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Expression of heme oxygenase-1 and regulation by cytokines in human osteoarthritic chondrocytes

Patricia Fernández[a], Maria Isabel Guillén[b], Francisco Gomar[c], Maria José Alcaraz[a,*]

[a]*Departamento de Farmacología, Facultad de Farmacia, Universidad de Valencia, Avda. Vicent Andrés Estellés s/n, Burjassot, 46100 Valencia, Spain*
[b]*Departamento de Química, Bioquímica y Biología Molecular, Universidad Cardenal Herrera CEU, Facultad de Ciencias Experimentales y de la Salud, Moncada, 46100 Valencia, Spain*
[c]*Departamento de Cirugía, Facultad de Medicina, Universidad de Valencia, Avda. Blasco Ibáñez, 15, 46010 Valencia, Spain*

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Abstract

Heme oxygenase-1 (HO-1) is implicated in the protection against tissue injury. We investigated the expression of this protein in cartilage sections and chondrocytes obtained from osteoarthritic patients. HO-1 was immunodetected in preparations from cartilage and also in chondrocytes cultured in the absence of stimulation. We found that HO-1 can be modulated by cytokines since the pro-inflammatory cytokines interleukin (IL)-1β, IL-17 and tumour necrosis factor-α (TNF-α) down-regulated this protein, whereas the anti-inflammatory cytokine IL-10 exerted the opposite effect. Our results suggest a role for HO-1 as part of protective mechanisms against tissue injury in human cartilage.

Keywords: Heme oxygenase-1; Human cartilage; Osteoarthritis; Chondrocyte; Cytokines; Nitric oxide

1. Introduction

Articular cartilage destruction is a key pathological characteristic of OA. Matrix turnover is solely dependent on chondrocytes, which are believed to be the main site of production of inflammatory mediators in human OA [1]. Pro-inflammatory cytokines up-regulate catabolic pathways inducing damage in the arthritic joint and particularly IL-1β is considered to be the main inducer of mediators causing cartilage degradation in rheumatoid arthritis and OA (reviewed in [2]). Thus, this cytokine can be considered as part of an autocrine pathway to modulate endogenous NO, PGE_2 and IL-6, which facilitate cartilage degradation and inhibit cartilage repair [3]. Other pro-inflammatory cytokines synergize with IL-1β to promote cartilage degradation. Up-regulation of TNF-α converting enzyme and increased TNF-α production are features of human OA-affected cartilage, where this cytokine may play a provokal role [4]. In addition, IL-17 stimulates proteoglycan and collagen II release and can be a potent upstream mediator of collagen breakdown in inflammatory joint diseases [5].

HO-1 is induced by a variety of stimuli causing cellular stress, including cytokines, mitogens, metals, reactive oxygen species, heat shock, radiations, hypoxia or hyperoxia. Several lines of evidence support that HO-1 is induced as an adaptative mechanism against injury elicited by these factors (reviewed in [6]). Thus, it has been a focus of interest to elucidate the regulation and function of this protein in human diseases.

It is well recognized that human osteoarthritic chondrocytes express NOS-2 and COX-2 and that these enzymes are up-regulated in response to pro-inflammatory cytokines. Nevertheless, possible protective mechanisms in cartilage are largely unknown and particularly the role of HO-1 has not been investigated. We have found that human chondrocytes express HO-1 and this protein

* Corresponding author. Tel.: +34-96-3544292; fax: +34-96-3544292.
E-mail address: maria.j.alcaraz@uv.es (M.J. Alcaraz).

Abbreviations: COX-2, cyclo-oxygenase-2; HO-1, heme oxygenase-1; IL, interleukin; NO, nitric oxide; NOS-2, nitric oxide synthase-2; OA, osteoarthritis; PGE_2, prostaglandin E_2; TNF-α, tumour necrosis factor-α.

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is subjected to regulation by pro- and anti-inflammatory cytokines.

2. Materials and methods

2.1. Cartilage samples and chondrocyte culture

Cartilage specimens were obtained from 10 patients with the diagnosis of advanced OA (10 females, 2 males, aged 71 ± 6 years, mean ± SEM) undergoing total knee joint replacement. Diagnosis was based on clinical, laboratory and radiological evaluation. Cartilage slices were removed from the femoral condyles and tibial plateaus and cut into small pieces. Chondrocytes were isolated by sequential enzymatic digestion: 1 hr with 0.1 mg/mL hyaluronidase (Sigma) followed by 18 hr with 2 mg/mL collagenase (type IA) (Sigma) at 37° in DMEM/Ham's F-12 (Sigma) containing penicillin (100 U/mL) and streptomycin (100 μg/mL). 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 5×10^5 cells/well in six-well plates. Cells were cultured in DMEM/Ham's F-12 supplemented with 10% human serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) in a humidified 5% CO_2 incubator at 37°. Chondrocytes were allowed to grow until nearly confluence and then incubated with IL-1β (100 or 200 U/mL), IL-17 (10 or 20 ng/mL), IL-10 (10 or 20 ng/mL), TNF-α (100 or 200 U/mL) (Biocarta) or the NO donor sodium nitroprussiate (SNP, 100 μM) (Sigma) for different times (24, 48 and 72 hr). Supernatants were collected, centrifuged and stored frozen at −70° until analysis. Cell lysates were obtained with lysis buffer (1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl, and 25 mM Tris, pH 7.4) and used for Western blot analysis. All experiments were performed with chondrocytes in primary culture. Nitrite, as index of nitric oxide production, and PGE_2 levels were determined in culture supernatants by a fluorometric method [7] and by radioimmunoassay [8], respectively.

2.2. Western blotting

HO-1, NOS-2 and COX-2 protein expression was studied in chondrocyte lysates. Equal amounts of protein, measured by the DC Bio-Rad protein reagent assay, were electrophoresed on a 12.5% polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Amersham Biosciences) for 90 min at 125 mA. Membranes were blocked with 3% (w/v) unfatted milk in 0.02 M phosphate-buffered saline (PBS), pH 7.0–0.1% Tween-20, and sequentially incubated with antibodies against HO-1 [9] (1/1,000), NOS-2 or COX-2 (1/1,000, Cayman Chem.) and peroxidase-conjugated goat anti-rabbit IgG (1/20,000, Dako). The immunoreactive bands were visualized using an enhanced chemiluminescence system (Amersham Biosciences).

2.3. Immunohistochemistry

To evaluate HO-1 expression in intact cartilage we cut full-thickness pieces of tissue from cartilage of four OA patients. A cartilage sample from a healthy multiorgan donor (male, 39 years) was also analyzed. The pieces were washed with PBS, fixed in 10% buffered formalin for 24 hr at 4°, embedded in paraffin and cross-sectioned (3-μm thick). After deparaffinization, the slides were treated for 5 min with 3% H_2O_2 to inhibit endogenous peroxidases and incubated for 30 min with 5% normal goat serum in PTB buffer (0.02 M PBS, pH 7.0, 0.1% Tween-20, 2% bovine serum albumin and 0.1% sodium azide) to block non-specific binding. HO-1 polyclonal antibody (1/50) in PTB containing 1.5% normal goat serum was incubated for 60 min at 37°. After washing, a peroxidase-conjugated goat anti-rabbit IgG (1/200) in PTB containing 1.5% normal goat serum was incubated for 30 min at 37°. 3,3′-Diaminobenzidine tetrahydrochloride (Sigma) was used as substrate to detect HO-1 positive cells. Negative staining control experiments were performed according to the above-described protocol, with omission of the primary antibody.

2.4. Statistical analysis

The results are presented as mean ± SEM; N represents the number of patients. The level of statistical significance was determined by ANOVA followed by Dunnett's *t*-test for multiple comparisons.

3. Results and discussion

HO-1 protein expression was observed in chondrocytes cultured without any stimulus for 24, 48 or 72 hr, as shown by Western blot experiments (Fig. 1). We have used the NO donor SNP as a positive control, which strongly induced this protein. Incubation of chondrocytes with pro-inflammatory cytokines IL-1β, IL-17 or TNF-α caused the attenuation of basal HO-1 expression at the three incubation times. Interestingly, the anti-inflammatory cytokine IL-10 enhanced HO-1 expression at 24 and 48 hr. Modulation of HO-1 expression was confirmed using two different concentrations of cytokines in 24 hr cultures (Fig. 2). Among the mediators playing a role in OA, NO may be responsible for many of IL-1β effects on cartilage. In this condition, an increased expression of NOS-2 and NO production by chondrocytes can be observed, with activation of metalloproteinases, induction of chondrocyte apoptosis, inhibition of collagen and proteoglycan synthesis, up-regulation of cytokines, etc. (reviewed in [10]). In our experiments, a high level of NOS-2 and COX-2 expression

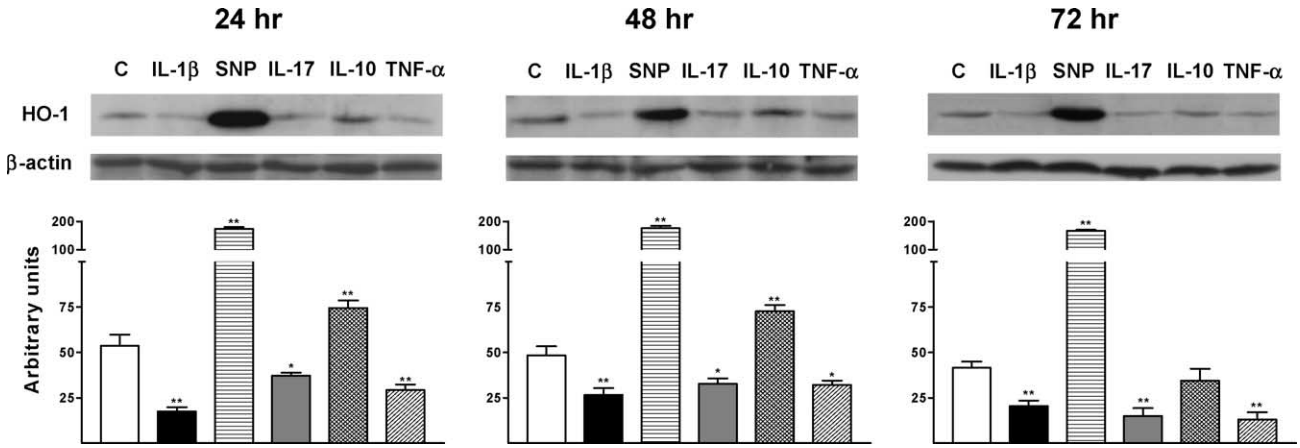

Fig. 1. Time course of HO-1 protein expression in human osteoarthritic chondrocytes. Cells were cultured for 24, 48 or 72 hr in the presence or absence of IL-1β (100 U/mL), IL-17 (10 ng/mL), IL-10 (10 ng/mL), TNF-α (100 U/mL) or SNP (100 μM). Lower panel shows relative densitometric intensities. Data are expressed as means ± SEM (N = 5 patients). $^{*}P < 0.05$, $^{**}P < 0.01$ with respect to control (C).

was achieved by IL-1β at the same concentrations able to modulate HO-1 (Fig. 3A and B), whereas IL-17 and TNF-α induced both proteins to a lower extent and with a higher effect on NOS-2. In contrast, IL-10 treatment did not induce these enzymes. Protein expression after cytokine treatment was paralleled by production of the corresponding metabolites, NO (measured as nitrite) and PGE_2. IL-1β treatment of chondrocytes resulted in the highest nitrite and PGE_2 levels in comparison with the other pro-inflammatory cytokines, IL-17 and TNF-α, whereas IL-10 did not stimulate the production of these inflammatory mediators.

Our study has also shown that HO-1 is present in cartilage sections from osteoarthritic patients (Fig. 4A and B), indicating that this protein is not the result of cellular stress during chondrocyte isolation and culture. HO-1 has also been detected in cartilage from a healthy donor (Fig. 4C), although a comparison is not possible due to the differences in type of cartilage, age, etc. Further studies would be needed to determine if there is a relationship between HO-1 expression and the evolution of OA.

It is known that exogenous NO released by NO donors or endogenous NO produced by inflammatory stimuli induce HO-1 expression in a number of cell types [9,11,12]. In human OA chondrocytes we have confirmed the induction

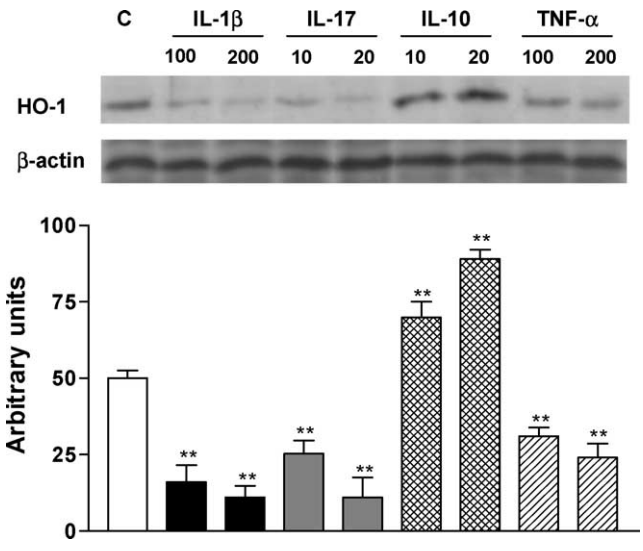

Fig. 2. Modulation of HO-1 protein expression by cytokines in human osteoarthritic chondrocytes. Cells were cultured for 24 hr in the presence or absence of IL-1β (100 and 200 U/mL), IL-17 (10 and 20 ng/mL), IL-10 (10 and 20 ng/mL) and TNF-α (100 and 200 U/mL). Lower panel shows relative densitometric intensities. Data are expressed as means ± SEM (N = 3 patients). $^{**}P < 0.01$ with respect to control (C).

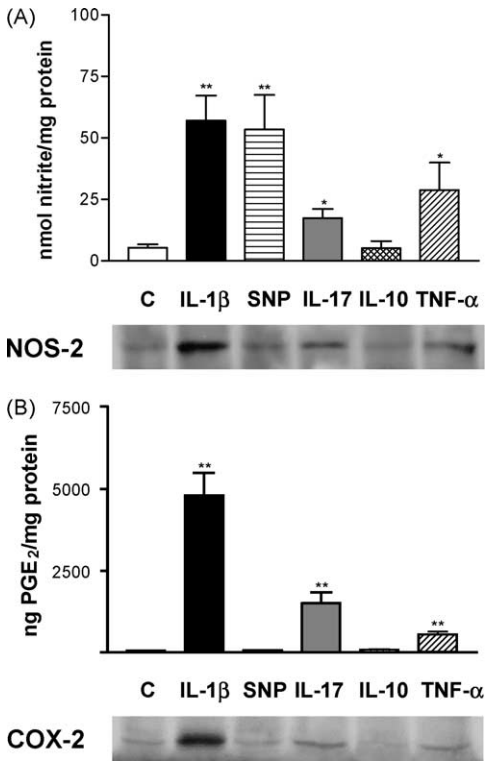

Fig. 3. Production of mediators and NOS-2 and COX-2 protein expression in human osteoarthritic chondrocytes. Cells were cultured for 24 hr in the presence or absence of IL-1β (100 U/mL), IL-17 (10 ng/mL), IL-10 (10 ng/mL), TNF-α (100 U/mL) or SNP (100 μM). Results are the mean ± SEM (N = 5). $^{*}P < 0.05$, $^{**}P < 0.01$ with respect to control (C).

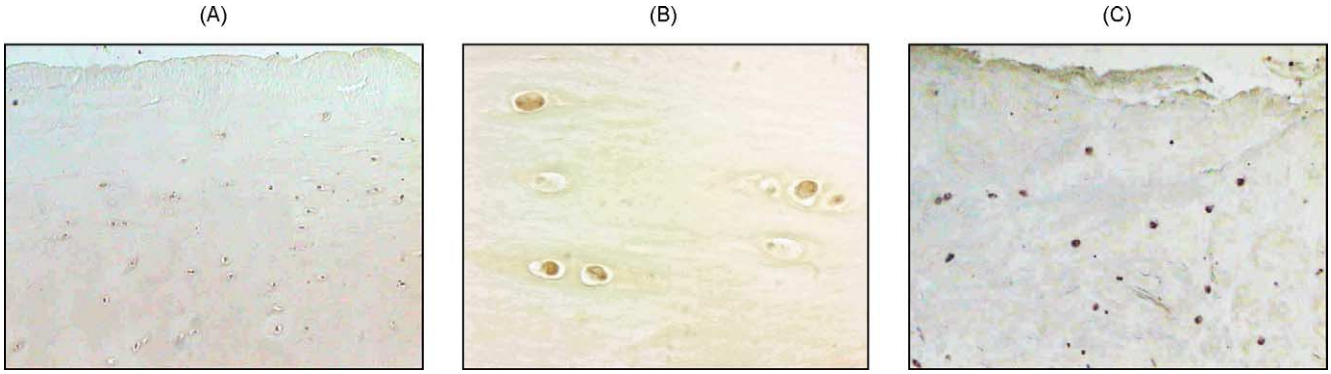

Fig. 4. Immunohistochemical analysis of HO-1 expression in human cartilage. Representative photomicrographs of cartilage sections immunostained using a polyclonal anti-HO-1 antibody. (A) OA cartilage, magnification 100×. (B) OA cartilage, magnification 600×. Three experiments were performed with tissue from four different patients. (C) Transversal section of meniscus from a healthy multiorgan donor.

of this protein by the NO donor SNP, however the high levels of NO generated by incubation with IL-1β did not cause an increase in HO-1 expression. In fact, we have observed the down-regulation of HO-1 by pro-inflammatory cytokines that are relevant in the pathogenesis of OA, indicating differences with respect to other cell systems where HO-1 induction by IL-1β and TNF-α has been reported [13].

On the other hand, a critical function of IL-10 is to limit inflammatory responses [14]. This cytokine inhibits IL-1β and TNF-α expression and is present in OA chondrocytes, where it may counteract their catabolic effects [15]. HO-1 induction may mediate the protective effects of IL-10 in osteoarthritic chondrocytes. To this respect, it is known that HO-1 inhibits IL-1β and TNF-α production, resulting in the control of experimental inflammation [16]. Our data suggest a role for HO-1 in the defence systems of human articular cartilage.

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