Rhein, a diacerhein-derived metabolite, modulates the expression of matrix degrading enzymes and the cell proliferation of articular chondrocytes by inhibiting ERK and JNK-AP-1 dependent pathways

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Abstract
Objective
To determine the effects of rhein on the expression of matrix metalloproteinases (MMP-1, -3, 13) and ADAMTs 4, 5 (a disintegrin and metalloproteinase with thrombospondin type-I repeat)/aggrecanases-1, -2 in interleukin-1-stimulated bovine articular chondrocytes, and to investigate the signalling pathways involved in the effects of the drug on gene expression and cell proliferation.

Methods
Bovine chondrocytes were treated with $10^{-4}$ M rhein for 18 h, followed by 10 ng/ml IL-1β for 30 min (cytoplasmic extracts) or 24 h (RNA extraction and EMSA). mRNA was assessed by RT-PCR for the expression of MMPs and aggrecanases, and the phosphorylation of MAP kinases was studied by Western blotting. NF-κB and AP-1 DNA binding were determined by gel retardation assay. The effects of inhibitors of these signalling pathways were compared to those of rhein. The proliferation of human chondrocytes and synoviocytes treated with the drug was also investigated.

Results
IL-1β-induced stimulation of the MMPs and aggrecanase-1 was markedly inhibited by rhein. The drug reduced IL-1β-induced NF-κB and AP-1 DNA binding, as well as the phosphorylation of ERK and JNK. Similar effects were produced by the specific inhibitors of these signalling pathways. In addition, rhein reduced the proliferation of both human chondrocytes and synoviocytes.

Conclusion
Our data indicate that rhein may reduce the deleterious effects of IL-1β on osteoarthritic cartilage through its effects on the ERK- and JNK-dependent pathways. Both its anti-catabolic and anti-proliferative properties may explain its value in the treatment of joint diseases.

Key words
Rhein, interleukin-1, osteoarthritis, chondrocytes, synoviocytes, matrix degrading enzymes, proliferation.
**Introduction**

Osteoarthritis (OA) is characterized by significant cartilage breakdown. Interleukin-1 (IL-1) has been shown to play a crucial role in the catabolic activity of the OA articular chondrocytes and in the degradation of articular cartilage (1, 2). This cytokine induces the expression of matrix-degrading enzymes, including several metalloproteases (MMPs) and the ADAMTs 4, 5 (a disintegrin and metalloprotease with a thrombospondin type-I repeat) / aggreganases 1, 2 (3). Furthermore, it downregulates the synthesis of the main components of cartilage, type II collagen and aggregan (4-6). OA Synovial fluids contain high levels of IL-1 (7), so that the cartilage homeostasis is altered in favour of the degradation.

In contrast to IL-1, several growth factors stimulate the synthesis of cartilage extracellular matrix components, hence promoting repair of the tissue. Among these, members of the TGF-β family, including TGF-β isoforms and BMPs (bone morphogenetic proteins), can up-regulate the expression of matrix molecules, whereas they inhibit the expression of several MMPs. For example, TGF-β1 counteracts most of the deleterious effects of IL-1, impacting both catabolic and anabolic aspects, thus suggesting that it could play a major role in the pathophysiology of OA and the repair potentialities of cartilage (8). Furthermore, in the rabbit OA model the expression of TGF-β receptor II was found to be dramatically reduced, so that the chondrocytes could become insensitive to the factor and therefore no longer counteract the IL-1-driven cartilage erosion (9). These data provide an explanation for the thus far apparently irreversible degradation processes of OA. Interestingly, this finding has received strong support from a study on transgenic mice that express a non-functional TGF-β type II receptor, in which the animals developed OA-like symptoms in their joints (10).

In view of this knowledge, it would be of interest to determine the effects of potential drugs for OA treatment and to re-examine the mechanism of action of well-established compounds, keeping in mind that both the inflammatory and degenerative aspects must be addressed when searching for new drugs that could act on OA cartilage. Rhein, the metabolite of diacerhein, is an anthraquinone that has been shown to reduce the severity of OA both in animal models and in human patients (11-18). The molecule is currently used in the treatment of OA (15) and was found to retard the evolution of adjuvant-induced arthritis in rats (19). The cellular mechanisms underlying its beneficial effects are not fully understood, although in vitro studies have revealed both enhancing effects of rhein on matrix synthesis and the inhibition of IL-1-induced expression of degrading enzymes in chondrocytes (20, 21). In contrast to conventional non-steroid anti-inflammatory drugs (NSAIDs), rhein does not inhibit the production of prostaglandin E by different cell types, including chondrocytes (22, 23). Nevertheless, the drug exhibits some anti-inflammatory and chondro-protective effects. In vitro, diacerhein and rhein inhibit the IL-1-induced stimulation of collagenase expression by rabbit articular chondrocytes, while they increase the synthesis of collagen and proteoglycans (20, 21). Furthermore, these compounds were found to stimulate the expression of TGF-β1 in chondrocytes (24), an effect that could contribute to its beneficial effect on cartilage, since TGF-β1 antagonizes most of the deleterious actions of IL-1 (25). In addition, rhein has been shown to reduce IL-1-induction of iNOS (inducible nitric oxide synthase) and to enhance COX2 (cyclo-oxygenase 2) production and activity (26).

To gain further insight into the mechanisms of rhein, we studied the effect of pharmacologically relevant concentrations of the drug on the signalling pathways and on the expression of genes related to the catabolism and anabolism of the extracellular matrix, using bovine articular chondrocytes (BAC) cultured under low oxygen tension and exposed to interleukin-1β (IL-1β). These cell culture conditions were chosen because of the hypoxic in situ environment of chondrocytes (27) and the fact that oxygen tension influences the response of...
these cells to IL-1β (28). Since rhein exhibits anti-tumor activity on several cancer cell lines (29), we also determined its effect on the proliferation of chondrocytes and synoviocytes.

Materials and methods

Culture and treatment of articular chondrocytes

Bovine articular chondrocytes from the knee joints of calves were enzymatically isolated (30). The cells were cultured in a 5% O2 atmosphere, using DMEM +10% fetal calf serum (FCS) previously equilibrated to 5% O2 by gas bubbling. Confluent cells were pre-treated with 10−4 M Rhein (Negma-Lerads, France) or its vehicle DMSO (dimethylsulfoxide) for 18 h, followed by incubation with IL-1β (10 ng/ml) for a further 30 min (cytoplasmic extracts) or 24 h (RNA extraction and EMSA).

Preparation of cytoplasmic and nuclear extracts

Following treatment, the chondrocytes were lysed in RIPA (Radio Immuno-Precipitation Assay) buffer to prepare cellular extracts for Western blotting (31). Alternatively, chondrocytes were lysed in hypotonic buffer to prepare cytoplasmic extracts for EMSA. The pellet was re-suspended in hypertonic buffer to obtain nuclear extracts (32, 33).

Western blot analysis of MAPKs

Cellular extracts were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride transfer membrane (PVDF, NEN Life Sciences Products). The membranes were treated with anti-phospho ERK1/ERK2 and anti-ERK1/2 antibodies (Upstate Biotechnology, Inc.), anti-phospho p38, anti-p38, anti-phospho JNK1/2 and anti-JNK antibodies (Cell Signalling Technologies) (31). Western blots were repeated at least three times with similar results. Representative blots are shown in the figures.

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were incubated in binding buffer for 30 min at 25°C with the cDNA probes, previously radio-labeled with 32P-γ-ATP (25 fmols) using T4 polynucleotide kinase (Life Technologies). The oligonucleotide probes NF-κB and AP1 were supplied by Life Technologies. Gel retardation assays were performed as previously described (34).

RNA extraction and real-time PCR conditions

Total RNA was extracted from the cellular pellet (35), and 1 μg of DNase I-treated total RNA was reverse transcribed. The resulting products were then diluted (1/100) before amplification. Oligonucleotide primers were designed from human or bovine sequences using Primer Express software (Applied Biosystems). Amplifications were carried out in triplicate. Serial dilutions of cDNA were also amplified to establish a standard curve and to determine the corresponding threshold cycle (CT). The 18S RNA gene was used as an endogenous control of the RNA amount in each sample. The relative gene expression was calculated using the 2−ΔΔCT method (36).

Use of inhibitors of the NF-κB, ERK and JNK pathways

The following inhibitors were used to block the NF-κB, ERK and JNK pathways, respectively: parthenolide (Sigma-Aldrich Co., St Quentin-Fallavier, France), U0126 (Promega, Charbonnières, France), and SP600125 (Calbiochem-VR, Strasbourg, France). The inhibitors were dissolved in DMSO and therefore the corresponding controls were included with the same amount of vehicle as the treated cultures.

DNA synthesis assay

Chondrocytes and synoviocytes were obtained from the joints of OA patients who were undergoing hip or knee replacement surgery. Chondrocytes were seeded at 1 × 104 cells/cm² in 6-well plates, whereas synoviocytes were plated at 2.5 × 105 cells/cm² in 12-well plates. Cells were grown to exponential phase in complete medium and incubated for 6 days in the presence or absence of rhein or its vehicle DMSO. The medium was changed every 2 days. [3H]thymidine (0.5 μCi/ml, 2200 Ci/mmol; Perkins Elmer) was added for the last 4 h of incubation. After two washes with cold PBS, the cell layers were treated with 5% trichloroacetic acid (TCA) at 4°C, washed twice with TCA solution and solubilized in 0.1 M NaOH for 30 min. The amount of [3H]thymidine incorporated was determined by liquid scintillation counting (37).

Statistical analysis

Experiments were performed at least three times and data from a representative experiment were taken and expressed as means ± SD (real time PCR) or ± SEM (DNA synthesis assay) of three wells per point. Statistical significance was determined by the Student’s t-test (*p < 0.05, **p < 0.011, ***p < 0.001).

Results

Rhein inhibits IL-1β-induced expression of matrix degrading enzymes

To determine the effect of rhein on the IL-1β-induced expression of matrix degrading enzymes, chondrocytes were treated with rhein for 24 h, and then with IL-1β (10 ng/ml) for a further 24 h. The mRNA levels of MMP-1, -3, -13 and aggrecanase-1 were significantly increased by IL-1β treatment (Figs. 1 and 2). In contrast, no significant change was observed in the mRNA level of aggrecanase-2, suggesting that the expression of this enzyme is not tightly controlled by the cytokine. IL-1β-induced stimulation of the MMPs and aggrecanase-1 was markedly inhibited by rhein at the concentration of 10−4 M. This concentration was chosen after previous studies using rhein at concentrations ranging from 10−4 M to 10−2 M, which showed the greatest effect at 10−4 M. Rhein’s inhibition of the effects of IL-1β was not caused by growth inhibition, since mRNA levels were normalized to the 18S mRNA as a reference. Similarly, no cytotoxicity of the drug at this concentration was observed, based on a Cytotox96® assay (Promega) (data not shown). No effect of rhein on aggrecanase-2 expression was found either in controls or IL-1β-treated cells.
Rhein blocks the phosphorylation of ERK, JNK, but not p38 kinase

We examined the effect of rhein on the phosphorylation of ERK, JNK and p38 kinase. Previous studies indicated that the activation of these kinases by IL-1β is an early event occurring at 0.25 – 1 h following addition of the cytokine (34). Consequently, a 30-min period was chosen to target ERK, JNK and p38. As shown in Figure 3, IL-1β markedly induced the phosphorylation of ERK, p38 and JNK. Rhein strongly inhibited IL-1β-induced ERK activation and, to a lesser extent the activation of JNK, but had no effect on p38. This finding suggests that the modulation of gene expression of chondrocytes by rhein involves the ERK and JNK signalling pathways.

Rhein inhibits IL-1β-induced NF-κB and AP-1 DNA binding

We then studied the consequences of rhein blockade of the IL-1β-phosphorylation of ERK and JNK on the downstream transactivating activity of NF-κB and AP-1, which have been implicated in the transcription of several inflammatory genes. It is also well known that the MAPK pathways influence AP-1 transactivation by increasing the abundance of AP-1 components and/or altering the phosphorylation of their subunits c-Jun and c-Fos. Thus, we performed EMSA experiments, using consensus cDNA probes for NF-κB and AP-1 and nuclear protein extracts from cultures pre-treated with rhein for 18 h and then with IL-1β for 24 h. It was found that IL-1β markedly enhanced the DNA binding of NF-κB and AP-1 (Fig. 4). Rhein alone did not modulate this binding, compared to the controls. In contrast, the drug significantly decreased the IL-1β-induced DNA complexes of both factors, especially that of AP-1. These results demonstrate that rhein blocks two major transcription factors, and therefore the transcriptional activity of several IL-1β-driven genes.

Comparative effects of rhein and inhibitors of NF-κB and MAPK on MMP expression

To gain further insight into the mecha-
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To ascertain the roles of NF-κB, ERK and JNK, we performed comparative experiments, using inhibitors of these pathways. Chondrocytes were treated for 24 h with 10^{-4} M rhein or 1% DMSO for 24 h. Some cultures without rhein were further incubated for 2 h with 10 μM parthenolide, 5 μM U0126 or 40 μM SP600125. Rhein- and inhibitor-treated cultures were then exposed to IL-1β (10 ng/ml) for 24 h. Appropriate concentrations of the inhibitors and incubation times were chosen after previous assays, to ensure efficiency without any cytotoxic effect (not shown). All of the inhibitors exerted the same inhibitory effect as rhein on IL-1β-induced MMP-1 and -3 mRNA expression, suggesting that the NF-κB, ERK and JNK pathways are involved in the modulation of these enzymes by the cytokine (Fig. 5). In contrast, inhibition of the JNK pathway did not produce the same effect as rhein on MMP-13 mRNA expression. Indeed, IL-1β stimulation was still observed in this case, which could be even greater than the IL-1β control. This suggests that only the NF-κB and ERK pathways are involved in the modulation of MMP-13 by IL-1β.

The profile of aggreganase mRNA levels was found to be similar to that of MMP-1 and MMP-3 (Fig. 6). The most effective inhibitor was parthenolide.

Fig. 3. Effect of Rhein on IL-1β-induced activation of MAPKs. Bovine chondrocytes were treated for 24 h in DMEM +1% FCS, with 10^{-4} M Rhein or 1% DMSO. They were further incubated for 2 h with 10 ng/ml IL-1β (10 ng/ml). The cytoplasmic extracts were prepared and subjected to Western Blot analysis using anti-phospho-ERK1/2 and anti-ERK2, anti-phospho-p38 and anti-p38 and anti-phospho-SAPK/JNK and anti-SAPK/JNK, as described in the Material and methods section. The protein-antibody complexes were visualized by chemoluminescence using HRP-conjugated secondary antibody. The results shown are representative of three independent experiments.

Fig. 4. Effect of Rhein on IL-1β-induced NF-κB and AP-1 DNA binding activity. Bovine chondrocytes were treated for 24 h in DMEM +1% FCS with 10^{-4} M Rhein or 1% DMSO. They were further incubated for 2 h with IL-1β (10 ng/ml). EMSA were performed as described in the Material and methods section. The autoradiography shown is representative of three independent experiments.
U0126 and SP600125 also markedly reduced the IL-1β-induced stimulation of aggrecanase-1 mRNA. These results indicate that all the three signalling elements are involved in the mechanism of IL-1 stimulation. Regarding aggrecanase-2, the findings are not as clear. First, IL-1β caused a significant but very small stimulation of mRNA production compared to that of aggrecanase-1. As we mentioned above, this weak enhancement is not always found, and seemed to depend on the experimental conditions. Rhein was found to abolish this effect. Inhibition of the NF-kB pathway by parthenolide did not suppress IL-1β-induced stimulation compared to the control containing parthenolide alone, but decreased the level observed in the IL-1β control with DMSO. In contrast, U0126 alone strongly enhanced the aggrecanase-2 mRNA level. The combination with IL-1β reduced that level, which was however as high as controls of IL-1β with DMSO. Finally, inhibition of the JNK pathway by SP600125 did not produce any significant change in the IL-1β modulation of aggrecanase-2 mRNA. From these results, we may tentatively conclude that IL-1β does not exert close control of aggrecanase-2 expression and that this modulation seems to implicate the ERK pathway rather than NF-kB and JNK. Although further investigation will be required to draw definite conclusions, it is clear that the regulation of aggrecanase-2 by IL-1β appears to be quite different from that of aggrecanase-1 and MMP-1 and -3.

Rhein inhibits the proliferation rates of both chondrocytes and synoviocytes

Fig. 5. Comparison of the effects of Rhein, NFκB inhibitor and MAPK inhibitors on MMP mRNA expression. Bovine chondrocytes were treated for 24 h in DMEM +1% FCS, with 10^−4 M Rhein or 1% DMSO. Then they were further incubated for 2 h with parthenolide (Parth, 10 μM), U0126 (5μM) or SP600 (SP600125, 40μM) followed by additional incubation with IL-1β (10 ng/ml) for 24 h. Total RNA was extracted and subjected to quantitative PCR to determine the steady-state mRNA levels of MMP-1, MMP-3, and MMP-13. Data were normalized to 18S mRNA and expressed as means ± SD of triplicate samples. The statistical significance of differences between control and treated cells was determined using the Student’s t-test (*p < 0.05, **p < 0.01, ***p < 0.001).
Discussion

The aim of this study was to investigate the intracellular signalling pathways of chondrocytes that could be involved in the modulatory effect of rhein on the deleterious effects induced by IL-1β on OA joints. As previous studies have shown that the in vitro response of articular chondrocytes to IL-1β was highly dependent on the oxygen level (27, 28), we used low oxygen tension to better mimic the conditions of the chondrocytes within cartilage. Rhein was found to reduce IL-1β-stimulated ERK and JNK kinase phosphorylation, as well as the transactivation activity of NF-κB and AP-1. This effect was associated with the down-regulation of MMP-1,-3,-13 and aggrecanase-1 gene expression. Our data are in agreement with a report showing that inhibition of IL-1β-stimulated MAP kinases, AP-1 and NF-κB transcription factors in bovine articular chondrocytes induced a down-regulation of MMP gene expression, including MMP-3 and MMP-13 (38). Several MMP gene promoters contain AP-1-responsive elements, and the role of AP-1 in the transcriptional activity of MMP genes has been well established (39, 40). The involvement of NF-κB, whose sites are not systematically found in all MMP promoters, is also supported by some evidence. For instance, NF-κB is involved in the regulation of MMP-1 and MMP-13 genes in chondrosarcoma cells (41). Blocking NF-κB activity by overexpression of its inhibitor IκBα was also found to reduce inflammation and tissue destruction by MMP-1 and MMP-3 in rheumatoid synovial tissue and dermal fibroblasts (42, 43). One possibility whereby NF-κB could play a role in MMP regulation may be through cross-talk with AP-1 in the nucleus (44). Our results are in agreement with a previous report showing a preventative effect of diacerhein and rhein on IL-1β-induced NF-κB activation in bovine chondrocytes (45). The inhibitory effect of rhein on IL-1β transactivation of NF-κB is likely to downregulate the expression of several pro-inflammatory genes whose promoters are driven by the factor, such as iNOS gene (46).

Among the enzymes that could participate in cartilage breakdown in OA, aggrecanase-1 and aggrecanase-2 (ADAMTs 4 and 5) have been hypothesized to play a key role in the early steps of proteoglycan degradation. Indeed, fragments of proteoglycans corresponding to the aggrecanase-specific site of cleavage have been detected in the synovial fluid of OA patients. Furthermore, knockout of the ADAMT 5 (aggrecanase 2) in mice prevents cartilage degradation in a murine model of OA (47). Interestingly, ours study demonstrates that IL-1β-induced expression of aggrecanase-1 is down-regulated by rhein in chondrocytes. However, in contrast to aggrecanase-1, aggrecanase-2 expression was found to be only slightly modulated by IL-1β and rhein was without effect on its expression. Such a differential control of aggrecanase-2 in comparison to aggrecanase-1 by IL-1 was previously reported by our laboratory (48). This finding appears to be in contradiction with the apparent key role of this enzyme in OA, deduced from the above experiment in the murine model of OA. No clear explanation for this discrepancy can be suggested at the moment. Further knowledge of the promoter structure of these enzyme genes will help in understanding their differential control by IL-1β and their respective roles in the degradation of OA cartilage.
In our study, IL-1β-induced AP-1 DNA binding was clearly shown to be reduced by rhein through inhibition of the ERK- and JNK-dependent molecular mechanism. Since expression of the subunit c-Jun of the AP-1 complex is not altered by rhein (20), it is likely that JNK phosphorylating activity is the major target whereby rhein decreases AP-1 transactivation. Interestingly, we report for the first time that rhein can inhibit the proliferation rates of both chondrocytes and synoviocytes. This effect is probably related to the decrease in ERK- and JNK-dependent AP-1 activation. Inhibition of the proliferation of joint cells could have benefits in terms of inhibiting the development of OA and RA (rheumatoid arthritis). Indeed, although synovial inflammation is rather limited in OA joints compared to RA, it is always present as a secondary process (49-51), even in the early stages, and has also been suggested to play a role in the pathophysiology of the disease. In RA joints the synoviocytes are characterized by proliferation, pannus formation, and the secretion of factors that promote inflammation, neovascularization, and cartilage degradation. Fibroblast-like synoviocytes in the rheumatoid synovium are similar to transformed cells and have been shown to proliferate in an anchorage-independent manner, to lack contact inhibition, and to express oncogenes and cell cycle proteins indicative of transformation. They exhibit both aggressive growth and invasiveness, so that some have described RA as a “cancer of the joint”, based on the fact that mortality rates for RA are similar to that for certain lymphomas and given the localized hyperplasia and angiogenesis observed in the pannus (52, 53). Furthermore, a deregulation of the transcription factors AP-1 and nuclear factor-kB, and of the tumour suppressor gene p53 have been implicated in the aggressive proliferative and invasive functions of fibroblast-like synoviocytes (54-57).

Of interest, rhein was found to inhibit TPA-induced AP-1 activation and cell transformation by blocking the JNK-dependent pathway (29). These results clearly demonstrated that rhein exerts a anti-tumoural function and provides a molecular explanation for previous reports indicating that the drug can suppress the growth of some cancer cells in rat liver (58), Ehrlich ascite tumour cells (59), and human glioma cells (60).

In conclusion, our present results suggest that rhein can exert beneficial effects on both OA articular cartilage and synovial membrane through inhibition of the NF-kB pathway and the ERK-, JNK-dependent transactivation of AP-1. This leads to a down-regulation of most of the degrading enzymes produced by chondrocytes and may help in reducing cartilage breakdown. Furthermore, with its anti-proliferative and anti-tumoural properties the drug may decrease the development of the inflammatory synovial tissue that accompanies both OA and RA joint disorders. However, the present in vitro findings do not provide evidence that these mechanisms could act in vivo, as in the clinical treatment of OA patients with rhein. Further exploration of the potential chondro-protective and anti-proliferation effects of the drug in both animal models and clinical settings seems warranted. In this context, the new MRI methods are of great interest, since they allow researchers to estimate cartilage erosion and the level of inflammation in the synovium.

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