Diacerein Reduces the Excess Synthesis of Bone Remodeling Factors by Human Osteoblast Cells from Osteoarthritic Subchondral Bone

JEAN-PIERRE PELLETIER, DANIEL LAJEUNESSE, PASCAL REBOUL, FRANÇOIS MINEAU, JULIO C. FERNANDES, PIERRE SABOURET, and JOHANNE MARTEL-PELLETIER

ABSTRACT. ***Objective.*** Although cartilage degradation characterizes osteoarthritis (OA), there is evidence that remodeling of subchondral bone in this disease is a contributing factor. Therapeutic strategies to modify the metabolism of subchondral bone osteoblasts may be indicated to treat OA. We studied the effects of diacerein and rhein on the metabolic and inflammatory variables of OA subchondral osteoblasts.

Methods. Human OA primary subchondral osteoblast cells were used. The effect of diacerein and rhein at therapeutic concentrations (5–20 μg/ml) was determined by osteoblast phenotypic factors, alkaline phosphatase, osteocalcin, and cAMP; on metabolic agents urokinase plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1), and insulin-like growth factor-1 (IGF-1); and on inflammatory mediators interleukin 6 (IL-6), prostaglandin E_2 (PGE_2), and cyclooxygenase-2 (COX-2).

Results. Diacerein and rhein did not affect either basal and $1,25(OH)_2D_3$ induced alkaline phosphatase or parathyroid hormone (PTH) stimulated cAMP formation. Conversely, they dose dependently and statistically inhibited $1,25(OH)_2D_3$ induced osteocalcin release, a situation explained by a reduction of mRNA levels for osteocalcin. Of the metabolic factors, they inhibited the production of uPA, with rhein showing slightly more potency; inhibitions of 69% and 57% were reached at the highest concentration (20 μg/ml) of rhein and diacerein, respectively. Both drugs also inhibited the PAI-1 level, albeit at a much lower level than for uPA. Interestingly, determination of the uPA/PAI-1 ratio revealed that both drugs inhibited it about 55%, suggesting a decrease in uPA activity. In contrast, IGF-1 levels only increased slightly when cells were treated with rhein but not with diacerein. A transient dose dependent effect was found on IL-6 production; an inhibition was noted at low drug concentrations, which returned to basal levels at the highest concentration tested. PGE_2 levels increased exponentially and were related to a concomitant increase in COX-2 levels in response to both drugs.

Conclusion. Our data indicate that diacerein and rhein do not appear to affect OA subchondral bone cells' basal cellular metabolism, yet both agents reveal a direct effect at reducing the synthetic activities of osteoblasts, which could be responsible for abnormal subchondral bone remodeling occurring during the course of OA. (J Rheumatol 2001;28:814-24)

Key Indexing Terms:
DIACEREIN OSTEOARTHRITIS SUBCHONDRAL BONE
REMODELING FACTORS OSTEOBLASTS

Among the many structural alterations that characterize osteoarthritis (OA), the morphological changes that take place at the subchondral bone level remain undefined, particularly with regard to the temporal relationship with the disease process[1-8], as well as the pathophysiological mechanisms involved in such changes[8-16]. Studies in both human and in experimental OA models have indicated that, in the early stages of the disease, the subchondral bone alterations are characterized by excessive bone resorption over bone formation[4-6,8]. At later stages, the balance seems to favor an increase in bone formation, leading to bone sclerosis and increased thickening of the subchondral bone plate, typical of what is observed in human OA[4-6]. Based on recent reports, however, this bone seemed hypomineralized[16]. Recently, it has been shown that OA subchondral bone explants and the corresponding osteoblast cells express a number of abnormal metabolic activities. Indeed, human OA subchondral osteoblasts were reported to have abnormal

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phenotypes, elevated alkaline phosphatase (ALPase) activity, increased release of osteocalcin, reduced parathyroid hormone (PTH) and prostaglandin E_2 (PGE_2) dependent cAMP formation, elevated urokinase plasminogen activator (uPA) and insulin-like growth factor-1 (IGF-1), and altered collagen metabolism[10-14]. Also recently reported was that these disease cells produce more interleukin 6 (IL-6) and PGE_2 levels than normal[17].

The pharmacological treatment of OA symptoms includes several different types of drugs that can be classified based on their mode of action. A large number are nonsteroidal antiinflammatory drugs (NSAID), which exert their action through their analgesic properties as well as by inhibiting cyclooxygenase (COX). Diacerein, a new and effective drug for the symptomatic treatment of OA, belongs to the anthraquinone chemical class. Its active metabolite is rhein. Its mechanism of action appears to be different from that described for classic NSAID: diacerein and rhein inhibit IL-1 production and activity and, depending on the cell type, either do not affect prostaglandin synthesis (macrophages) or stimulate its synthesis (chondrocytes)[18-21]. In OA cartilage and synovial membrane cells, diacerein inhibits the IL-1 synthesis and reduces the levels of IL-1 receptors and IL-1ß converting enzyme (ICE, or caspase-1)[20-23]. This drug and its main metabolite, rhein, have also shown the capacity to reduce the level of IL-1ß induced metalloproteases and nitric oxide by chondrocytes[20,24]. In OA animal models, diacerein has demonstrated protective effects on cartilage matrix degradation[25-28]. In clinical trials, its oral administration was associated with symptomatic improvement in patients with OA[29-31].

We examined whether diacerein and rhein, in addition to their action on cartilage and synovial membrane, also act on the metabolism of OA subchondral bone osteoblasts. We examined the effects of these agents on the osteoblast factors most likely to be involved in the remodeling of subchondral bone.

MATERIALS AND METHODS

Specimen selection. Femoral condyles were recovered under sterile conditions from 18 patients aged 62 to 84 years (mean ± SD 71 ± 10 yrs; 14 women/4 men) undergoing total knee replacement and who were classified as having OA by the American College of Rheumatology clinical criteria[32]. No patient had received medication that would interfere with bone metabolism.

Subchondral bone osteoblast culture. The overlying cartilage was removed and the trabecular bone tissue dissected from the subchondral bone plate. All manipulations were performed under a magnifying microscope to ensure complete removal of cartilage and trabecular bone.

Primary cell cultures were prepared as described[10]. Briefly, bone samples were cut into small pieces of 2 mm^2 before sequential digestion in 1 mg/ml collagenase type I (Sigma-Aldrich, Oakville, ON, Canada) in HAMF12/DMEM media (Sigma-Aldrich) without serum at 37°C for 4 h. After being washed, the digested bone pieces were cultured in BGFb media (Sigma-Aldrich) containing 20% fetal bovine serum (FBS) (BioWhittaker, Walkersville, MD, USA). This medium was replaced every 2 days until cells were observed in the petri dishes. At this point, the culture medium was replaced with fresh media containing 10% FBS. At confluence, cells were passaged once, grown until confluency (about 5 days) in HAMF12/DMEM media containing 10% FBS, then assayed.

Effects of diacerein and rhein. Except for cAMP and COX-2 determinations, cell conditioning was performed for the last 2 days of culture. Forty-eight hours prior to determination, the medium was replaced with fresh media in HAMF12/DMEM containing 1% insulin-transferrin-selenium mixture (ITS) (Sigma-Aldrich). For determination of ALPase activity and osteocalcin release, cells were also preincubated at 37°C for 24 h in the presence of 50 nM $1,25(OH)_2D_3$ (Sigma-Aldrich) in the same culture medium containing 2% charcoal-stripped FBS. Fresh culture medium containing $1,25(OH)_2D_3$ was then added in the presence or absence of 5 to 20 μg/ml of diacerein or rhein. At the end of the incubation, the medium was collected and frozen at –80°C prior to assay. Cells were then washed twice with phosphate buffered saline (PBS), pH 7.4, and solubilized in the buffer consisting of 100 mM glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 1% Triton X-100, pH 10.5, for 60 min with agitation at 4°C.

Osteocalcin was determined using a specific enzyme immunoassay (Biomedical Technologies, Stoughton, MA, USA) with a detection limit of 0.5 ng/ml. Cellular ALPase activity was determined as the release of *p*-nitrophenol hydrolyzed from *p*-nitrophenyl phosphate (12.5 mM final concentration) at 37°C for 30 min after solubilizing the cells in the buffer. Protein determination was performed by the bicinchoninic acid method[33]. The uPA, plasminogen activator inhibitor-1 (PAI-1), and IL-6 levels were all determined by specific ELISA: American Diagnostica Inc. (Greenwich, CT, USA) for uPA (sensitivity 10 pg/ml) and PAI-1 (sensitivity 50 ng/ml); R&D Systems Inc. (Minneapolis, MN, USA) for IL-6 (sensitivity 0.7 pg/ml). PGE_2 was determined using a PGE_2 enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI, USA). This assay uses the competition between PGE_2 and a PGE_2-acetylcholinesterase conjugate (PGE_2 tracer) for a limited amount of PGE_2 monoclonal antibody (sensitivity was 9 pg/ml).

Northern blot analysis of osteocalcin mRNA levels was performed using confluent cells incubated for their last 48 h of culture in HAMF12/DMEM containing 2% charcoal-stripped FBS in the presence of 50 nM $1,25(OH)_2D_3$ and in the presence or absence of 20 μg/ml of either diacerein or rhein. Cells were washed twice then extracted following the procedure of Chomczynski and Sacchi[34]. Ten micrograms of total RNA were size-fractionated on 1.0% agarose gel containing 1.2 M formaldehyde and transferred to nylon membranes. The membranes were UV autocross-linked, prehybridized at 42°C for 20 h, and hybridized at 42°C overnight with a digoxigenin (DIG) labeled DNA probe for osteocalcin [phBGP/Sac complementary DNA (cDNA)], generously provided by Dr. J.M. Wozney and T. Celeste (Genetics Institute, Inc., Cambridge, MA, USA). The DIG-uridine triphosphate probe was performed using a random primed DNA labeling kit following the protocol provided by the company (Boehringer-Mannheim, St. Laurent, Québec, Canada). Filters were washed once for 60 min in 2 × SSC/1 × Denhardt's solution at 42°C, twice in 1 × SSC/0.1% sodium dodecyl sulfate (SDS) for 30 min each at 65°C, and twice in 0.1 × SSC/1% SDS at room temperature before exposure to Kodak X-Omat films for 1 h at room temperature. Hybridizing signals on the blots were analyzed quantitatively by densitometric scanning of autoradiograms. The filters were then reprobed with cDNA for glyceraldehyde phosphate dehydrogenase (GAPDH) to monitor loading between samples.

For cAMP determination, cells were preincubated at 37°C for 24 h in HAMF12/DMEM containing 1% ITS mixture and 2% charcoal-stripped FBS. Fresh culture medium was then added in the presence or absence of 20 μg/ml of diacerein or rhein and cells incubated at 37°C for 48 h. At the end of the incubation period, 1 mM of 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich), a phosphodiesterase inhibitor, was added for 10 min followed by incubation for 5 min with 100 nM human PTH fragment 1–34 (Peninsula Laboratories, Belmont, CA, USA), and the reaction was stopped with 3% perchloric acid (final concentration). cAMP levels were then evaluated by radioimmunoassay (Diagnostic Products, Los Angeles, CA, USA).

For IGF-1, cells were preincubated and incubated in the presence or

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absence of 20 μg/ml diacerein and rhein, as for cAMP. The IGF-1 levels were determined by a specific ELISA (Diagnostics Systems Laboratories, Webster, TX, USA); sensitivity was 0.03 ng/ml.

To determine the COX-2 level, a Western blot analysis was performed as described[20]. Cells were incubated 24 h in the presence or absence of 5 to 20 μg/ml diacerein and rhein, extracted in the RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin and pepstatin, 1% NP-40, 1 mM sodium orathovanadate, and 1 mM NaF), the protein determined, and Western immunoblot performed. Briefly, 25 μg of cellular protein extract was subjected to 12% sodium dodecyl sulfate-polycrylamide gel electrophoresis under reducing conditions, and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada). After blocking with SuperBlock™ blocking buffer (Pierce, Rockford, IL, USA) in Tris buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) and washing, the membranes were incubated overnight at 4°C with the primary antibody in the blocking buffer as above, and 0.5% Tween 20. The antibody used was a rabbit polyclonal anti-human COX-2 (Oxford Biomedical Research, Oxford, MI, USA; 1:2500 dilution). A second anti-rabbit antibody [horseradish peroxidase conjugated (Pierce); 1:20,000 dilution] was subsequently incubated with the membrane for 1 h at room temperature and then washed extensively with TTBS (20 mM Tris-HCl, 150 mM NaCl), pH 7.5, 0.1% Tween® 20 at room temperature. After incubation with SuperSignal™ Ultra Chemiluminescent substrate (Pierce), membranes were prepared for autoradiography and exposed to Kodak X-Omat film.

Data analysis. Data are expressed as the mean ± SEM of independent specimens. Statistical analysis was performed by analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparisons test. A difference of 0.05 was considered significant.

RESULTS

Effect of diacerein and rhein on cell biomarkers of OA subchondral osteoblasts. Studies with human OA subchondral osteoblasts have shown that these cells have abnormal metabolic features compared to normal osteoblasts[10,11]. We tested whether diacerein and rhein could alter the expression/cell biomarkers of OA subchondral osteoblasts, namely ALPase activity, osteocalcin release, and hormonal dependent cAMP production. Figure 1 shows that ALPase activity in these cells responded to $1,25(OH)_2D_3$ challenge (n = 8), as expected from human osteoblasts, with a 1.5-fold increase over basal values. Neither diacerein nor rhein at doses ranging from 5 to 10 μg/ml affected ALPase activity under basal and hormonal stimulation. At 20 μg/ml, both drugs reduced basal and $1,25(OH)_2D_3$ induced ALPase by about 20%. In contrast, $1,25(OH)_2D_3$ induced osteocalcin secretion (n = 8) was dose dependently reduced by both diacerein and rhein (Figure 2). Rhein inhibited $1,25(OH)_2D_3$ induced osteocalcin release by 35% and diacerein by 23% at the maximal dose. This reduction in osteocalcin release was due to a significant reduction in osteocalcin mRNA levels in response to either diacerein or rhein. Figure 3 shows a representative Northern blot analysis of osteocalcin mRNA levels in cells treated or not with 20 μg/ml rhein or diacerein. Both drugs reduced osteocalcin expression in OA subchondral osteoblasts [relative osteocalcin to GAPDH ratios — 1.25 ± 0.08, 0.88 ± 0.4 ($p < 0.01$ vs basal) and 0.93 ± 0.03 ($p < 0.05$ vs basal) for basal, rhein, and diacerein treatments, respectively (n = 3 cell preparations)]. PTH stimulation of OA osteoblasts induced a 4-fold increase in cAMP formation (n = 7) in these cells (Figure 4). Diacerein and rhein had no significant effect on either basal or PTH stimulated cAMP formation.

Effect of diacerein and rhein on metabolic capacities of OA subchondral osteoblasts. OA is characterized by bone sclerosis that may be due to either enhanced bone formation, reduced bone resorption, or both. A key element initiating

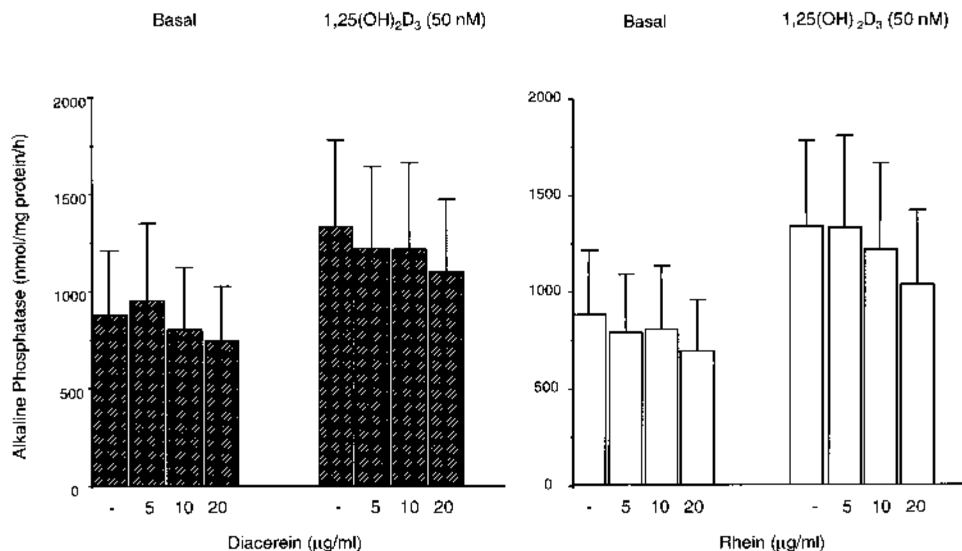

Figure 1. Dose dependent effect of diacerein and rhein on ALPase activity by primary human subchondral osteoblasts of patients with OA. Confluent cells were incubated for their last 2 days of culture in HAMF12/DMEM media in the presence of 2% charcoal treated FBS with or without 50 nM $1,25(OH)_2D_3$, and in the presence of increasing doses of diacerein and rhein. ALPase activity was determined by the hydrolysis of p-nitrophenyl phosphate into p-nitrophenol. Values are the mean ± SEM of 8 cell preparations (n = 8 patients).

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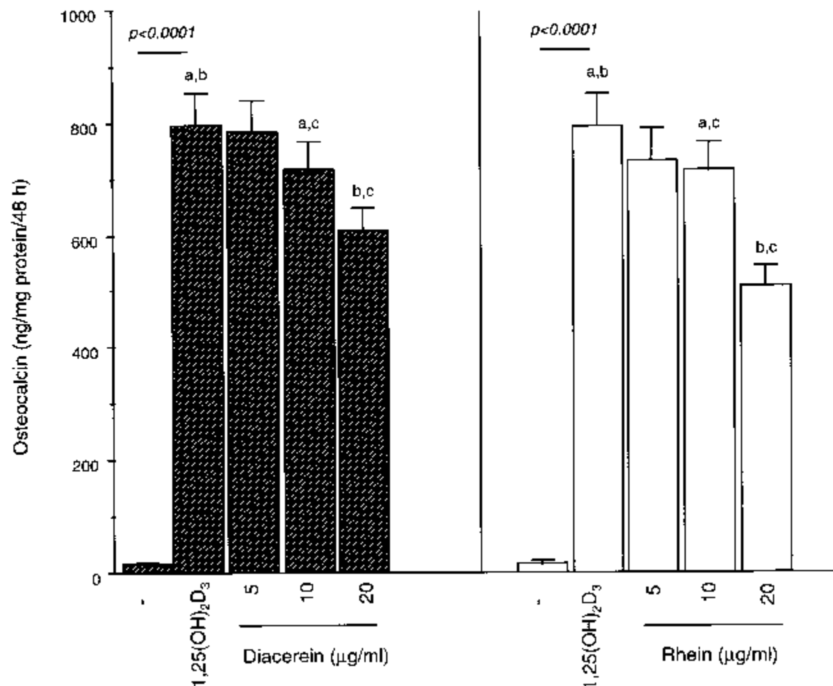

Figure 2. Dose dependent effect of diacerein and rhein on osteocalcin release by primary human subchondral osteoblasts of patients with OA. Confluent cells were incubated for their last 2 days of culture in HAMF12/DMEM media in the presence of 2% charcoal treated FBS with or without 50 nM $1,25(OH)_2D_3$, and in the presence of increasing doses of diacerein and rhein. Osteocalcin release was determined on aliquots of conditioned media by enzyme immunoassay. Values are the mean ± SEM of 8 cell preparations (n = 8 patients). ANOVA indicates a significant difference of p < 0.0001. Subtest analyses indicate significant differences of p < 0.01 between assays sharing the same letter.

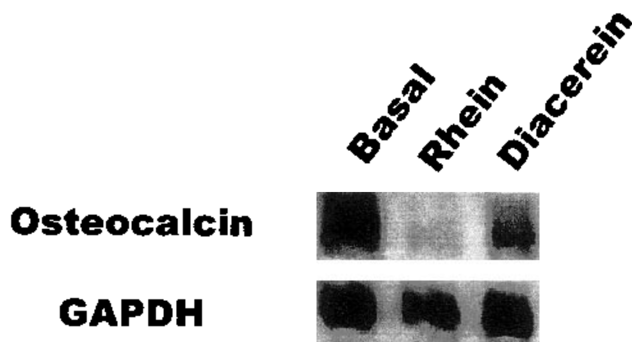

Figure 3. Representative Northern blot analysis of the effect of diacerein and rhein on osteocalcin mRNA levels by primary human subchondral osteoblasts of patients with OA. Confluent cells were incubated for their last 2 days of culture in HAMF12/DMEM media in the presence of 2% charcoal treated FBS with 50 nM $1,25(OH)_2D_3$, and in the presence of 20 µg/ml of either diacerein or rhein. After solubilizing cells with Trizol, 10 mg total RNA was electrophoresed and osteocalcin mRNA was detected by a specific probe labeled with DIG. Membranes were then reprobed with a cDNA for GAPDH to assess similar loadings between samples. Densitometric analysis of the osteocalcin mRNA to GAPDH mRNA ratios is the mean ± SEM of 3 patients with OA.

local bone remodeling is the uPA/plasmin system, which can link bone resorption with bone formation by locally stimulating other protease systems and releasing trapped growth factors in bone specimens. IGF-1 is also well recognized as promoting bone cell growth and osteoblast proliferation. Hence, we next evaluated the role of diacerein and rhein on the metabolic capacities of OA subchondral osteoblasts by evaluating the uPA, PAI-1, and IGF-1 levels produced by these cells. Both diacerein and rhein had significant dose dependent inhibitory effects on the production of uPA by these cells (Figure 5). Rhein induced a slightly higher inhibition than diacerein at all doses tested. Similarly, rhein dose dependently reduced PAI-1 levels (Figure 6), albeit much less than for uPA levels, whereas the effect of diacerein on this variable was found only at the highest dose tested. Interestingly, as shown in Table 1, the uPA/PAI-1 ratio in these cells was significantly reduced (ANOVA, p < 0.0001) to similar levels by both rhein and diacerein at all doses tested. This suggests that although each drug affected these systems slightly differently, the resulting general effect of the 2 drugs was similar. Rhein slightly yet significantly increased IGF-1 production, while diacerein was without significant effect (Figure 7).

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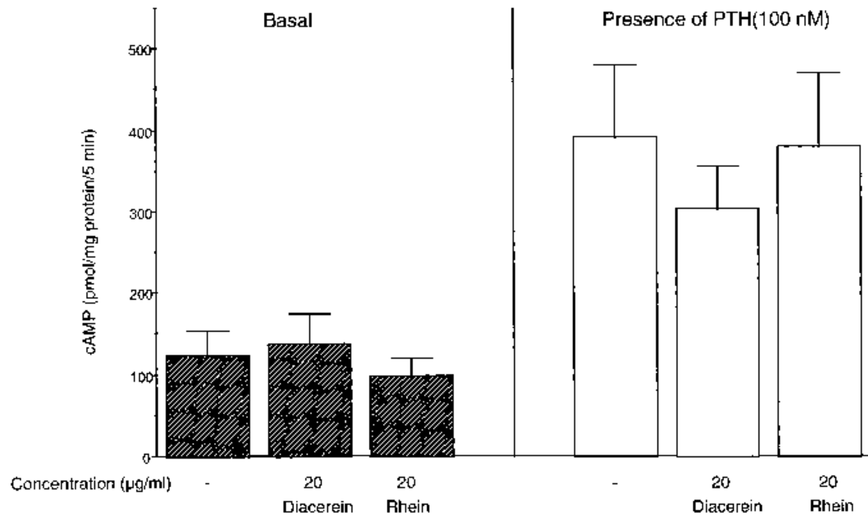

Figure 4. Effect of diacerein and rhein on the production of cyclic AMP (cAMP) by primary human subchondral osteoblasts of patients with OA. Confluent cells were incubated in the presence or not of 100 nM PTH fragment 1–34 and cAMP was determined on total extracts by radioimmunoassay. Results are the mean ± SEM of 7 cell preparations (n = 7 patients).

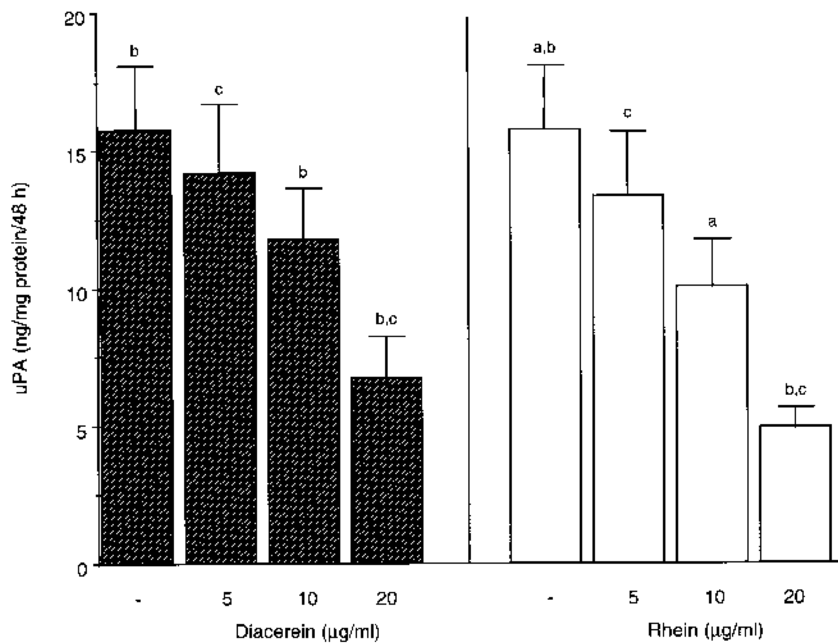

Figure 5. Dose dependent effect of diacerein and rhein on the production of uPA by primary human subchondral osteoblasts from patients with OA. Confluent cells were incubated for their last 2 days of culture in HAMF12/DMEM media without FBS but in the presence of 1% insulin-transferrin-selenium mixture. Levels of uPA released by cells were determined on aliquots of conditioned media by ELISA. Values are mean ± SEM of 8 cell preparations (n = 8 patients). ANOVA indicates a significant difference of $p < 0.0001$. Subtest analyses indicate significant differences between assays sharing the same letter: a, $p < 0.001$; b, $p < 0.01$, and c, $p < 0.05$.

Effect of diacerein and rhein on the production of inflammatory mediators by OA subchondral osteoblasts. At the clinical level, OA is characterized by pain, swelling, and inflammation in the affected joints. Although synoviocytes may be mostly responsible for this occurrence, the role of osteoblasts, which can produce cytokines locally, has not been fully evaluated, nor has the possible role of antiinflammatory drugs in this process. We reported that IL-6 and PGE_2 levels are increased in OA subchondral osteoblasts[17]. Figure 8 shows that diacerein and rhein had transient dose dependent inhibitory effects on IL-6 production. At low concentrations (5 and 10 μg/ml), IL-6 release was reduced

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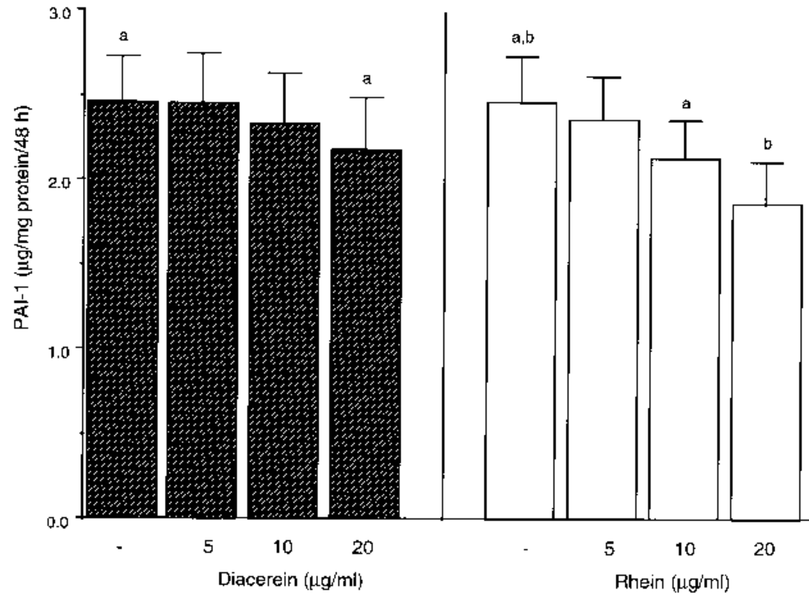

Figure 6. Dose dependent effect of diacerein and rhein on the production of PAI-1 by primary human subchondral osteoblasts from patients with OA. Confluent cells were incubated for their last 2 days of culture in HAMF12/DMEM media without FBS but in the presence of 1% ITS mixture. Levels of PAI-1 released by cells were determined on aliquots of conditioned media by ELISA. Values are mean ± SEM of 8 cell preparations (n = 8 patients). ANOVA indicate a significant difference of $p < 0.0001$. Subtest analyses indicate significant differences between assays sharing the same letter: a and b, $p < 0.01$.

Table 1. Effect of diacerein and rhein on the uPA/PAI-1 ratio of human OA subchondral osteoblasts. Data are expressed as mean ± SEM of 8 independent experiments.

Dose, µg/ml	Diacerein		Rhein
0		$6.41 \pm 0.65 \times 10^{-3}$	
5	$5.73 \pm 0.8 \times 10^{-3}$		$5.58 \pm 0.08 \times 10^{-3}$
10	$4.95 \pm 0.35 \times 10^{-3}$*		$4.60 \pm 0.60 \times 10^{-3}$*
20	$3.00 \pm 0.56 \times 10^{-3}$**		$2.71 \pm 0.31 \times 10^{-3}$**
	$p < 0.0001$†		$p < 0.0001$†

Post hoc test using Tukey-Kramer multiple analysis test: †$p < 0.0001$ by ANOVA analysis of variance; *$p < 0.01$ between drug treatment and basal level; **$p < 0.001$ between drug treatment and basal level.

whereas at the highest dose (20 µg/ml), IL-6 levels returned to basal values. In contrast, PGE_2 levels increased exponentially in response to dose dependent increases in both rhein and diacerein, although rhein was the most potent in stimulating PGE_2 release in these cells (Figure 9A). This was directly related to an increase in COX-2 protein in these cells, as detected by Western blot analysis (Figure 9B).

DISCUSSION

Diacerein is an effective agent for the treatment of human OA[29-31,35] and in animal models of this disease[25-28,36]. However, although the IL-1 system has been shown to be implicated as one of the action mechanisms[20,21], other potential targets remain to be determined. We have extended our understanding of diacerein and rhein's potential mechanism of action on human OA subchondral osteoblasts. As reported, alterations in osteoblast cell markers and metabolic activities may be responsible for the observed sclerosis in OA[8-16]. Studies indicate a potential role of bone tissues in the initiation and/or progression of OA[1-8,11]. Based on these findings, it seems that therapeutic strategies aimed at modifying bone cell responses may be indicated to treat OA. We studied the effect of diacerein and rhein on OA osteoblast variables to determine whether these agents could alter pathways likely involved in subchondral bone remodeling in this disease.

Diacerein and rhein had selective effects on $1,25(OH)_2D_3$-dependent pathways of OA subchondral

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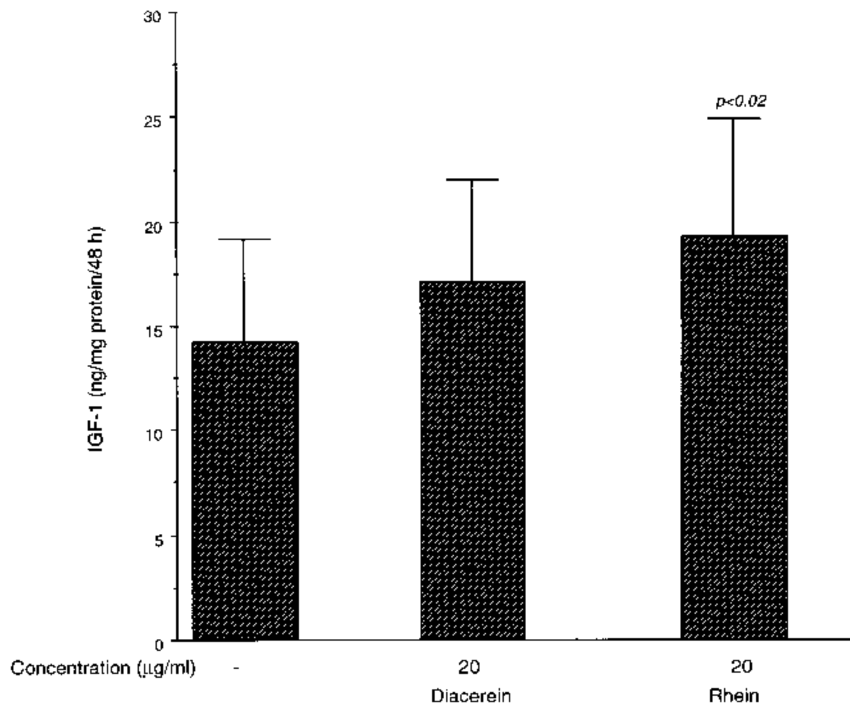

Figure 7. Effect of diacerein and rhein on the production of IGF-1 by primary human subchondral osteoblasts from patients with OA. Confluent cells were incubated for their last 2 days of culture in HAMF12/DMEM media without FBS but in the presence of 1% ITS mixture. Levels of IGF-1 released by cells were determined on aliquots of conditioned media by ELISA. Values are mean ± SEM of 4 cell preparations (n = 4 patients).

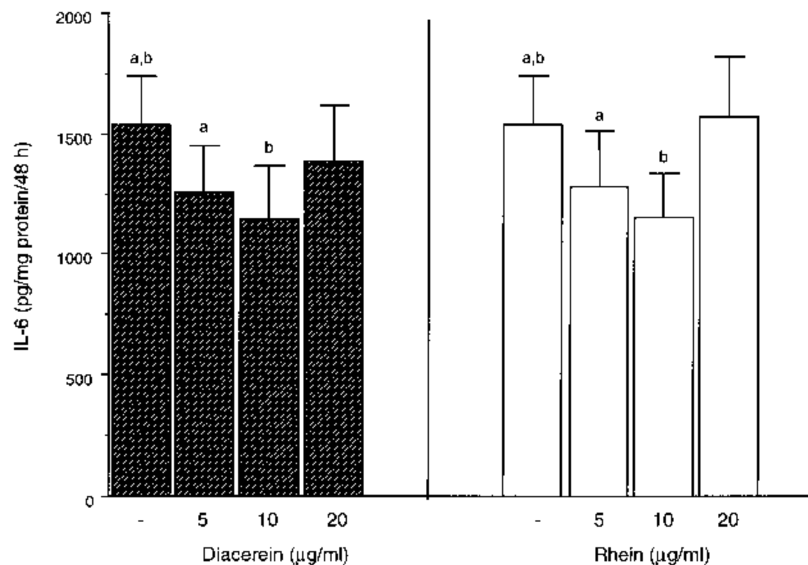

Figure 8. Dose dependent effect of diacerein and rhein on the production of IL-6 by primary human subchondral osteoblasts from patients with OA. Confluent cells were incubated for their last 2 days of culture in HAMF12/DMEM media without FBS but in the presence of 1% ITS mixture. Levels of IL-6 released by cells were determined on aliquots of conditioned media by ELISA. Values are mean ± SEM of 8 cell preparations (n = 8 patients). ANOVA analyses indicate a significant difference of $p < 0.0001$ for diacerein and $p < 0.001$ for rhein. Subtest analyses indicate significant differences between assays sharing the same letter: a and b, $p < 0.01$.

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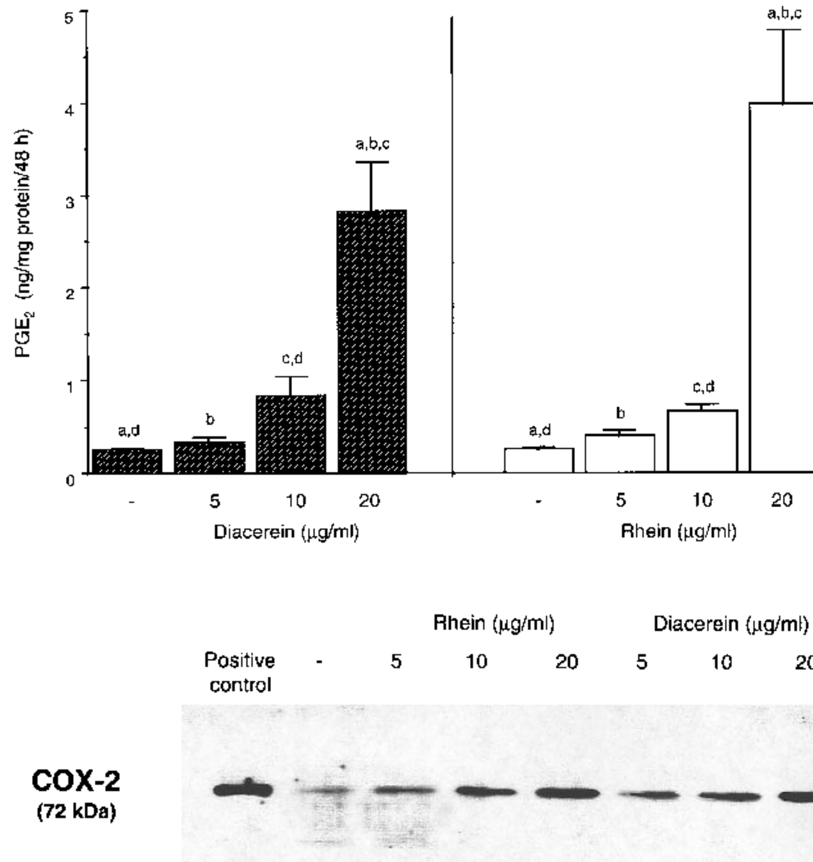

Figure 9. Dose dependent effect of diacerein and rhein on the production of PGE_2 and COX-2 by primary human subchondral osteoblasts from patients with OA. Confluent cells were incubated for their last 2 days of culture in HAMF12/DMEM media without FBS but in the presence of 1% ITS mixture of 8 cell preparations. (A) Levels of PGE_2 released by cells were determined on aliquots of conditioned media by ELISA. Values are mean ± SEM. ANOVA indicates a significant difference of $p < 0.0001$. Subtest analyses indicate significant differences between assays sharing the same letter: a, b, c, d, $p < 0.01$. (B) Representative Western blot analysis of COX-2 levels in subchondral osteoblasts from a patient with OA.

osteoblasts. ALPase activity was comparable to published data[10], and showed a typical response to hormonal challenge in these diseased cells. However, neither diacerein nor rhein affected this activity. In contrast, diacerein and rhein showed dose dependent inhibitory effects on $1,25(OH)_2D_3$ induced osteocalcin release. This is a very peculiar observation, since $1,25(OH)_2D_3$ signaling *per se* does not seem to be responsible for this situation, as $1,25(OH)_2D_3$ induced ALPase activity was not modified by these treatments, as was $1,25(OH)_2D_3$ induced osteocalcin, arguing in favor of direct activity of these drugs on osteocalcin expression. Indeed, osteocalcin mRNA levels were directly reduced by diacerein and rhein treatments, suggesting a selective role of these drugs on this pathway. As $1,25(OH)_2D_3$ was directly added to cells in *in vitro* culture, it would be surprising that either drug affected these levels to such extent as to modify osteocalcin expression without altering ALPase activity. Abnormally elevated osteocalcin levels have been observed in the bones of patients with OA even at sites distant from weight bearing joints[37,38] and we previously observed high osteocalcin levels in conditioned media from OA subchondral osteoblasts[10]. Hence, the inhibitory effect of these drugs is interesting, as osteocalcin is believed to be involved in the local modulation of bone formation. Indeed, osteocalcin knockout mice show better bones and increased variables of bone formation than wild-type animals, suggesting that osteocalcin retards bone formation/mineralization[39]. These data would suggest that diacerein and rhein prevent this effect. Of note, although OA bone tissue is sclerotic[1-3,7], it is also undermineralized[12,13,38,40], a situation that may be explained by osteocalcin levels. In humans, osteocalcin levels are elevated in high turnover situations, hence representing one of the coupling elements involved in this process. In contrast, neither diacerein nor rhein affected PTH dependent cAMP formation by these cells. This would indicate that not all cell markers of the osteoblast phenotype in these cells were modified by these drugs. Combined with no difference in cell growth/density (data not shown), this

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would suggest that at the doses used these drugs were not deleterious for osteoblasts. Conversely, other variables were affected more severely, indicating selective effects on specific pathways.

Rhein had a very small but significant stimulatory effect on IGF-1 levels. This finding is most interesting as it suggests that this drug could potentiate cell growth and/or differentiation of osteoblasts. However, as human OA osteoblasts have already been shown to have increased IGF-1 levels compared to normal osteoblasts[10], this could suggest that this agent acts on yet other pathways involved in the regulation of IGF-1 levels besides those affected by the disease *per se*. This small effect on IGF-1 levels could probably not stimulate osteocalcin levels in these cells, whereas the direct effect of diacerein and rhein on osteocalcin mRNA levels would translate into a more sustained inhibition. Conversely, uPA and PAI-1 levels were dose dependently inhibited by both diacerein and rhein, suggesting that both agents could affect bone remodeling via these systems. Indeed, the overall effect was a strong reduction in the uPA/PAI-1 ratio in these cells, a situation that would reduce overall uPA activity and hence contribute in retarding bone resorption. This reduction in uPA/PAI-1 activity could then translate into more mineralized bone formed, and hence reverse the trend observed in patients with OA[12,13,38,40]. In this respect, it is noteworthy that IL-6 levels were also reduced by both drugs at low or therapeutic concentrations. IL-6 is a key cytokine in bone remodeling as it directly stimulates osteoclasts to resorb bone[41,42]. Therefore, reducing IL-6 levels would contribute to curbing bone resorption. These findings are well in line with the report of Moore, *et al*[27], who showed that diacerein reduces IL-6 and other cytokines such as IL-1α and tumor necrosis factor (TNF)-α produced by surrounding granulomas at cartilage subcutaneous implant sites in a mouse model.

PGE_2 levels were markedly stimulated by both diacerein and rhein. A similar situation has been observed with human OA chondrocytes, and COX-2 levels were increased in these cells[20] as was also observed here. This could then be directly responsible for the increase in PGE_2 levels. Prostaglandins have very important roles in bone remodeling, as they can act both as bone inducing and bone resorbing agents, depending on their levels. Indeed, at lower levels prostaglandins are known to recruit mesenchymal osteoblast progenitor cells, whereas at higher doses they tend to stimulate bone resorption[43-45]. This effect is modulated by corticosteroid levels, which affect the capacity of PGE_2 to stimulate collagen type I formation by osteoblasts[44], a situation observed *in vivo* in OA bone tissue[12,13]. This could suggest that at lower doses, a stimulatory effect on bone formation could be observed, whereas as the concentration of the drugs increases, chances of inducing bone resorption increase. Furthermore, PGE_2 has recently been shown by us[17] and others[46,47] to elicit the synthesis of IL-6 in osteoblasts. As both diacerein and rhein stimulated PGE_2 levels several-fold, IL-6 levels would be expected to increase. However, this situation was not observed since IL-6 levels dropped in the presence of low doses of both drugs, but at the highest dose IL-6 levels returned to basal values. This could suggest that both diacerein and rhein are potent inhibitors of basal IL-6 production, and that they can prevent the stimulatory effect of PGE_2. Hence, the potential direct inhibition of IL-6 production by both agents coupled to their effect on PGE_2 production, which would increase IL-6 levels, would result in small effects, if any, as observed here. This would indicate that rhein and diacerein have to act on a potent inhibitory pathway of IL-6 production to overrule the regulation of PGE_2 [46,47]. The nature of this pathway remains to be investigated.

As both diacerein and rhein increase PGE_2 and IGF-1 and decrease osteocalcin levels, this may indicate that these agents may be promoting bone turnover when used *in vivo*. Bone sclerosis in OA is not due to a decrease in bone resorption, as indicators are not overly modified in these patients[13], but more likely to a role on collagen matrix deposition/mineralization, as proposed by Mansell and Bailey[12]. Therefore, the observed increase in PGE_2 levels in response to diacerein and rhein could promote bone resorption[41] and IGF-1 release[48], while lower osteocalcin levels would promote bone formation/mineralization. Indeed, OA subchondral osteoblasts produce 2 to 3-fold higher PGE_2 levels than normal human subchondral osteoblasts (unpublished observation), which could in part explain bone sclerosis, while increasing PGE_2 levels several-fold as observed with diacerein and rhein would lead to enhanced resorptive capacities, as observed with rodent models[43-45]. Furthermore, the decrease in uPA in response to these agents would retard bone formation by preventing the release of trapped growth factors, and prevent further sclerosis in this tissue. Indeed, as the OA bone tissue is undermineralized[12,13], reducing bone cell proliferation (reduction in uPA) while promoting bone mineralization (reduction in osteocalcin) would favor a more mineralized bone tissue in OA.

Our results indicate a potential direct beneficial role for both diacerein and rhein on the abnormal metabolic activities of OA subchondral osteoblasts, whereas these drugs do not overly affect cell integrity or phenotypic cell markers.

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