-HO-1 for 30 min with K-LISA™ IKK β Inhibitor screening kit (Calbiochem EMD Bioscience, Darmstadt, Alemania). NF- κ B binding to DNA was quantified by ELISA in nuclear extracts from cells stimulated with IL-1 β (10 ng/ml) in the presence or absence of LV-HO-1 for 1 h, using the Nuclear Extract Kit Active Motif for nuclei extraction followed by Trans AM™ NF- κ B kit (purchased from Active Motif Europe, Rixensart, Belgium), according to the manufacturer's recommendations.

2.10. Statistical analysis

The data were analyzed by Kruskal–Wallis test and the Dunn's post test using the GraphPad Prism 5 software (Graph Pad Software, La Jolla, CA, USA). A *p*-value of less than 0.05 was considered to be significant.

3. Results

3.1. Induction of HO-1 by CoPP and effects on mineralization and senescence-associated β -galactosidase activity

Fig. 1 shows the time-course of HO-1 protein induction by CoPP. Cell stimulation with IL-1 β for 4 h down-regulated HO-1 protein

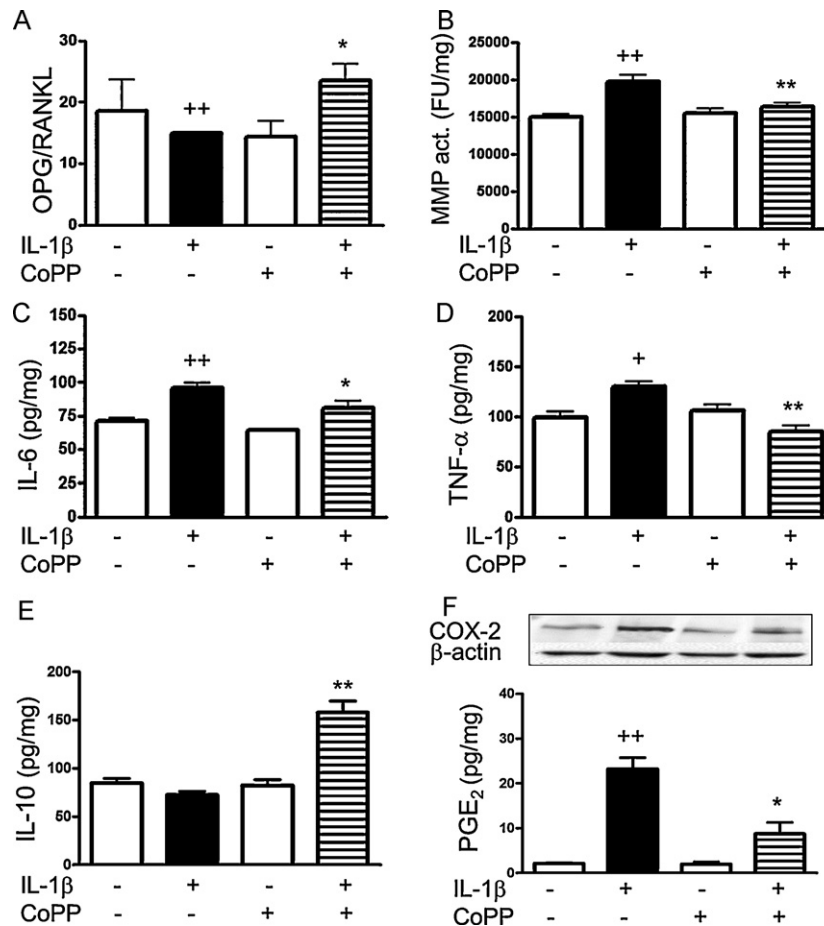


Fig. 5. Effects of CoPP on the production of mediators. (A) Osteoprotegerin and RANKL were measured by ELISA and the figure shows the ratio osteoprotegerin (OPG)/RANKL, (B) MMP activity was measured by fluorometry and expressed as fluorescence units per mg of protein, (C) IL-6, (D) TNF- α and (E) IL-10, were measured by ELISA, (F) PGE₂ was measured by RIA and COX-2 protein expression was analyzed by Western blotting. Mediators were determined in cell culture supernatants after 24 h incubation of OA osteoblasts with CoPP in the presence or absence of IL-1 β . IL-10 was measured after 4 h incubation. COX-2 protein was analyzed in cell extracts after 24 h incubation in the presence or absence of IL-1 β . Data represent mean \pm S.E.M. *n* = 6. +*p* < 0.05, ++*p* < 0.01 with respect to nonstimulated cells; **p* < 0.05, ***p* < 0.01 with respect to IL-1 β .

expression whereas CoPP ($10 \mu\text{M}$) counteracted this effect. This concentration of CoPP was selected due to its strong HO-1 induction and lack of toxicity [14,15]. HO-1 protein was up-regulated by CoPP or CoPP + IL-1 β in a time-dependent manner with maximal effects at 24 h.

Deposition of a mineralized matrix can reflect bone formation activity. Fig. 2A shows that IL-1 β reduced mineral deposition in OA osteoblasts whereas CoPP treatment in the presence of IL-1 β induced mineralization assessed by the Alizarin red method. Quantitative analysis of Alizarin red staining confirmed these results, as IL-1 β reduced it to $0.49 \pm 0.03 \mu\text{g}/\text{mg}$ protein from $0.84 \pm 0.04 \mu\text{g}/\text{mg}$ protein in nonstimulated cells ($p < 0.01$). Values for CoPP-treated cells were of $0.73 \pm 0.02 \mu\text{g}/\text{mg}$ protein in nonstimulated cells and $1.11 \pm 0.05 \mu\text{g}/\text{mg}$ protein in the presence of IL-1 β ($p < 0.01$ versus IL-1 β).

We investigated in OA osteoblasts the levels of senescence-associated β -galactosidase activity, a widely used biomarker of senescence. Fig. 2B shows that osteoblast stimulation with IL-1 β enhances β -galactosidase activity ($34.2 \pm 0.5\%$ positive cells) with respect to nonstimulated cells ($11.0 \pm 0.4\%$, $p < 0.01$). CoPP treatment decreased senescence-associated β -galactosidase activity

in IL-1 β -stimulated osteoblasts ($16.0 \pm 0.7\%$, $p < 0.05$) but did not affect this activity in nonstimulated cells ($11.0 \pm 0.4\%$, $p > 0.05$).

3.2. Effects of CoPP treatment on gene expression

To study the influence of HO-1 induction by CoPP on osteoblast osteogenesis potential, we examined the mRNA expression of a number of osteoblast differentiation and mineralization genes. As shown in Fig. 3, CoPP increased the production of collagen 1A1 and collagen 1A2 either in the presence or absence of IL-1 β . In cells stimulated with IL-1 β , CoPP treatment significantly increased mRNA levels of osteocalcin, bone morphogenetic protein 2 (BMP2) and Runx2. In addition, the expression of genes central to osteoclastogenesis was also regulated by CoPP with enhancing effects on osteoprotegerin without modification of RANKL. Fig. 4 shows that CoPP strongly induced the mRNA expression of HO-1 in OA osteoblasts, either in the presence or absence of IL-1 β . This cytokine showed a tendency to decrease HO-1 mRNA levels with respect to basal expression. Cell stimulation with IL-1 β induced COX-2 and microsomal PGE synthase-1 (mPGES-1) mRNA, which

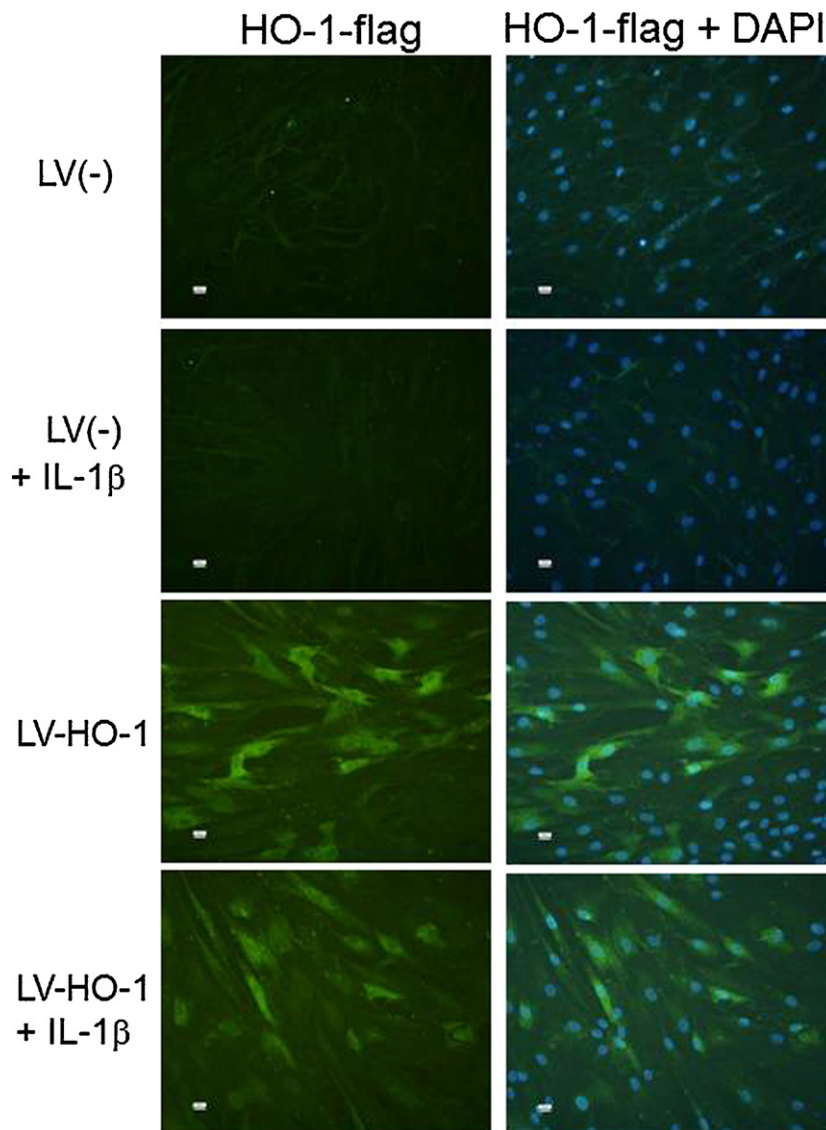


Fig. 6. LV-HO-1 transduction of OA osteoblasts. Cells were transduced with LV-HO-1 or the empty vector LV(-) and incubated for 24 h in the presence or absence of IL-1 β . Immunocytochemical analysis was performed using an anti-flag specific antibody to determine the expression of HO-1 induced by transduction. Cell nuclei were counterstained with DAPI. Fluorescence micrographs representative of three separate experiments.

was significantly decreased in the presence of CoPP. In addition, mRNA expression of MMP-1, MMP-2 and MMP-3 was enhanced by IL-1 β and down-regulated by CoPP treatment. We have also shown a tendency to increase mRNA expression of human telomerase reverse transcriptase (hTERT) in osteoblasts incubated with CoPP. Interestingly, the expression of the senescence marker caveolin-1 was significantly enhanced by IL-1 β and down-regulated by CoPP.

3.3. Effects of CoPP treatment on production of mediators

Stimulation of OA osteoblasts with IL-1 β for 24 h reduced the ratio osteoprotegerin/RANKL (Fig. 5A) and increased the release of MMP activity (Fig. 5B) and pro-inflammatory cytokines IL-6 (Fig. 5C) and TNF- α (Fig. 5D) into the culture medium. CoPP treatment significantly prevented the effects of IL-1 β . The production of the anti-inflammatory cytokine IL-10 was not modified after 24 h (data not shown) but this cytokine was significantly increased after 4 h incubation by CoPP treatment of cells in the presence of IL-1 β (Fig. 5E). In addition, COX-2 expression and PGE₂ production were enhanced by IL-1 β after 24 h incubation (Fig. 5F) whereas CoPP significantly reduced them.

3.4. Effects of LV-HO-1 on gene expression

To confirm that CoPP effects on OA osteoblasts were due to the induction of HO-1 by this molecule, we performed other series of experiments using cells transduced with LV-HO-1. Fig. 6 shows the expression of HO-1 dependent on lentiviral transduction (revealed by an anti-flag antibody). Our results indicate that OA osteoblast transduction with LV-HO-1 effectively resulted in the up-regulation of HO-1 protein in nonstimulated cells as well as in the presence of IL-1 β .

We examined the effects of LV-HO-1 transduction on gene expression. As shown in Fig. 7, HO-1 up-regulation significantly increased the expression of osteoblast differentiation and mineralization marker genes, as observed with CoPP treatment. In addition, the modification of osteoprotegerin and RANKL mRNA by LV-HO-1 was similar to that caused by CoPP. Senescence markers, COX-2, mPGES-1, MMP-1, MMP-2 and MMP-3 gene expression were also modified according to the results obtained with CoPP (Fig. 8).

3.5. Effects of LV-HO-1 on production of mediators

Consistent with the results obtained using CoPP, we found an increased osteoprotegerin/RANKL ratio in cells transduced with

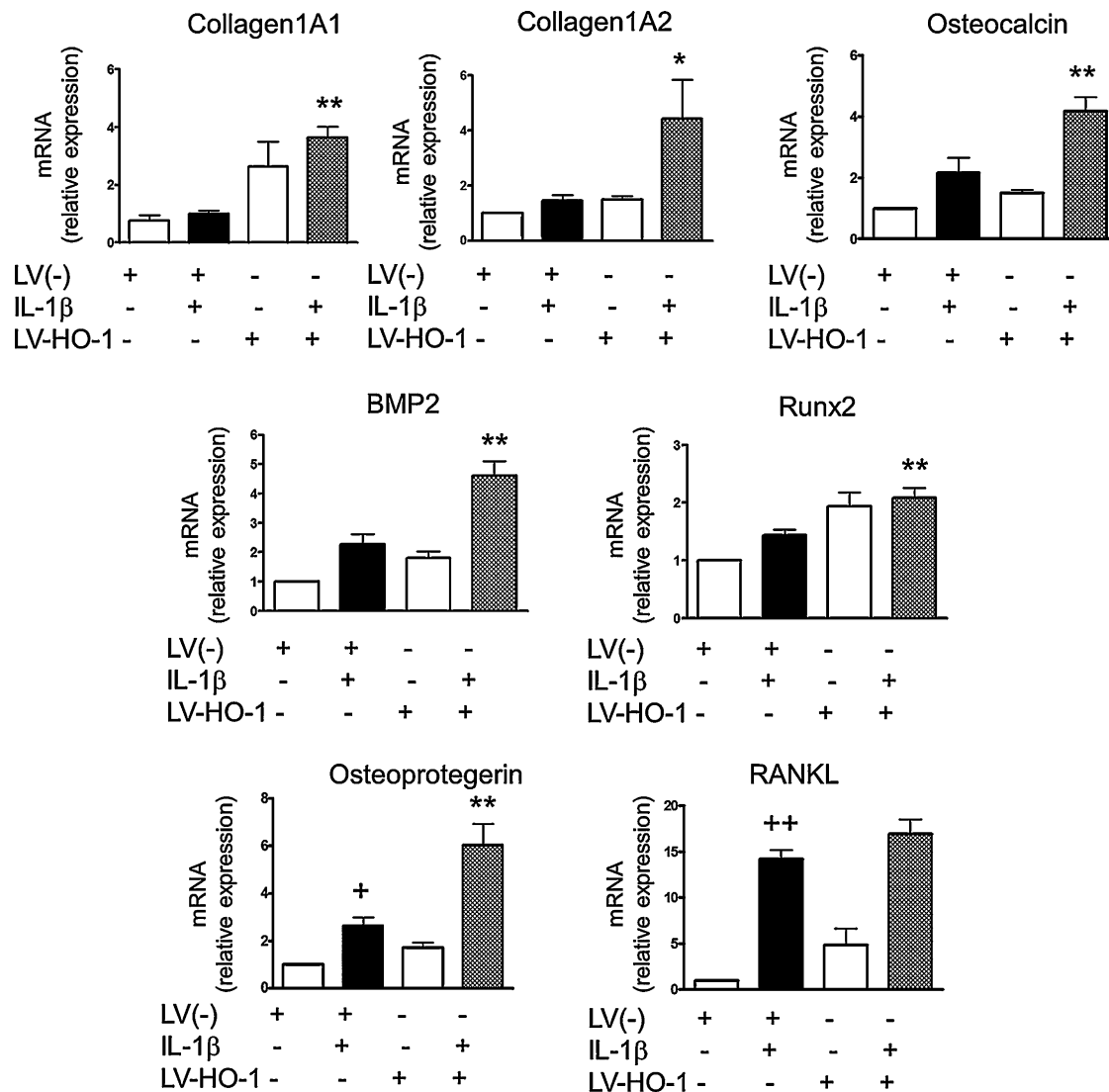


Fig. 7. Effects of LV-HO-1 on mRNA expression of osteogenesis-related genes. Real-time PCR analysis was performed in OA osteoblasts incubated with CoPP and IL-1 β for 16 h. Data represent mean \pm S.E.M., $n = 6$. + $p < 0.05$, ++ $p < 0.01$ with respect to nonstimulated cells; * $p < 0.05$, ** $p < 0.01$ with respect to IL-1 β .

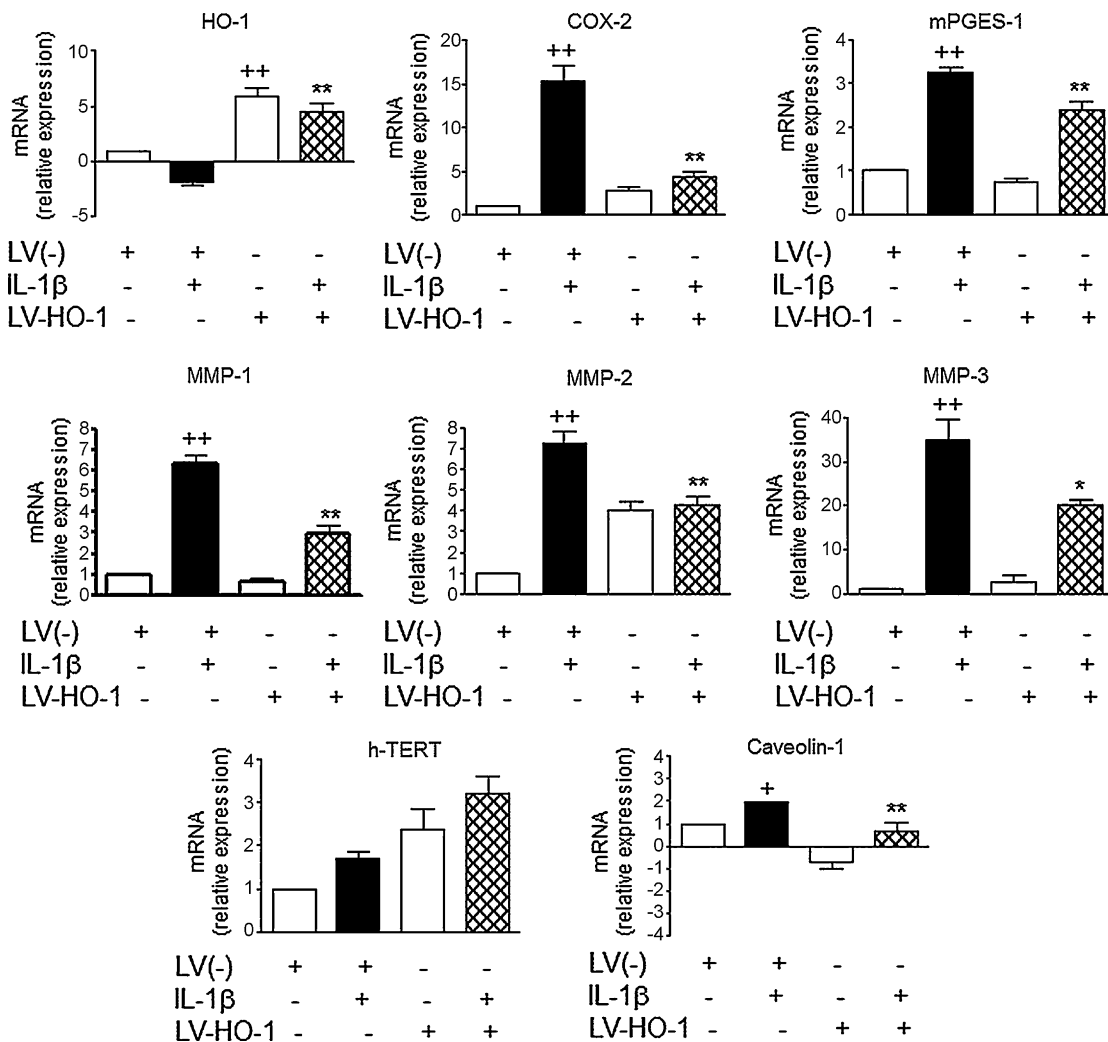


Fig. 8. Effects of LV-HO-1 on mRNA expression of genes related with inflammation or senescence. Real-time PCR analysis was performed in OA osteoblasts incubated with CoPP and IL-1 β for 3 h. Data represent mean \pm S.E.M., $n = 6$. $+p < 0.05$, $++p < 0.01$ with respect to nonstimulated cells; $*p < 0.05$, $**p < 0.01$ with respect to IL-1 β .

LV-HO-1 and stimulated with IL-1 β (Fig. 9A), as well as a lower production of MMP activity, IL-6, TNF α and PGE $_2$ (Fig. 9B, C, D, F). In addition, IL-10 levels were significantly increased after HO-1 transduction in cells stimulated with IL-1 β (Fig. 9E).

3.6. Effects of LV-HO-1 on NF- κ B

To further investigate the consequences of HO-1 overexpression in human OA osteoblasts, we assessed the effects of LV-HO-1 on NF- κ B activation which plays a key role in the regulation of IL-1 β -induced inflammatory genes. A marked activation of NF- κ B was detected after IL-1 β cell stimulation and HO-1 significantly attenuated this process (Fig. 10A). In addition, we observed that IL-1 β significantly increased I κ B α phosphorylation (Fig. 10B) whereas osteoblast transduction with LV-HO-1 tended to reduce the effect of this cytokine although without reaching statistical significance.

4. Discussion

Abnormal bone tissue remodeling in OA can be dependent on the imbalance between osteoblast and osteoclast functions, with a significant contribution of soluble factors such as cytokines, chemokines and degradative enzymes [33,34]. Pro-inflammatory cytokines inhibit osteogenic differentiation of mesenchymal stem

cells [35] and activate osteoblasts to release cytokines and chemokines involved in the local regulation of bone metabolism and osteoclast stimulation [36,37]. In particular, IL-1 β is involved in the pathological modulation of bone cell metabolism [38]. Our results indicate that HO-1 induction counteracts the effects of this cytokine on OA osteoblasts.

Matrix mineralization is a final step of osteoblast differentiation and plays a critical role in maintaining the mechanical integrity of the calcified tissues. HO-1 induction by CoPP positively regulated the osteogenic function of OA osteoblasts and was able to promote gene expression and mineralization. Similar effects on osteoblast differentiation markers were observed in cells transduced with LV-HO-1 confirming that CoPP effects were dependent on HO-1 induction. Both the mineral and organic components of bone matrix contribute to its material properties. We have shown that HO-1 promotes collagen 1 expression in osteoblasts without alteration in the ratio of collagen 1A1 to collagen 1A2 expression that would retard mineralization in OA [3]. HO-1 also positively affects osteocalcin, BMP2 and Runx2 expression in the presence of IL-1 β . Nevertheless, it has been reported that HO-1 up-regulation inhibits the maturation and mineralization of osteoblasts from the calvaria of fetal rats in the absence of cell stimulation [21]. The discrepancies with the present study may be due to differences in species, cell type and culture conditions. Our results in cells from diseased human tissue are in agreement with the effects of HO-1

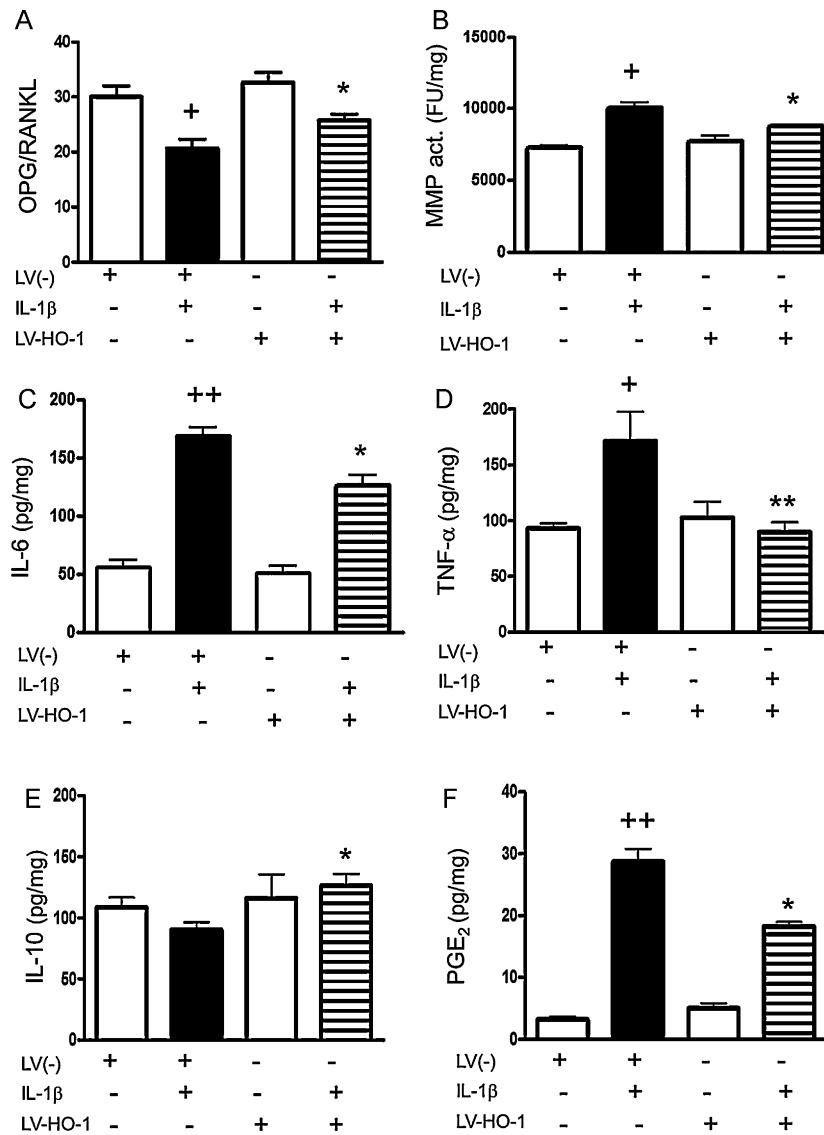


Fig. 9. Effects of LV-HO-1 on the production of mediators. (A) Osteoprotegerin and RANKL were measured by ELISA and the figure shows the ratio osteoprotegerin (OPG)/RANKL, (B) MMP activity was measured by fluorometry and expressed as fluorescence units per mg of protein, (C) IL-6, (D) TNF- α and (E) IL-10, were measured by ELISA, (F) PGE₂ was measured by RIA. Mediators were determined in cell culture supernatants after 24 h incubation of OA osteoblasts with CoPP in the presence or absence of IL-1 β . IL-10 was measured after 4 h incubation. Data represent mean \pm S.E.M., $n = 6$. + $p < 0.05$, ++ $p < 0.01$ with respect to nonstimulated cells; * $p < 0.05$, ** $p < 0.01$ with respect to IL-1 β .

on osteogenesis in human bone marrow-derived mesenchymal stem cells [18].

The balance between osteoprotegerin and RANKL determines osteoclast function and is an important mechanism in the pathogenesis of bone disease that result from bone resorption (reviewed in [39,40]). We have demonstrated that IL-1 β decreases the ratio osteoprotegerin/RANKL in OA osteoblasts with respect to nonstimulated cells whereas HO-1 induction reverted this process.

It is known that IL-1 β and oxidative stress elicit premature senescence in OA chondrocytes and stress-induced caveolin-1 may mediate senescence and OA progression [41]. Our data indicate that HO-1 down-regulates senescence markers in OA osteoblasts thus suggesting that HO-1 could be a strategy to prevent senescence-associated osteoblast alterations.

We have shown that HO-1 induction significantly reduced the production of pro-inflammatory cytokines such as TNF α and IL-6 whereas IL-10 levels were enhanced. IL-6 is produced by nonstimulated OA osteoblasts [42] and up-regulated upon cell stimulation by different agents. This cytokine plays an important role in osteoclast recruitment and bone resorption [43]. High levels

of IL-6 mRNA expression have been demonstrated in female patients with fragility fracture and have been related to alterations in bone formation surface relative to resorption surface and fracture propensity as this cytokine inhibits human osteoblast differentiation and mineral deposition [44]. Our results also indicate that HO-1 induction decreases PGE₂ production in OA osteoblasts stimulated with IL-1 β , an effect dependent on the inhibition of COX-2 and mPGES-1 expression. In mice, COX-2 may play a critical role in bone resorption stimulated by 1,25-dihydroxy vitamin D₃ and parathyroid hormone [45] whereas mPGES-1 has been suggested as a new target for the treatment of inflammatory bone disease [46]. PGE₂ exhibits a dual role and may be a mediator of both bone resorption and bone formation [47] by mechanisms mediated by EP2/EP4 [48] or EP4 receptors [49], respectively. This eicosanoid is involved in the mechanism of IL-1-mediated osteoclast-like cell formation induced by pro-inflammatory cytokines [50,51] and enhances IL-6 production in human OA osteoblasts [52] leading to the suppression of osteoprotegerin production [53]. In addition, PGE₂ has been shown to induce MMP-1 [54].

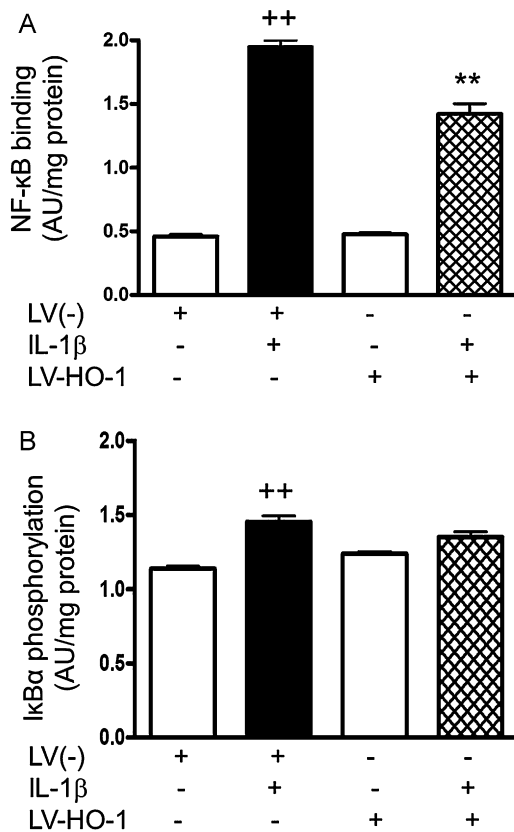


Fig. 10. Effects of LV-HO-1 on NF- κ B activation in OA osteoblasts. (A) NF- κ B binding to DNA, B: I κ B α phosphorylation. Cells were stimulated with IL-1 β for 1 h (A) or 30 min (B). NF- κ B binding to DNA and I κ B α phosphorylation were determined by ELISA in nuclear and cytosolic fractions, respectively. Data are expressed as mean \pm S.E.M. of independent cultures with cells from 3 different donors. AU = arbitrary units. ⁺⁺ p < 0.01 with respect to nonstimulated cells, ^{**} p < 0.01 with respect to IL-1 β .

MMPs may play complex roles in bone metabolism [55]. MMP-2-dependent degradation of bone matrix may be involved in bone resorption induced by IL-1 β [56]. We have shown that HO-1 induction down-regulates the expression of MMP-1, MMP-2 and MMP-3 stimulated by IL-1 β . Our results thus suggest that HO-1 induction in human OA osteoblasts could down-regulate inflammatory responses related to osteolysis [57].

In the present study, we have extended our observations on HO-1 regulation of the transcription factor NF- κ B in OA chondrocytes [18]. Our data indicate that HO-1 reduces NF- κ B activation induced by IL-1 β in OA osteoblasts, which may contribute to the inhibitory effects observed on the production of inflammatory and degradative mediators. Although further studies will be needed to elucidate the mechanisms involved, our results have revealed a protective role for HO-1 in human OA osteoblasts suggesting possible applications in pathological bone loss. Therefore, HO-1 may be a therapeutic strategy aimed at modifying not only cartilage degradation but also bone alterations.

Acknowledgements

This work has been funded by research grants: SAF2010-22048 and RETICEF RD07/0013/2001 (Spanish Ministry of Science and Innovation, ISCIII, FEDER), and Prometeo2010-047 (Generalitat Valenciana). V. Clérigues was supported by a fellowship from Generalitat Valenciana. The authors thank Prof. A. Cuadrado (Alberto Sols Institute, UAM, Madrid, Spain) for his help with preparation of LV-HO-1.

References

- [1] Goldring MB, Goldring SR. Osteoarthritis. *J Cell Physiol* 2007;213:626–34.
- [2] Li B, Aspden RM. Mechanical and material properties of the subchondral bone plate from the femoral head of patients with osteoarthritis or osteoporosis. *Ann Rheum Dis* 1997;56:247–54.
- [3] Couchourel D, Aubry I, Delalandre A, Lavigne M, Martel-Pelletier J, Pelletier JP, et al. Altered mineralization of human osteoarthritic osteoblasts is attributable to abnormal type I collagen production. *Arthritis Rheum* 2009;60:1438–50.
- [4] Sanchez C, Deberg MA, Piccardi N, Msika P, Reginster JY, Henrotin YE. Osteoblasts from the sclerotic subchondral bone downregulate aggrecan but upregulate metalloproteinases expression by chondrocytes. This effect is mimicked by interleukin-6, -1beta and oncostatin M pre-treated non-sclerotic osteoblasts. *Osteoarthritis Cartilage* 2005;13:979–87.
- [5] Hopwood B, Gronthos S, Kuliwaba JS, Robey PG, Findlay DM, Fazzalari NL. Identification of differentially expressed genes between osteoarthritic and normal trabecular bone from the intertrochanteric region of the proximal femur using cDNA microarray analysis. *Bone* 2005;36:635–44.
- [6] Hopwood B, Tsykin A, Findlay DM, Fazzalari NL. Microarray gene expression profiling of osteoarthritic bone suggests altered bone remodelling, WNT and transforming growth factor-beta/bone morphogenic protein signalling. *Arthritis Res Ther* 2007;9:R100.
- [7] Kumarasinghe DD, Perilli E, Tsangari H, Truong L, Kuliwaba JS, Hopwood B, et al. Critical molecular regulators, histomorphometric indices and their correlations in the trabecular bone in primary hip osteoarthritis. *Osteoarthritis Cartilage* 2010;18:1337–44.
- [8] Boileau C, Tat SK, Pelletier JP, Cheng S, Martel-Pelletier J. Diacerein inhibits the synthesis of resorptive enzymes and reduces osteoclastic differentiation/survival in osteoarthritic subchondral bone: a possible mechanism for a protective effect against subchondral bone remodelling. *Arthritis Res Ther* 2008;10:R71.
- [9] Dodge GR, Poole AR. Immunohistochemical detection and immunochemical analysis of type II collagen degradation in human normal, rheumatoid, and osteoarthritic articular cartilages and in explants of bovine articular cartilage cultured with interleukin 1. *J Clin Invest* 1989;83:647–61.
- [10] Lee YM, Fujikado N, Manaka H, Yasuda H, Iwakura Y. IL-1 plays an important role in the bone metabolism under physiological conditions. *Int Immunol* 2010;22:805–16.
- [11] Lisignoli G, Toneguzzi S, Pozzi C, Piacentini A, Riccio M, Ferruzzi A, et al. Proinflammatory cytokines and chemokine production and expression by human osteoblasts isolated from patients with rheumatoid arthritis and osteoarthritis. *J Rheumatol* 1999;26:791–9.
- [12] Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997;336:1066–71.
- [13] Alcaraz MJ, Fernandez P, Guillen MI. Anti-inflammatory actions of the heme oxygenase-1 pathway. *Curr Pharm Des* 2003;9:2541–51.
- [14] Guillen MI, Megias J, Gomar F, Alcaraz MJ. Heme oxygenase-1 regulates catabolic and anabolic processes in osteoarthritic chondrocytes. *J Pathol* 2008;214:515–22.
- [15] Garcia-Arnanis I, Guillen MI, Castejon MA, Gomar F, Alcaraz MJ. Heme oxygenase-1 down-regulates high mobility group box 1 and matrix metalloproteinases in osteoarthritic synoviocytes. *Rheumatology* 2010;49:854–61.
- [16] Rawlinson SC, Zaman G, Mosley JR, Pittsillides AA, Lanyon LE. Heme oxygenase isozymes in bone: induction of HO-1 mRNA following physiological levels of mechanical loading in vivo. *Bone* 1998;23:433–6.
- [17] Chae HJ, Chin HY, Lee GY, Park HR, Yang SK, Chung HT, et al. Carbon monoxide and nitric oxide protect against tumor necrosis factor-alpha-induced apoptosis in osteoblasts: HO-1 is necessary to mediate the protection. *Clin Chim Acta* 2006;365:270–8.
- [18] Barbagallo I, Vanella A, Peterson SJ, Kim DH, Tibullo D, Giallongo C, et al. Overexpression of heme oxygenase-1 increases human osteoblast stem cell differentiation. *J Bone Miner Metab* 2010;28:276–88.
- [19] Kook YA, Lee SK, Son DH, Kim Y, Kang KH, Cho JH, et al. Effects of substance P on osteoblastic differentiation and heme oxygenase-1 in human periodontal ligament cells. *Cell Biol Int* 2009;33:424–8.
- [20] Zwerina J, Tzima S, Hayer S, Redlich K, Hoffmann O, Hanslik-Schnabel B, et al. Heme oxygenase 1 (HO-1) regulates osteoclastogenesis and bone resorption. *FASEB J* 2005;19:2011–3.
- [21] Lin TH, Tang CH, Hung SY, Liu SH, Lin YM, Fu WM, et al. Upregulation of heme oxygenase-1 inhibits the maturation and mineralization of osteoblasts. *J Cell Physiol* 2010;222:757–68.
- [22] Westacott CI, Webb GR, Warnock MG, Sims JV, Elson CJ. Alteration of cartilage metabolism by cells from osteoarthritic bone. *Arthritis Rheum* 1997;40:1282–91.
- [23] Pradel W, Mai R, Gedrange T, Lauer G. Cell passage and composition of culture medium effects proliferation and differentiation of human osteoblast-like cells from facial bone. *J Physiol Pharmacol* 2008;59(Suppl. 5):47–58.
- [24] Megias J, Guillen MI, Clerigues V, Rojo AI, Cuadrado A, Castejon MA, et al. Heme oxygenase-1 induction modulates microsomal prostaglandin H synthase-1 expression and prostaglandin E(2) production in osteoarthritic chondrocytes. *Biochem Pharmacol* 2009;77:1806–13.
- [25] Sanchez C, Deberg MA, Bellahcnee A, Castronovo V, Msika P, Delcour JP, et al. Phenotypic characterization of osteoblasts from the sclerotic zones of osteoarthritic subchondral bone. *Arthritis Rheum* 2008;58:442–55.
- [26] Rojo AI, Sagarra MR, Cuadrado A. GSK-3beta down-regulates the transcription factor Nrf2 after oxidant damage: relevance to exposure of neuronal cells to oxidative stress. *J Neurochem* 2008;105:192–202.

- [27] Mietz H, Esser JM, Welsandt G, Kociok N, Hueber A, Jousseaume A, et al. Latanoprost stimulates secretion of matrix metalloproteinases in tenon fibroblasts both in vitro and in vivo. *Invest Ophthalmol Vis Sci* 2003;44:5182–8.
- [28] Kasai K, Banba N, Hishinuma A, Matsumura M, Kakishita H, Matsumura M, et al. 15-Deoxy-Delta(12,14)-prostaglandin J(2) facilitates thyroglobulin production by cultured human thyrocytes. *Am J Physiol Cell Physiol* 2000;279:C1859–6.
- [29] Daouti S, Latario B, Nagulapalli S, Buxton F, Uziel-Fusi S, Chirn GW, et al. Development of comprehensive functional genomic screens to identify novel mediators of osteoarthritis. *Osteoarthritis Cartilage* 2005;13:508–18.
- [30] Gossel M, Modder UI, Atkinson EJ, Lerman A, Khosla S. Osteocalcin expression by circulating endothelial progenitor cells in patients with coronary atherosclerosis. *J Am Coll Cardiol* 2008;52:1314–25.
- [31] Tat SK, Pelletier JP, Verges J, Lajeunesse D, Montell E, Fahmi H, et al. Chondroitin and glucosamine sulfate in combination decrease the pro-resorptive properties of human osteoarthritis subchondral bone osteoblasts: a basic science study. *Arthritis Res Ther* 2007;9:R117.
- [32] Li X, Afif H, Cheng S, Martel-Pelletier J, Pelletier JP, Ranger P, et al. Expression and regulation of microsomal prostaglandin E synthase-1 in human osteoarthritic cartilage and chondrocytes. *J Rheumatol* 2005;32:887–95.
- [33] Lisignoli G, Toneguzzi S, Piacentini A, Cristino S, Grassi F, Cavallo C, et al. CXCL12 (SDF-1) and CXCL13 (BCA-1) chemokines significantly induce proliferation and collagen type I expression in osteoblasts from osteoarthritic patients. *J Cell Physiol* 2006;206:78–85.
- [34] Sakao K, Takahashi KA, Mazda O, Arai Y, Tonomura H, Inoue A, et al. Enhanced expression of interleukin-6, matrix metalloproteinase-13, and receptor activator of NF-kappaB ligand in cells derived from osteoarthritic subchondral bone. *J Orthop Sci* 2008;13:202–10.
- [35] Lacey DC, Simmons PJ, Graves SE, Hamilton JA. Proinflammatory cytokines inhibit osteogenic differentiation from stem cells: implications for bone repair during inflammation. *Osteoarthritis Cartilage* 2009;17:735–42.
- [36] Lisignoli G, Piacentini A, Toneguzzi S, Grassi F, Cocchini B, Ferruzzi A, et al. Osteoblasts and stromal cells isolated from femora in rheumatoid arthritis (RA) and osteoarthritis (OA) patients express IL-11, leukaemia inhibitory factor and oncostatin M. *Clin Exp Immunol* 2000;119:346–53.
- [37] Gowen M, Chapman K, Littlewood A, Hughes D, Evans D, Russell G. Production of tumor necrosis factor by human osteoblasts is modulated by other cytokines, but not by osteotropic hormones. *Endocrinology* 1990;126:1250–5.
- [38] Kim CH, Kang BS, Lee TK, Park WH, Kim JK, Park YG, et al. IL-1beta regulates cellular proliferation, prostaglandin E2 synthesis, plasminogen activator activity, osteocalcin production, and bone resorptive activity of the mouse calvarial bone cells. *Immunopharmacol Immunotoxicol* 2002;24:395–407.
- [39] Wada T, Nakashima T, Hiroshi N, Penninger JM. RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med* 2006;12:17–25.
- [40] Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch Biochem Biophys* 2008;473:139–46.
- [41] Dai SM, Shan ZZ, Nakamura H, Masuko-Hongo K, Kato T, Nishioka K, et al. Catabolic stress induces features of chondrocyte senescence through overexpression of caveolin 1: possible involvement of caveolin 1-induced down-regulation of articular chondrocytes in the pathogenesis of osteoarthritis. *Arthritis Rheum* 2006;54:818–31.
- [42] Chenoufi HL, Diamant M, Rieneck K, Lund B, Stein GS, Lian JB. Increased mRNA expression and protein secretion of interleukin-6 in primary human osteoblasts differentiated in vitro from rheumatoid and osteoarthritic bone. *J Cell Biochem* 2001;81:666–78.
- [43] Manolagas SC. Role of cytokines in bone resorption. *Bone* 1995;17:63S–7S.
- [44] Tsangari H, Findlay DM, Zannettino AC, Pan B, Kuliwaba JS, Fazzalari NL. Evidence for reduced bone formation surface relative to bone resorption surface in female femoral fragility fracture patients. *Bone* 2006;39:1226–35.
- [45] Okada Y, Lorenzo JA, Freeman AM, Tomita M, Morham SG, Raisz LG, et al. Prostaglandin G/H synthase-2 is required for maximal formation of osteoclast-like cells in culture. *J Clin Invest* 2000;105:823–32.
- [46] Blackwell KA, Hortschansky P, Sanovic S, Choudhary S, Raisz LG, Pilbeam CC. Bone morphogenetic protein 2 enhances PGE(2)-stimulated osteoclast formation in murine bone marrow cultures. *Prostaglandins Other Lipid Mediat* 2009;90:76–80.
- [47] Gao Q, Xu M, Alander CB, Choudhary S, Pilbeam CC, Raisz LG. Effects of prostaglandin E2 on bone in mice in vivo. *Prostaglandins Other Lipid Mediat* 2009;89:20–5.
- [48] Suzawa T, Miyaura C, Inada M, Maruyama T, Sugimoto Y, Ushikubi F, et al. The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: an analysis using specific agonists for the respective EPs. *Endocrinology* 2000;141:1554–9.
- [49] Yoshida K, Oida H, Kobayashi T, Maruyama T, Tanaka M, Katayama T, et al. Stimulation of bone formation and prevention of bone loss by prostaglandin E EP4 receptor activation. *Proc Natl Acad Sci USA* 2002;99:4580–5.
- [50] Akatsu T, Takahashi N, Udagawa N, Imamura K, Yamaguchi A, Sato K, et al. Role of prostaglandins in interleukin-1-induced bone resorption in mice in vitro. *J Bone Miner Res* 1991;6:183–9.
- [51] Lader CS, Flanagan AM. Prostaglandin E2, interleukin 1alpha, and tumor necrosis factor-alpha increase human osteoclast formation and bone resorption in vitro. *Endocrinology* 1998;139:3157–64.
- [52] Massicotte F, Lajeunesse D, Benderdour M, Pelletier JP, Hilal G, Duval N, et al. Can altered production of interleukin-1beta, interleukin-6, transforming growth factor-beta and prostaglandin E(2) by isolated human subchondral osteoblasts identify two subgroups of osteoarthritic patients. *Osteoarthritis Cartilage* 2002;10:491–500.
- [53] Liu XH, Kirschenbaum A, Yao S, Levine AC. Cross-talk between the interleukin-6 and prostaglandin E(2) signaling systems results in enhancement of osteoclastogenesis through effects on the osteoprotegerin/receptor activator of nuclear factor-kappaB (RANK) ligand/RANK system. *Endocrinology* 2005;146:1991–8.
- [54] Kim CH, Park YG, Noh SH, Kim YK. PGE2 induces the gene expression of bone matrix metalloproteinase-1 in mouse osteoblasts by cAMP-PKA signaling pathway. *Int J Biochem Cell Biol* 2005;37:375–85.
- [55] Mosig RA, Dowling O, DiFeo A, Ramirez MC, Parker IC, Abe E, et al. Loss of MMP-2 disrupts skeletal and craniofacial development and results in decreased bone mineralization, joint erosion and defects in osteoblast and osteoclast growth. *Hum Mol Genet* 2007;16:1113–23.
- [56] Kang BS, Park YG, Cho JY, Kim JK, Lee TK, Kim DW, et al. Interleukin-1 and tumor necrosis factor-alpha induce collagenolysis and bone resorption by regulation of matrix metalloproteinase-2 in mouse calvarial bone cells. *Immunopharmacol Immunotoxicol* 2003;25:347–64.
- [57] Lavigne P, Shi Q, Lajeunesse D, Dehnade F, Fernandes JC. Metabolic activity of osteoblasts retrieved from osteoarthritic patients after stimulation with mediators involved in periprosthetic loosening. *Bone* 2004;34:478–86.