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Effects of rhein on human articular chondrocytes in alginate beads

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Abstract

This study was designed to investigate the effects of rhein, the active metabolite of diacerhein, on the metabolic functions of human chondrocytes cultured in alginate beads.

Enzymatically isolated osteoarthritic (OA) chondrocytes were cultured in alginate beads in a well-defined culture medium for 12 days. Rhein was tested in a range of concentrations comprised between 10^{-7} and 4×10^{-5} M, in the presence or absence of 10^{-10} M IL-1β. Interleukin (IL)-6 and -8, macrophage inflammatory protein (MIP-1β), stromelysin-1 (MMP-3), aggrecan (AGG), tissue inhibitor of metalloproteinases-1 (TIMP-1), prostaglandin E_2 (PGE_2) and nitric oxide (NO) productions were assayed. Cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) mRNA steady-state levels were also quantified. In the basal condition, 10^{-5} M rhein increased by 46.5% the production of AGG, decreased by 17–30% the production of IL-6, MMP-3, NO and MIP-1β but enhanced by 50% the production of PGE_2. IL-1β increased IL-6, IL-8, MIP-1β, NO, PGE_2 and MMP-3 productions, but inhibited AGG and TIMP-1 synthesis. Rhein partially reversed the effect of IL-1β on TIMP-1 and NO production, had no effect on AGG, IL-6 and MIP-1β production, but up-regulated the IL-1β stimulated PGE_2 production. The COX-2 and iNOS mRNA levels and IL-8 production were not modified by rhein.

Overall, these results contribute to explain the clinical efficiency of rhein and give new information on its mechanisms of action.

Keywords: Cartilage; Chondrocytes; Cytokines; Osteoarthritis; Rhein

1. Introduction

Osteoarthritis (OA) is a complex disease characterized by bone remodelling, synovium inflammation and cartilage loss. The mechanisms that lead to cartilage degradation primarily involve an excessive production of matrix metalloproteinases (MMPs), including collagenases and stromelysins [1]. Their levels are found to be elevated in the synovial fluid and serum of OA patients. Chondrocytes, but also synovium cells, largely contribute to this enhancement, by secreting high levels of MMPs in response to cytokines, primarily IL-1β, TNFα and oncostatin M. These cytokines act synergically, not only to enhance pro-MMPs secretion, but also to modulate the activation/inhibition system, leading to an increase of the proteolytic activity in cartilage [2]. Among these cytokines, IL-1β is considered as the most active in OA. *In vitro*, IL-1β modifies the normal metabolic functions of chondrocytes, and provokes an imbalance between the catabolic and anabolic events, leading to an excess of cartilage resorption [2,3].

The main objectives in the management of OA are to reduce symptoms, to minimize functional disability and to limit disease progression. Until now, several drugs used in the treatment of OA have been demonstrated to be

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Abbreviations: ACL, anterior cruciate ligament; AGG, aggrecan; BSA, bovine serum albumin; CM, cell-associated matrix; COX, cyclooxygenase; DMEM, Dulbecco's Modified Eagle's Medium; DTT, dithiothreitol; EASIA, enzyme amplified sensitivity immunoassay; FRM, further removed matrix; GADPH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; iNOS, inductible nitric oxide synthase; LDH, lactate deshydrogenase; MIP, macrophage inflammatory protein; MMLV-RT, murine leukaemia virus reverse transcriptase; MMP, matrix metalloproteases; NAD, nicotinamide adenine dinucleotide; NO, nitric oxide; OA, osteoarthritis; PAI, plasminogen activator inhibitor; PCR, polymerase chain reaction; PGE_2, prostaglandin E_2; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteases; TNF, tumor necrosis factor; uPA, urokinase plasminogen activator.

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symptom-modifying drugs that induce symptomatic relief, but few of these drugs have been investigated for their effects on the structural changes of the tissue. Diacerhein is a symptom-modifying drug, which has been recently reported to possess structure-modifying effects [4]. However, little information is available about the mechanisms of action that sustain these effects. Diacerhein is a low molecular weight heterocyclic compound that is entirely transformed by desacetylation during the absorption process in rhein, the active metabolite found in plasma and synovial fluid [5,6].

In vitro, short-term treatment (72 hr) with high concentrations of rhein or diacerhein (3.33×10^{-5} and 6.66×10^{-5} M) down-regulated, at transcriptional and/or post-transcriptional levels, NO production by human OA chondrocytes in monolayer [7]. Further, rhein up-regulated IL-1β stimulated PGE_2 and COX-2 protein synthesis. After 6 days of incubation, synthesis of glycosaminoglycan (i.e. ^{35}S-suphate incorporation) and collagens (i.e. ^{3}H-proline labeling) by rabbit chondrocytes in monolayer culture was increased by 10^{-6} M diacerhein, whereas collagenolytic activity released into the culture medium was concentration-dependently decreased in the range of 10^{-7} to 10^{-4} M [8]. The positive effect of diacerhein on cartilage matrix formation was explained by its stimulating effect on TGF-β1 and TGF-β2 gene expression and by its inhibiting action on IL-1β secretion [9–14].

In an accelerated canine model of OA in which sensory input from the ipsilateral hind limb was interrupted by L4-S1 dorsal root ganglionectomy prior to anterior cruciate ligament (ACL) transection, diacerhein (15–20 mg/kg once daily for 8 weeks) did not significantly modify the severity of cartilage lesions, the collagenolytic activity and the proteoglycan level contained in OA cartilage [15]. In a less rapidly progressive canine model, in which OA was induced by ACL only, diacerhein (40 mg/kg for 8 months) has been shown to slow down the progression of cartilage lesions as estimated by an arthroscopic grading scale [16]. However, 32 weeks after ACL transection, the mean proteoglycan concentration of the OA cartilage and the level of collagenolytic activity in cartilage extracts were not significantly different in the diacerhein group compared to the placebo. In an ovine model of OA induced by menisectomy, diacerhein (25–50 mg/kg daily for 9 months) failed to significantly improve the gross morphological and histological severity of cartilage lesions [17].

In several randomized, double blind, placebo-controlled clinical trials of 2–6 duration months, diacerhein significantly reduced the pain and functional impairment in patients with hip or knee OA [18–22]. Recently, a 3-year, placebo-controlled trial (ECHODIAH) including 507 patients with primary OA of the hip, has demonstrated that the occurrence of radiographic progression (i.e. joint space narrowing measured by a graduated magnifying lens) of at least 0.5 mm during the study was significantly lower and occurred later in the diacerhein (100 mg daily) group as compared with the placebo group [4].

For the first time, this study investigated the 12 days effect of rhein on the metabolism of chondrocytes cultured in alginate beads in which, chondrocytes keep their phenotype stable overall the culture duration. This work provided new mechanisms of action of rhein, and contributed to explain the clinical observations.

2. Materials and methods

2.1. Chondrocytes culture in alginate beads

Cartilage was obtained from the knees (femoral condyles, tibial plateau and patellar cap) of cadavers immediately after death being excised from the superficial and medium layers of cartilage and avoiding the calcified layer. The experiments were performed with osteoarthritic chondrocytes isolated from cartilage specimens coming from six different donors (two males and four females) with a mean age of 48 (38–59) years.

Cartilage was cut into small fragments and then subjected to sequential enzymatic digestions with hyaluronidase, pronase and collagenase as described previously [23]. The cells were then filtered through a nylon mesh with a pore diameter of 70 μm, and then washed three times with sterile water 0.9% NaCl. Cells viability was estimated by trypan blue exclusion test and in all cases was superior to 95%. Chondrocytes were suspended in 1.2% low viscosity alginate (Sigma–Aldrich) in 0.155 M NaCl solution at a density of 4×10^6 cells/mL, which was slowly passed through a 25 gauges needle in a dropwise fashion into a 102 mM $CaCl_2$ solution (Sigma–Aldrich). After instantaneous gelation, the beads were allowed to polymerize further for 10 min in this solution. Thereafter, they were washed with a saline solution and nine beads containing each approximately 50,000 cells were cultured in 1 mL of culture medium per well in a 24-well plate. Culture medium was DMEM supplemented with 1% ITS+ (ICN Biomedicals), 10 mM HEPES (Biowhittaker Europe), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Biowhittaker Europe), 200 μg/mL glutamine (Biowhittaker Europe), 50 μg/mL ascorbic acid (Sigma–Aldrich), and 2 mM proline (Invitrogen). ITS+ is a premixed cell growth system containing in 1 mL: 0.625 mg insulin, 0.625 mg transferrin, 0.625 μg selenious acid, 0.125 g bovine serum albumin (BSA) and 0.535 mg linoleic acid. Cells were maintained in this culture medium (wash-out medium) for 48 hr as a precaution against *in vivo* contamination with drugs that donors might have taken before death. After this wash-out period, culture medium was changed and the drugs added. Culture medium was then changed every 3 days and the collected supernatants were kept at −20° until analysis. Chondrocytes were cultured in alginate beads for a period of 12 days following the wash-out.

At the end of the culture, alginate beads were dissolved in 1 mL 0.1 M citrate for 10 min, and centrifuged at 1200 *g* for 10 min. The supernatant containing macromolecules originated from the further removed matrix (FRM) compartment, and the pellet, containing cells with their associated matrix (CM), were then collected and separately analyzed. These two fractions were studied separately. The cell pellets were washed with PBS (Biowhittaker Europe), and then homogenized in 1 mL of PBS by ultrasonic dissociation (three pulses of 10 s, 50 W/cm^2) at 4° for DNA assay. CM and FRM were kept at −20° until analysis.

2.2. Treatments

Chondrocytes were cultured for 12 days in the absence or in presence of 10^{-10} M human IL-1β (Roche Pharmaceuticals) and with or without rhein (Negma Laboratories). Fresh IL-1β and rhein were added at each medium change every 3 days. Rhein was tested at concentrations ranging among 10^{-7} to 4×10^{-5} M, was first dissolved in NaOH 0.1 M, and then diluted in culture medium to achieve the required final concentration. After an oral administration of diacerhein in healthy volunteers, the peak concentration (C_{max}) of rhein found in the plasma was 10^{-5} M. Following daily administration of oral diacerhein 50 mg every 12 hr for 1 month, a pilot study conducted in three arthritic patients revealed that rhein could reach the synovial fluid at concentrations in a range (10^{-6} to 10^{-5} M) very close to that observed in plasma [5,6]. Chondrocytes coming from six different donors have been included. Four out the six have been used for the analysis of AGG, cytokines, PGE_2, MMP-3, TIMP-1 and NO synthesis and the two other for the measurement of COX-2 and iNOS mRNA levels. Three wells were used for each concentration of the drug and for the corresponding controls.

2.3. DNA assay

The DNA content was measured using a fluorimetric method [24]. This measurement ensures elimination of result variations caused by the different number of chondrocytes in each culture.

2.4. LDH release assay

The cell viability was estimated by the ratio of lactate deshydrogenase (LDH) released in the culture supernatant. In a 96-well plate, 50 μL of Tris 0.01 M buffer, pH 8.5, containing 0.1% BSA and lactate 800 mM and 50 μL of a solution 1.6 mg/mL INT (*p*-iodonitrotetrazolium violet, Sigma–Aldrich), 4 mg/mL NAD (Roche Pharmaceuticals) and 0.4 mg/mL phenazine methosulfate (Sigma–Aldrich) were added to 100 μL of culture supernatant, FRM or CM (before any freezing). The absorbance at 492 nm was read after 5 min of incubation at room temperature. The calibration was performed with LDH isolated from rabbit muscle. The cell death score was obtained by calculating the ratio: $100 - (100/(\text{LDH CM} + \text{FRM} + \text{supernatant}) \times \text{LDH CM})$.

2.5. Quantitative real-time RT–PCR

RNA was isolated by the Promega SV Total RNA Isolation System, after 72 hr of treatment. Cells (3×10^6) were lysed and the samples were processed following the manufacturer's protocol. RNA (2 μg) was reverse transcribed in a volume of 30 μL containing 1X RT buffer (50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT), 10 mM DTT, 1 mM dNTPs, 500 ng oligo(dT)15 primer, 140 U M-MLV reverse transcriptase and 40 U RNaseOUT (Invitrogen) for 60 min at 37°. The reaction was stopped by incubation at 95° for 5 min and by adding 100 μL of H_2O.

PCR reaction was performed using the LightCycler-FastStart DNA master Sybr Green I (Roche Diagnostics). This reaction mixture was provided as a 10-fold stock solution containing FastStart Taq DNA polymerase, reaction buffer, dNTP mix, 10 mM $MgCl_2$ and Sybr Green I dye. For each LightCycler PCR a mastermix of the following reaction components was prepared in a final volume of 18 μL: 2 μL LightCycler-FastStart DNA master Sybr Green I, 1.6 μL of 25 mM $MgCl_2$, 0.5 μM sense and antisense primers (GADPH sense TTGGTATCGTGGAAGGACTCA, antisense TGTCATCATATTTGGCAGGTTT; iNOS sense CCATGGAACATCCCAAATAC, antisense TCTGCATGTACTTCATGAAGG; COX-2 sense TTCAAATGAGATTGTGGGAAAA, antisense AGATCATCTCTGCCTGAGTATCTT).

The mastermix was loaded into glass capillary (Roche Diagnostics) and 2 μL of PCR template was added. The template source was either 3 ng first-strand cDNA or purified DNA standard.

Amplification was performed with a spectrofluorometric thermal cycler (LightCycler, Roche Diagnostics). After an initial denaturation step at 95° for 10 min, amplification was performed using 40 cycles of denaturation (95° for 15 s), annealing (temperature gradient: 68–58° with a decrement of 0.5° per cycle during 20 cycles, continued by 20 cycles at 58°, for 5 s) and extension (72° for 15 s). For each run, a standard curve was generated from purified DNA ranging from 10^6 to 10 copies of the cDNA of interest. To standardize mRNA levels, we amplified glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a house-keeping gene as internal control. Normalized gene expression was calculated as the ratio between sample and GAPDH cDNA copy number.

After amplification, a final melting curve was recorded by cooling the PCR mixture to 65° for 30 s and then slowly heating it to 95° at 0.1°/s. Fluorescence was measured continuously during the slow temperature rise to monitor the dissociation of the double strands DNA. Specificity of the expected COX-2 and iNOS products was demonstrated

by melting curves analysis. Amplification products performed in the LightCycler were checked by electrophoresis on 1.5% ethidium bromide stained agarose gel. The estimated size of the amplified fragments matched the calculated size.

2.6. Prostaglandin (PG) E_2 radioimmunoassay

PGE_2 was assayed in the different compartments (culture supernatants, FRM and CM) without previous extraction according to a previously described radioimmunoassay [25]. In this assay, polyclonal antiserum obtained from rabbit does not cross-react with other prostanoids (TxB_2, 6-keto-PGF1α, PGA_2) or fatty acids (arachidonic, linoleic or oleic acids). ^{3}H-Labeled PGE_2 was purchased from New England Nuclear and the standard molecule (PGE_2) from Sigma–Aldrich. Intra and interassay coefficients of variation were 6 and 10%, respectively. The limit of detection of the RIA was 20 pg/mL.

2.7. Immunoassays for AGG, IL-6, IL-8, MIP-1β, MMP-3 and TIMP-1

Aggrecans (AGG), interleukin (IL)-6, -8, macrophage inflammatory protein (MIP-1β), tissue inhibitor of metalloproteinases-1 (TIMP-1) and stromelysin-1 (MMP-3) were measured in the different culture compartments (culture supernatants, FRM and CM), by specific enzyme amplified sensitivity immunoassays (EASIAs, Biosource Europe). The assays were based on the oligonal system in which several monoclonal antibodies were directed against distinct epitopes of the molecules. In the case of MMP-3, the antibodies were directed against pro-MMP-3, activated-MMP-3 and MMP-3 bound to TIMP-1 and TIMP-2. The intra and interassay coefficients of variation were less than 5% for all immunoassays.

2.8. Nitric oxide (NO) assay

Nitrite and nitrate are stable end products of nitric oxide. Nitrate is reduced to nitrite by the addition in the supernatant of nitrate reductase (0.25 U/mL, Roche Pharmaceuticals) for 20 min at 37°. Nitrite concentrations in conditioned culture supernatants were determined by a spectrophotometric method based upon the Griess reaction [26]. The absorption was measured at 540 nm. Sodium nitrite ($NaNO_2$) was used for calibration.

2.9. Calculation and statistical analysis

The results (mean ± SEM) were expressed as the concentration of AGG, MMP-3, TIMP-1, IL-6, IL-8, MIP-1β, NO and PGE_2 in the culture supernatants, CM and FRM per microgram of DNA. The total production was obtained by the addition of the quantities found in the culture supernatant, in the CM and the FRM. The total quantities found in the CM and the FRM correspond to the cumulative amounts over 12 days. Mean values were compared for each culture condition using the *U*-test of Mann–Whitney with a limit of significance at $P < 0.05$. The concentration-dependency was demonstrated by calculating the Spearman's correlation coefficient (r).

3. Results

3.1. Effects of rhein on DNA content and LDH release

The content of DNA (about 3.5 μg per well) increased by 38% over the culture duration and was not affected by the presence of IL-1β (10^{-10} M) or rhein (10^{-7} to 2×10^{-5} M) (Table 1). In the basal conditions and in the presence of 10^{-10} M IL-1β, the cell death score, estimated by the cumulated amount of LDH released from the cells during 12 days of culture, was below 3%. At concentrations ranging between 10^{-7} and 10^{-5} M, rhein did not significantly affect the cell death score or the DNA content (Fig. 1). In the presence of IL-1β, 10^{-5} M of rhein slightly increased cell death score, but this score remained below 10%. However, at concentrations superior to 10^{-5} M, rhein showed a cytotoxicity characterized by a concentration-dependent release of LDH ($r = 0.985$, $P < 0.0001$ without IL-1β; $r = 0.993$, $P < 0.0001$ with IL-1β). A decrease of DNA content (Fig. 1 and Table 1) is also observed at the concentration of 4×10^{-5} M of rhein.

3.2. Effects of rhein on AGG production

In alginate culture model, AGG production was primarily immobilized in the alginate matrix. Seventy percent of the newly synthesized AGG were found in the FRM, 28% in the CM and only 2% in the supernatant. After 12 days of culture, rhein at the plasmatic concentration (C_{max}; 10^{-5} M) enhanced by 46.5% the total AGG production. This effect was concentration-dependent in the range of concentrations comprised between 10^{-7} and 3.33×10^{-6} M ($r = 0.94$, $P = 0.0165$), reached a maximum at 3.33×10^{-6} M, and then slightly decreased at the highest

Table 1
Content of DNA (μg) per well after a 12 days culture period, in presence or absence of 10^{-10} M IL-1β

	Basal	+ IL-1β
Control	3.78 ± 0.46	3.16 ± 0.60
10^{-7} M rhein	3.61 ± 0.37	3.12 ± 0.34
3.33×10^{-7} M rhein	3.63 ± 0.22	3.34 ± 0.17
10^{-6} M rhein	3.58 ± 0.19	3.65 ± 0.50
3.33×10^{-6} M rhein	3.72 ± 0.34	3.11 ± 0.12
10^{-5} M rhein	3.70 ± 0.10	3.08 ± 0.22
2×10^{-5} M rhein	3.45 ± 0.12	2.67 ± 0.48
4×10^{-5} M rhein	3.17 ± 0.23	2.23 ± 0.07*

* $P < 0.05$.

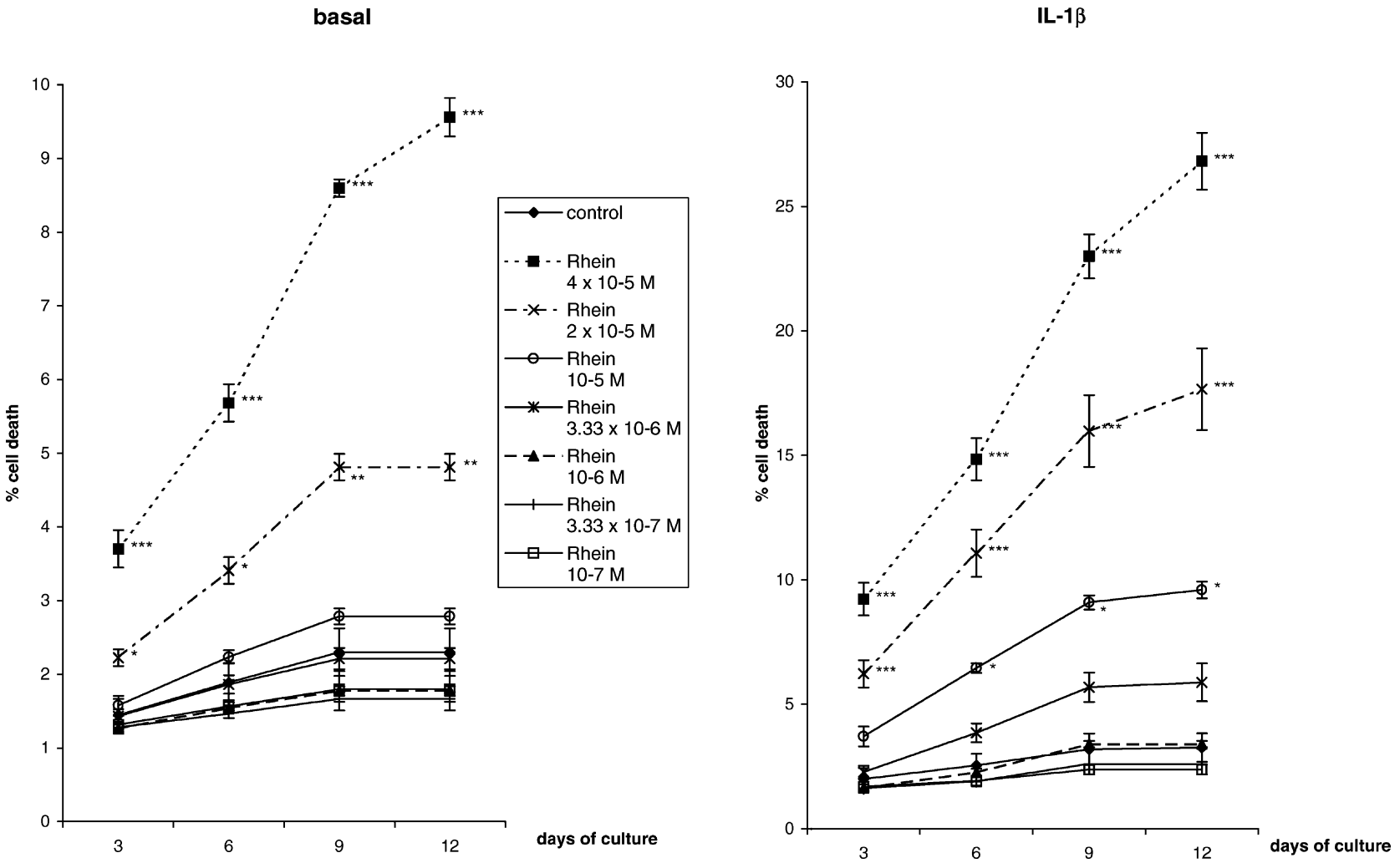

Fig. 1. Cumulative percentage (sum of the number of cell death at the end of a 3-day culture period) of cell death in alginate chondrocyte culture maintained for 12 days in the absence or in the presence of 10^{-10} M IL-1β and with or without increased concentrations of rhein (◆, control; ■, rhein 4×10^{-5} M; ×, rhein 2×10^{-5} M; ○, rhein 10^{-5} M; ✱, rhein 3.33×10^{-6} M; ▲, rhein 10^{-6} M; |, rhein 3.33×10^{-7} M; and □, rhein 10^{-7} M). Results are represented by the mean ± SEM of four independent cultures performed with chondrocytes coming from different donors. Here $*$ indicates $P < 0.05$, $**$ indicate $P < 0.01$, and $***$ indicate $P < 0.001$.

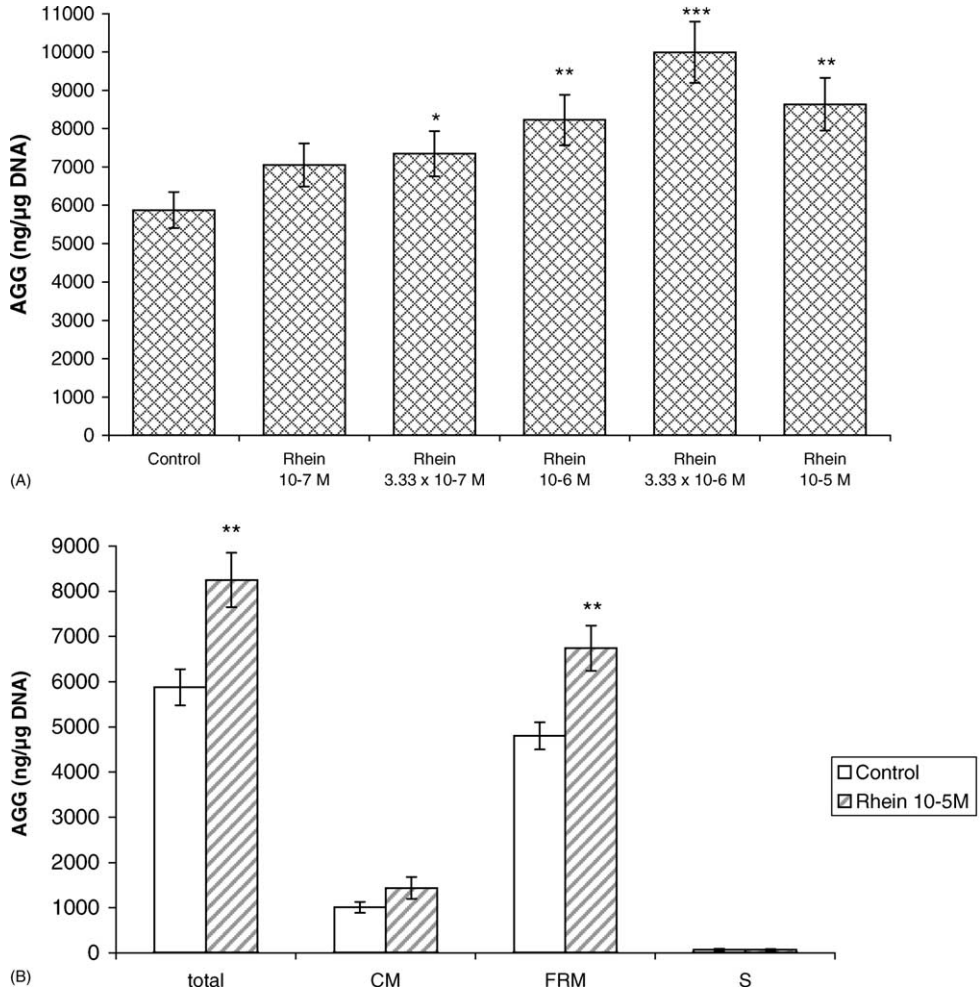

Fig. 2. (A) Effect of rhein on the total AGG production at the end of 12 days of culture under basal condition (in the absence of IL-1β). (B) Effect of 10^{-5} M rhein on the amount of AGG accumulated in the supernatant (S), the pericellular (CM) and further removed (FRM) matrix after 12 days of culture in alginate beads. Results are expressed as the percentage of the control, and represented by the mean ± SEM of four independent cultures performed with chondrocytes coming from different donors. Here ** indicate $P < 0.01$.

concentration (Fig. 2A). The effect of rhein was primarily observed in the FRM compartment (Fig. 2B). IL-1β drastically depressed AGG production (basal production 5877 ± 317 ng/µg DNA vs. IL-1 treated group 939 ± 46 ng/µg DNA). Rhein had no significant effect on the IL-1β inhibitory effect whatever the concentration tested (data not shown).

3.3. Effects of rhein on TIMP-1 and MMP-3 production

In the basal condition, human chondrocytes released 225 ± 3.5 ng/µg DNA/12 days of TIMP-1. When 10^{-10} M IL-1β was added to the culture medium, TIMP-1 production was reduced by 83%. At the concentrations tested, rhein did not modify the basal TIMP-1 production, but partially reversed the IL-1β inhibitory effect. In the presence of rhein 10^{-5} M, TIMP-1 production by IL-1 treated chondrocytes was doubled. The basal production of MMP-3 by human OA chondrocytes was 6349 ± 362 ng/µg DNA/12 days. At 10^{-5} M, rhein reduced by 17% the basal MMP-3 production. No significant effect was observed at the lowest concentrations of rhein. In alginate beads model, IL-1β increased MMP-3 production only during the first 3 days of treatment (551 ± 16 ng/µg DNA in basal and 1270 ± 126 ng/µg DNA with IL-1β), and after the IL-1β-stimulating effect disappeared. At day 3, rhein 10^{-5} M significantly decreased this IL-1β-simulated production by 34% ($P < 0.01$). Consequently, the presence of IL-1β profoundly increased the MMP-3/TIMP-1 ratio (43.5 compared to 12 in basal conditions, Fig. 3). Rhein (10^{-5} M) significantly decreased the MMP-3/TIMP-1 ratio in the basal conditions and in the presence of IL-1β (Fig. 3).

3.4. Effects of rhein on IL-6, IL-8 and MIP-1β production

In the basal condition, OA chondrocytes constitutively produced in average 10.3 ± 0.5 ng/µg DNA of IL-6, 26.7 ng/µg DNA of IL-8 and 81.4 ± 1.4 pg/µg DNA of MIP-1β. Most of the newly synthesized IL-6 and MIP-1β (96.7% for IL-6, and 96.3% for MIP-1β) was released in

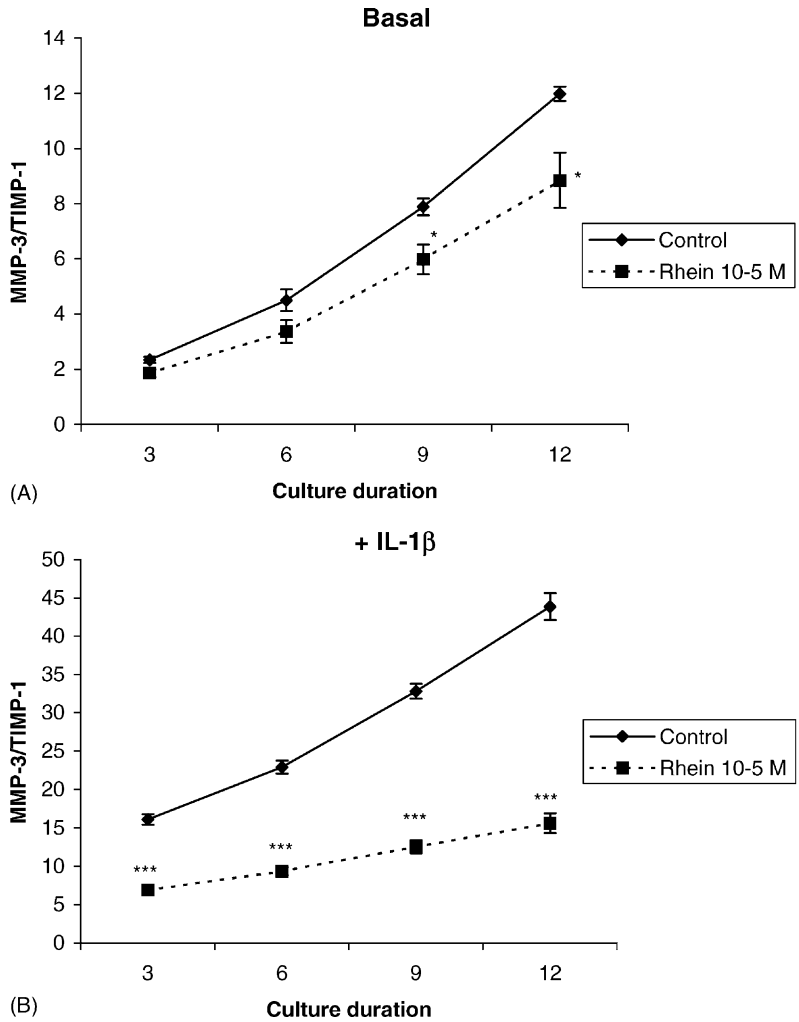

Fig. 3. Effect of 10^{-5} M rhein on MMP-3/TIMP-1 ratio obtained after 3, 6, 9 or 12 days of culture in the absence (A) or in the presence (B) of 10^{-10} M IL-1β. The MMP-3/TIMP-1 ratios were calculated on the base of cumulated amounts of MMP-3 and TIMP-1 produced by human chondrocytes after 3, 6, 9 or 12 days of culture in alginate bead. The values are the mean ± SEM of four independent cultures performed with chondrocytes coming from different donors. Here $*$ indicates $P < 0.05$ and $***$ indicate $P < 0.001$.

the culture supernatant whereas, 23% of the IL-8 produced was retained in the alginate beads. IL-1β stimulated IL-6, IL-8 and MIP-1β production by 13, 11 and 39 times, respectively. In the basal conditions, rhein significantly decreased IL-6 production at the concentration of 3.33×10^{-6} and 10^{-5} M ($r = -0.91$, $P = 0.0015$) (Fig. 4A) but had no effect on IL-8. MIP-1β production was also inhibited by rhein at concentrations ranging between 10^{-7} and 10^{-5} M (Fig. 4B). In the presence of IL-1β, rhein did not significantly modify IL-6, -8 and MIP-1β production.

3.5. Effects of rhein on PGE_2 production

In the basal condition, PGE_2 was produced in low levels (227 ± 16.9 pg/μg DNA), and was all released in the culture supernatants. This production was significantly enhanced by rhein at the concentration of 10^{-5} M (Fig. 5A). The stimulating effect of rhein on PGE_2 production was more marked and concentration-dependent in the presence of 10^{-10} M IL-1β ($r = 0.984$, $P = 0.0004$). At 10^{-5} M, rhein increased by three times the IL-1β stimulated PGE_2 production (Fig. 5B) but did not modulate COX-2 mRNA level (Fig. 6) suggesting that rhein regulated PGE_2 production at a post-transcriptional level.

3.6. Effects of rhein on NO production

In the basal condition, chondrocytes produced in mean 32 nmoles/μg DNA/12 days of NO_2/NO_3. IL-1β (10^{-10} M) increased by three times the basal NO_2/NO_3 production. In the absence or in the presence of IL-1, rhein decreased NO production in a concentration-dependent manner (without IL-1β, $r = -0.972$, $P = 0.0244$; with IL-1β, $r = -0.966$, $P = 0.0017$). At the concentration of 10^{-5} M, rhein reduced both basal and IL-1β stimulated production of NO by 50 and 45%, respectively (Fig. 7). Rhein did not affect iNOS mRNA level (data not shown).

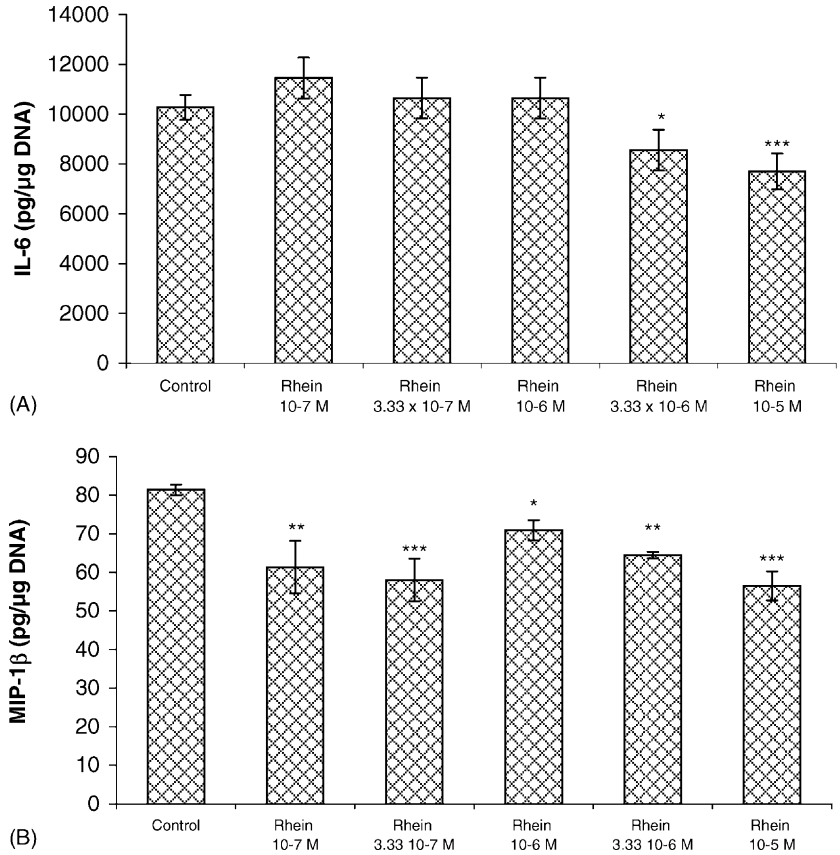

Fig. 4. Effects of increase in amounts of rhein on the basal production (in the absence of IL-1β) of IL-6 (A) and MIP-1β (B) by human OA chondrocytes. Results are represented by the mean ± SEM of four independent cultures performed with chondrocytes coming from different donors. Here ∗ indicates $P < 0.05$, ∗∗ indicate $P < 0.01$, and ∗∗∗ indicate $P < 0.001$.

4. Discussion

Until now, *in vitro* observations were limited to the 3 first days of treatment with rhein using human or animal chondrocytes cultured in monolayer. In this work, chondrocytes were cultured in alginate beads in order to keep stable their phenotype, for at least 12 days of culture [27]. The alginate bead model is highly recommended for studying cartilage matrix formation, because it allows the separate analysis of the pericellular and the interterritorial matrix (or further removed matrix). Interestingly, we have demonstrated that rhein strongly increased in a concentration-dependent manner the amount of aggrecan contained in the alginate bead, supporting the concept that rhein could promote cartilage matrix formation. As previously suggested by Felisaz *et al*. [9], this stimulating effect of rhein on aggrecan synthesis could be secondary to the over expression of TGF-β1 and -β2 by chondrocytes themselves. *In vitro*, TGF-βs enhance the expression of type II collagen and aggrecan by isolated chondrocytes as well as by cartilage explants [28,29]. In parallel, rhein depressed MMP-3 synthesis and increased TIMP-1 production by IL-1β treated chondrocytes suggesting that rhein could counteract some of the IL-1 effects on cartilage catabolism. These results corroborate the Tamura's study demonstrating that rhein suppressed the IL-1α-induced production of pro-MMPs-1, -3, -9 and -13 but increased TIMP-1 synthesis by rabbit chondrocytes in monolayer [30]. In parallel, Tamura and Ohmori [31] have shown that rhein decreased the total activity of MMPs in the culture supernatants and reduced proteoglycan degradation. Taken together, these observations suggest that rhein might have a protective effect on proteoglycan degradation *via* the suppressive effect of rhein on the production and on the activity of pro-MMPs. This duality led to a strong decrease of the MMP-3/TIMP-1 ratio showing that rhein can also counteract catabolic events. The antagonism of rhein towards IL-1 can be explained by its capacity to decrease the number of IL-1 receptors on OA chondrocytes [12]. Nevertheless, rhein was not efficient against the overall IL-1 effects suggesting that other more specific regulatory features are involved in the rhein activity. Since it was demonstrated that NO mediates the stimulatory effect of IL-1β on metalloproteinases synthesis [32,33], the inhibition of NO by rhein has been considered as another possible mechanism of action. As NO synthesis blockage by NO synthase inhibitors reduces the stimulating effect of IL-1 on metalloproteinases synthesis, it is probable that the effect of rhein on MMP-3 production results from the inhibition of NO synthesis. The inhibition of NO synthesis

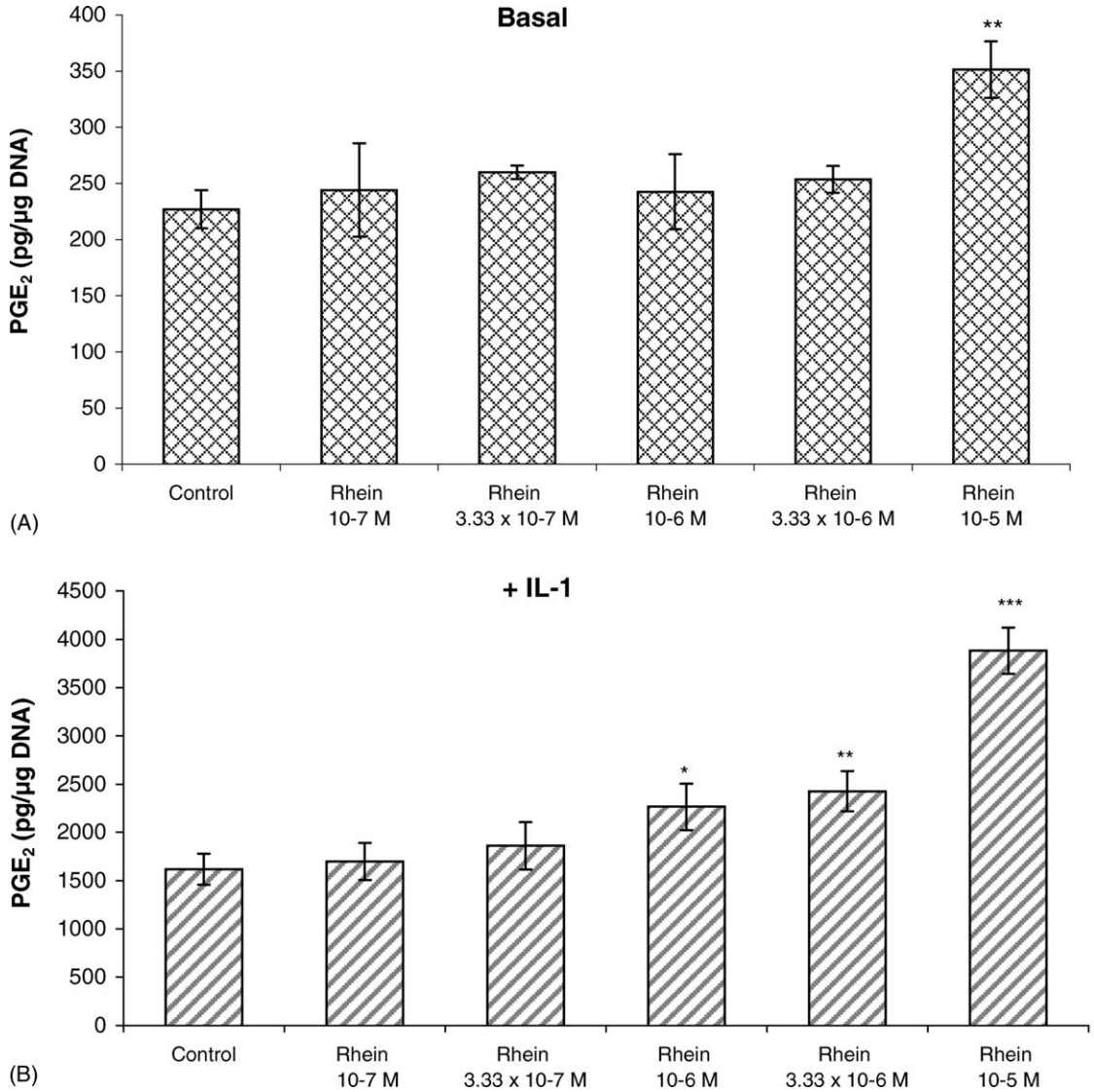

Fig. 5. Effect of rhein on the total production of PGE_2 by human OA chondrocytes cultured for 12 days in alginate beads with (B) or without (A) 10^{-10} M IL-1β. The results are represented by mean ± SEM of four independent cultures performed with chondrocytes coming from different donors. Here ∗ indicates $P < 0.05$, ∗∗ indicate $P < 0.01$, and ∗∗∗ indicate $P < 0.001$.

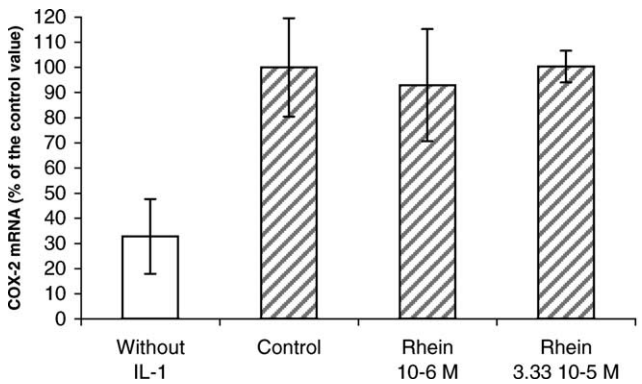

Fig. 6. Effect of rhein on IL-1β stimulated COX-2 mRNA quantified by real-time RT–PCR. The mRNA copy numbers were normalized against the corresponding copy number of GADPH mRNA. The results are represented by mean ± SD of two independent cultures performed with chondrocytes coming from different donors.

by rhein has been previously observed by Pelletier *et al.* [7] on both OA chondrocytes and cartilage explants. Rhein was also demonstrated to decrease arthritis activity and NO level in plasma of rat with adjuvant-induced arthritis, suggesting that the effect of rhein on arthritis is related to the inhibition of NO synthesis [34].

For the first time, a drug was demonstrated to reduce the basal production of MIP-1β by human OA chondrocytes. MIP-1β is a chemokine found to be elevated in the synovial fluid of OA compared to normal or RA patients [35]. This chemokine is chemoattractive for macrophages and activated neutrophils. Furthermore, MIP-1β was demonstrated to stimulate MMP-3 synthesis by chondrocytes [36]. Therefore, it is possible that in our culture system, the inhibitory effect of rhein on MMP-3 production was secondary to the decrease of MIP-1β synthesis by OA chondrocytes.

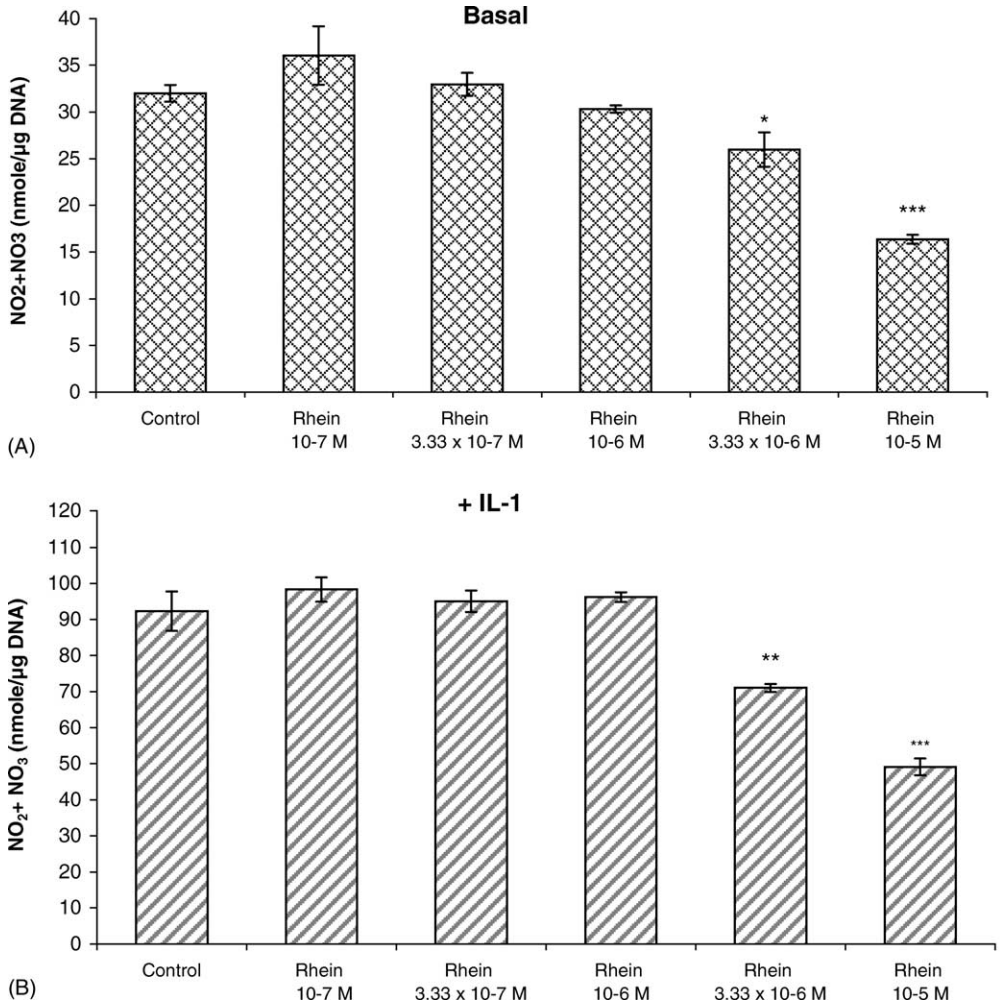

Fig. 7. Effect of rhein on the total production of NO_2 by human OA chondrocytes cultured for 12 days in alginate beads with (B) or without (A) 10^{-10} M IL-1β. The results are represented by mean ± SEM of four independent cultures performed with chondrocytes coming from different donors. Here ∗ indicates $P < 0.05$, ∗∗ indicate $P < 0.01$, and ∗∗∗ indicate $P < 0.001$.

Studies have shown that diacerhein and rhein increased PGE_2 but did not modulate COX-2, phospholipase A_2, or 5-lipoxygenase protein production [37–39]. Our results support this finding as we showed that rhein concentration-dependently increased both basal and IL-1β-stimulated PGE_2 production. Furthermore, we demonstrated that rhein did not modify COX-2 mRNA levels, even in the presence of IL-1, suggesting that rhein up-regulated PGE_2 production at a post-transcriptional level. Two possible hypothesis can be put forward to explain the action of rhein on PGE_2 synthesis:

(1) Rhein acts as an antioxidant and this prolongs the cyclooxygenase lifetime. Some arguments support this hypothesis. Firstly, rhein structure possesses scavenger groups, such as phenolic function, for reactive oxygen species. Secondly, COX-2 is an endoperoxide synthase forming a tyrosyl radical, which regulates its activity. To scavenge this radical could prolong its activity and then increase the PGE_2 production.

(2) We have reported that rhein is a potent inhibitor of NO. NO was demonstrated to down-regulate PGE_2 synthesis by chondrocytes. Therefore, the overproduction of PGE_2 could be secondary to the inhibition of NO synthesis [40].

It is of note that, in contrast and as previously reported [27], nonsteroidal anti-inflammatory drugs (NSAIDs) fully block PGE_2 synthesis by chondrocytes cultured in similar conditions. This finding clearly indicates that these two classes of drugs act by different pathways on pain and inflammation in OA. Nevertheless, some questions remain on the clinical impact of PGE_2 over expression. It is now well accepted that the release of high levels of PGE_2 at the inflammatory site contributes to inflammatory pain. Nevertheless, rhein has been shown to relieve chronic pain in knee and hip OA [18–22]. On the other hand, PGE_2 could act as a regulator of the inflammatory reaction, by decreasing IL-1 secretion and activity [41]. Furthermore, PGE_2 has been reported to enhance proteoglycans and collagen

type II synthesis, by stimulating IGF-1 release by chondrocytes [42,43], and so could exert cartilage protective properties.

Finally, we have observed that rhein at concentrations over 10^{-5} M and in the presence of a high concentration of IL-1β, induced cytotoxicity. This cytotoxicity has never been reported in humans. One explanation could be that rhein *in vivo* is metabolized to glucurono and sulfo-conjugate [44]. Furthermore, the cytotoxic effect reported herein, has been observed at concentrations of rhein superior to those found in the synovial fluid of arthritic patients after daily administration of diacerhein 50 mg.

From this work, we can conclude that rhein may act on different biochemical factors involved in OA pathophysiology. This drug stimulates aggrecan production and promotes matrix formation, decreases the production of some proinflammatory mediators and correct the MMPs/TIMPs imbalance by stimulating TIMP-1 synthesis and decreasing MMP-3 production by OA chondrocytes. Furthermore, rhein counteracts some of the deleterious effects of IL-1β, one of the most active cytokine in OA. Overall, these results contribute to explain the clinical efficiency of rhein [18–22] and give new informations on its mechanisms of action.

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