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# Rosiglitazone Induces Interleukin-1 Receptor Antagonist in Interleukin-1 –Stimulated Rat Synovial Fibroblasts via a Peroxisome Proliferator–Activated Receptor / –Dependent Mechanism

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**Objective.** To study the potency of 2 peroxisome proliferator–activated receptor (PPAR) agonists, 15-deoxy-<sup>12,14</sup>-prostaglandin J<sub>2</sub> (15-deoxy-PGJ<sub>2</sub>) and rosiglitazone, to modulate the expression of interleukin-1 receptor antagonist (IL-1Ra) in rat synovial fibroblasts.

**Methods.** Levels of messenger RNA for IL-1Ra and PPAR isotypes (α, β, γ) were assessed by real-time polymerase chain reaction in rat synovial fibroblasts exposed to 10 ng/ml of IL-1. PPAR levels were assessed by Western blotting and secreted IL-1Ra levels by immunoassay. The potency of PPAR agonists and the PPAR/agonist GW-501516 on IL-1Ra levels was tested in the range of 1–10 nM and at 100 pM, respectively. The contribution of PPAR to the effects of rosiglitazone on IL-1Ra secretion was examined either by its overexpression or by inhibition using wild-type or dominant-negative constructs and the antagonist GW-9662 (10 nM), respectively. The dominant-negative strategy was also performed to investigate the possible contribution of PPARγ and NF-κB activation.

**Results.** IL-1–induced IL-1Ra production was increased by 10 nM rosiglitazone but was reduced dose-dependently by 15-deoxy-PGJ<sub>2</sub>. Both agonists lowered IL-1 secretion, but rosiglitazone alone reduced the imbalance of IL-1/IL-1Ra toward basal levels. Enhancement of IL-1–induced IL-1Ra production by rosiglitazone was not affected by PPAR overexpression or by its inhibition with dominant-negative PPAR or GW-9662. Inhibition of NF-κB was also ineffective against rosiglitazone but abolished the stimulating effect of IL-1 on IL-1Ra. All PPAR isotypes were expressed constitutively in rat synoviocytes, but PPARα decreased dramatically upon IL-1 exposure, whereas PPARβ remained stable. Dominant-negative PPARγ abolished the enhancement of IL-1Ra by rosiglitazone, whereas GW-501516 reproduced the effect of rosiglitazone on IL-1Ra secretion.

**Conclusion.** Rosiglitazone stimulates IL-1Ra production by a PPARγ mechanism in activated rat synovial fibroblasts, further contributing to its potential antiarthritic properties and opening new perspectives for the modulation of inflammatory genes by specific PPAR agonists in articular cells.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by persistent synovitis, with synovial hypertrophy secondary to dysregulated proliferation of synovial cells, and infiltration by peripheral blood mononuclear cells. Resident as well as infiltrating cells produce numerous inflammatory cytokines, including tumor necrosis factor (TNF) and interleukin 1 (IL-1), which play a central role in the pathogenesis of RA (1–4). Thus, agents that reduce the production and/or activity of IL-1 and TNF are now used in rheumatology practices for the treatment of severe inflammatory diseases (5–7).

The production and biologic activity of IL-1 are highly regulated events (8), ranging from the control of IL-1 gene expression, protein synthesis, and processing by IL-1–converting enzyme (caspase 1) to the variable expression of cell surface receptors and their soluble counterparts or the neutralization of the effects of IL-1 by a natural receptor antagonist (IL-1Ra). The IL-1 receptor family is composed of 2 receptors that have different physiologic roles: the type 1 receptor (IL-1RI), which transduces a signal upon IL-1 binding, and the type 2 receptor (IL-1RII), which fails to transduce signal and has been called a “decoy” receptor (8). When IL-1 binds to IL-1RI, a complex is formed, which then binds to the IL-1R accessory protein, which is essential to IL-1 signaling through the formation of a high-affinity binding complex (9). Indeed, IL-1RI is incapable of signal transduction on its own (10), whereas the high-affinity complex further recruits the adaptor protein MyD88 and the IL-1R–associated kinase to generate intracellular events through signaling pathways that are not restricted to IL-1. Modulating IL-1 at the presignaling level, for example, by interfering with its binding on specific membrane receptors, is therefore a promising approach that has high therapeutic relevance (6).

IL-1Ra, a member of the IL-1 gene family, is the first natural antagonist identified in cytokine receptor studies that inhibits the effects of IL-1 on target cells because of its inability to recruit IL-1R accessory protein upon binding to IL-1RI

(11). As a consequence, IL-1Ra can have both systemic and cellular effects by inhibiting IL-1 and IL-1. Furthermore, IL-1Ra binds to IL-1RII with an affinity that is 100–500 times lower than its affinity to bind to IL-1RI, thus contributing to the reduction of IL-1 signal transduction (12). Despite the close affinity of IL-1 and IL-1Ra for IL-1RI, an excess of IL-1Ra is required to inhibit IL-1 activity (8), and the imbalance between IL-1Ra and IL-1 may predispose to the perpetuation of inflammation in human (13) and experimental (14) arthritis. Indeed, both systemic infusion of large amounts of IL-1Ra (11) and local overexpression of IL-1Ra by gene transfer (15) reduced the severity of experimental arthritis and the progression of experimental osteoarthritis, respectively. Similarly, mice deficient in the IL-1Ra gene were shown to spontaneously develop a chronic inflammatory arthropathy that resembled RA (16), further highlighting the pivotal role of IL-1Ra in counterbalancing the activity of IL-1 in the body.

Peroxisome proliferator-activated receptors (PPARs; isotypes  $\alpha$ ,  $\beta$ ,  $\gamma$ ) are ligand-inducible nuclear transacting factors (17). PPAR heterodimerizes with retinoid X receptor (18) and binds to peroxisome proliferator-activated receptor response element (PPRE) located in the promoter region of PPAR target genes. These lipid-sensitive receptors can be activated in a variable isotype-specific manner by natural fatty acids, leukotrienes, prostaglandins, and some synthetic agonists, including the antidiabetic drugs (thiazolidinediones [TZDs]), which have recently emerged as modulators of inflammation (17,19,20). Indeed, several recent studies have shown that activation of PPAR  $\gamma$ , either by endogenous ligands, such as

15-deoxy-<sup>12,14</sup>-prostaglandin J<sub>2</sub> (15-deoxy-PGJ<sub>2</sub>), or by

TZD had antiinflammatory potencies in rodent models of

arthritis (21–24). At the cellular level, 15-deoxy-PGJ<sub>2</sub> and TZD were shown to inhibit the transcriptional induction of genes thought to contribute to joint pathology, such as TNF (19,20), IL-1 (20), gelatinase B (19), inducible nitric oxide synthase, and matrix metalloproteinase 13 (25). PPAR  $\beta$  is the less well-characterized PPAR isotype, despite its ubiquitous expression. It appears to take part in reverse transport of cholesterol, wound healing, cell proliferation, and apoptosis, although its contribution to inflammation remains largely unknown (26). A highly selective PPAR  $\beta$  agonist has been synthesized only very recently (27).

We previously demonstrated that PPARs  $\alpha$  and  $\beta$  were expressed constitutively in rat synovial fibroblasts and that PPAR  $\alpha$  agonists inhibited TNF and IL-1 gene expression through interaction with the NF- $\kappa$ B signaling pathway (28). However, we hypothesized that the antiarthritic potency of PPAR ligands could also be supported by their ability to interfere with cytokine presignaling, either by increasing receptor shedding or, in the case of IL-1, by the synthesis of its natural antagonist. Consistent with our hypothesis, a recent study showed that TZD enhanced IL-1Ra secretion in monocytic cells stimulated with phorbol myristate acetate (PMA) (29), further suggesting that PPAR agonists could contribute to cytokine neutralization in addition to inhibiting their expression.

Therefore, we studied the ability of 2 PPAR agonists, the natural low binding-affinity compound

15-deoxy-PGJ<sub>2</sub> and the synthetic high binding-affinity TZD rosiglitazone, to modulate IL-1Ra in rat synovial fibroblasts stimulated with homologous IL-1. This cell type was shown to be unable to produce enough IL-1Ra to counteract IL-1 during the course of RA (13,30) and to be highly responsive to IL-1, which plays a key pathophysiologic role in arthritis (1). Under these experimental conditions, we showed an opposite effect of PPAR agonists on the expression and secretion of IL-1Ra as well as on the balance between IL-1 and IL-1Ra. We also explored the mechanisms supporting the induction of IL-1Ra by rosiglitazone. Overexpression of wild-type or dominant-negative forms of PPAR  $\alpha$ , as well as the use of a specific PPAR  $\alpha$  antagonist, failed to modify the impact of rosiglitazone on IL-1Ra secretion. Rosiglitazone-induced IL-1Ra secretion was unaffected by transfection with a dominant-negative vector of NF- $\kappa$ B, whereas it was suppressed in the presence of a dominant-negative vector of PPAR  $\beta$ . A PPAR  $\beta$ -dependent induction of IL-1Ra secretion by rosiglitazone was further supported by the stimulating effect of the PPAR  $\beta$  agonist GW-501516 and the high and invariable expression of PPAR  $\beta$  in IL-1-stimulated cells.

## MATERIALS AND METHODS

**Isolation and culture of synovial fibroblasts.** Synovial tissues were obtained surgically from the knee joints of male Wistar rats weighing 130–150 gm (Charles River, L'Arbresle, France) that had been killed under anesthesia. Synovial fibroblasts were obtained by sequential digestion with Pronase and collagenase B (Roche Molecular Biochemicals, Meylan, France) as described previously (28). The cells were washed 2 times in phosphate buffered saline (PBS) and cultured to confluence in 75-cm<sup>2</sup> flasks at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The medium used was Dulbecco's modified Eagle's medium–Ham's F12 supplemented with L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 g/ml), amphotericin B (50 ng/ml), and 10% heat-inactivated fetal calf serum (FCS) (Invitrogen, Cergy-Pontoise, France). Synovial cells were subcultured under similar conditions and were used between passages 3 and 6, which corre-

**RNA extraction and real-time polymerase chain reaction (PCR) analysis.** Total RNA from cultured synoviocytes was isolated using TRIzol (Invitrogen). Two micrograms of total RNA was reverse-transcribed for 90 minutes at 37°C using hexamer random primers and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen). To quantify IL-1Ra, PPAR $\alpha$ , PPAR $\beta$  and PPAR $\gamma$  mRNA expression, a real-time quantitative PCR was performed using LightCycler (Roche Molecular Biochemicals) technology.

Primer sequences and annealing temperatures were as follows: for IL-1Ra, 5'-GACCTTCTACCTGAGGAACAA-CCA-3' (forward) and 5'-AAGAACACATTCCGAAAGT-CAATAGG-3' (reverse) at 62°C; for PPAR $\alpha$ , 5'-ACTATGGA-GTCCACGCATGTGA-3' (forward) and 5'-TTGT-GGTACGCCAGCTTTAGC-3' (reverse) at 55°C; for PPAR $\beta$ , 5'-CCAGCAGCTGCACAGACCTCTC-3' (forward) and 5'-ATCTGCAGCTGGTCCAGCA-3' (reverse) at 58°C; for PPAR $\gamma$ , 5'-GCTGGTCGATACTACTGGAGATC-3' (forward) and 5'-CACAAATGCCATCAGGTTTGG-3' (reverse) at 55°C; and for S29, 5'-AGATGGGTCACCAGCAGCTCTACTG-3' (forward) and 5'-AGACGCGCAAGAGCGAGAA-3' (reverse) at 59°C.

PCR was performed with the SYBR Green Master Mix system (Qiagen, Courtaboeuf, France). Melting curve analysis was performed after amplification to determine the melting temperature of the specific PCR products, and product sizes were examined on a 2% agarose gel stained with ethidium bromide (0.5 g/ml). Each run included standard dilutions and positive and negative reaction controls. The mRNA level of the gene of interest and of the ribosomal protein S29, which was chosen as the housekeeping gene, were determined in parallel for each sample. Results were expressed as the normalized ratio of the mRNA level of each gene of interest to that of the S29 gene.

**Transient transfection experiments.** Synovial fibroblasts were seeded in 6-well plates at  $5 \times 10^5$  cells/well and grown to 80% confluence. Cells were transfected with either

500 ng of a PPAR $\alpha$  expression vector (pcDNA3.1 PPAR $\alpha$ ; a generous gift from Dr. H. Fahmi, Centre Hospitalier de l'Université de Montréal, Montreal, Quebec, Canada), 500 ng of a dominant-negative vector of PPAR $\alpha$  (PPAR $\alpha$  mutated in mutants), cells were grown and maintained in 1% FCS (low FCS) culture medium.

**Study design.** Synoviocytes were cultured in low FCS culture medium in the presence or absence (vehicle alone,

0.1% of DMSO final concentration) of PPAR ligands, which were added at the same time as 10 ng/ml of interleukin-1 (Sigma, St. Quentin Fallavier, France). The level of messenger RNA (mRNA) for IL-1Ra was determined 12 hours after IL-1 challenge, whereas the level of secreted IL-1Ra was assayed in supernatants at 24 hours. Expression of PPAR isotypes was studied on synoviocytes stimulated with 10 ng/ml of IL-1 for 6 hours (mRNA analysis) or for 12 hours (protein analysis). The PPAR ligands used were rosiglitazone (1, 3, or

10  $\mu$ M; Cayman Chemical, Ann Arbor, MI), 15-deoxy-PGJ<sub>2</sub> (1, 3, or 10  $\mu$ M; Calbiochem, Meudon, France), the PPAR antagonist GW-9662 (10  $\mu$ M; Cayman Chemical), and the PPAR $\alpha$  agonist GW-501516 (100 pM; Alexis Biochemicals, Paris, France).

by Gurnell et al [32]; a generous gift from Dr. M. T. Corvol,

Unité Mixte de Recherche INSERM 530, Université Paris V, Paris, France), 500 ng of a dominant-negative vector of PPAR $\beta$  (PPAR $\beta$  mutated in the loop preceding the AF-2 domain [Glu<sup>411</sup>Pro] as described by Grimaldi et al [33]; a generous gift from Dr. P. A. Grimaldi, INSERM U470, Université de Nice-Sophia Antipolis, Nice, France), 500 ng of a dominant-negative vector of NF- $\kappa$ B (NF- $\kappa$ B mutated [Ala<sup>32</sup>, Ala<sup>36</sup>]; Clontech, Palo Alto, CA), or 1  $\mu$ g of PPRE-Luc and 1  $\mu$ g of NF- $\kappa$ B-Luc (a generous gift from Dr. H. Fahmi) for gene reporter assays.

Transfections were performed for 2 hours using 10  $\mu$ l of polyethyleneimine reagent (Euromedex, Souffelweyer-sheim, France) in 1 ml of complete medium. Twenty-four hours after transfection, cells were stimulated with IL-1 for 24 hours in the presence or absence of PPAR agonists. **Gene reporter activity.** After transient transfection with plasmid reporter as described above, cells were harvested in Cell-Culture Lysis Reagent (Promega, Charbonnières, France) before measurement of luciferase activity according to the recommendations of the manufacturer (Promega). **Western blot analysis of PPAR isotypes.** Cells exposed to IL-1 were washed twice with ice-cold PBS and scraped off the flask in cold lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mg/ml of leupeptin, and 1 mM phenylmethyl-sulfonyl fluoride). Cells were disrupted by sonication and centrifuged at 3,000 revolutions per minute for 10 minutes. The supernatants were collected, and the protein concentration was determined by an assay based on the method of Bradford. Protein samples (25  $\mu$ g) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) and electroblotted onto polyvinylidene difluoride membrane. After 1 hour in blocking buffer (Amersham Bio-sciences, Orsay, France), membranes (Immobilon; Waters, St. Quentin-en-Yvelines, France) were blotted overnight at 4°C with antibodies against PPAR $\alpha$  (Tebu, Le Perray-en-Yvelines, France), against PPAR $\beta$  or PPAR $\gamma$  (generous gifts from Professor Michel Dauca, Université Henri Poincaré, Vandoeuvre-les-Nancy, France), and against  $\beta$ -actin (Sigma); all antibody dilutions were 1:1,000. After 3 washings with Tris buffered saline (TBS)-Tween, the blot was incubated with anti-rabbit IgG conjugated with horseradish peroxidase (Cell Signaling, Beverly, MA) at a dilution of 1:2,000 in blocking buffer for 1 hour at room temperature. After 4 washings with TBS-Tween, protein bands were detected by chemiluminescence with the Phototope Detection system according to the manufacturer's instructions (Cell Signaling).

**Quantitative determination of IL-1Ra and IL-1 in cell culture medium.** Levels of IL-1Ra and IL-1 were measured in culture supernatants according to the manufacturer's instructions (BioSource International, Camarillo, CA, and R&D Systems, Minneapolis, MN, respectively), using solid-phase sandwich enzyme-linked immunosorbent assays (ELISAs). Absorbance was read at 450 nm on a microplate reader (Multiscan; Labsystems, Montigny-le-Bretonneux, France). The limits of detection were 12 pg/ml for IL-1Ra and

5 pg/ml for IL-1. The assays showed no cross-reactivity between rat IL-1, IL-1 $\beta$ , and IL-1Ra (manufacturers' data). Positive controls provided by the manufacturers were used in each experiment.

**Statistical analysis.** Results are expressed as the mean  $\pm$  SD of at least 3 assays. Comparisons were made by analysis of variance, followed by Fisher's protected least significant difference post hoc test using StatView version 5.0 software (SAS Institute, Cary, NC). *P* values less than 0.05 were considered significant.

## RESULTS

**Effects of PPAR agonists on IL-1Ra expression in IL-1-stimulated synoviocytes.** Preliminary experiments using an MTT assay showed no modification of cell viability and proliferation in rat synovial fibroblasts treated with IL-1 (10 ng/ml) and/or PPAR agonists in the concentration range tested. Under the experimental conditions we used, untreated cells showed a basal level of mRNA for IL-1Ra that was unaffected by rosiglitazone (10  $\mu$ M) but was reduced by 15-deoxy-PGJ<sub>2</sub> at the same concentration (Figure 1A). Similar effects on basal secretion of IL-1Ra were observed (Figure 1B).

Stimulation with IL-1 induced IL-1Ra mRNA expression; this effect was decreased by 15-deoxy-PGJ<sub>2</sub>, but slightly increased by rosiglitazone (Figure 1A). Rosiglitazone also increased (2.5-fold) the stimulating effect of IL-1 on IL-1Ra secretion, while 15-deoxy-PGJ<sub>2</sub> suppressed the IL-1Ra level below the control level in IL-1-stimulated cells (Figure 1B). Previous dose-ranging experiments showed that rosiglitazone stimulated IL-1-induced IL-1Ra secretion only at the highest concentration used (10  $\mu$ M), whereas 15-deoxy-PGJ<sub>2</sub> had a close dose-related inhibitory effect, and that other agonists of PPAR isotypes (troglitazone at 1, 3, or 10  $\mu$ M) and (Wy-14,643 at 1, 10, or 100  $\mu$ M) had no effect on this parameter (data not shown).

**Effects of PPAR agonists on IL-1 production.** To investigate effects of PPAR ligands on IL-1 secretion, experimental conditions were adapted as follows: after 12 hours of stimulation with exogenous rat IL-1, culture medium was replaced by fresh medium without IL-1 to check for endogenous IL-1 production in the culture supernatant. No spontaneous secretion of IL-1 was detected under these conditions, but IL-1 challenge increased both IL-1Ra and IL-1 levels (Table 1), although to a different extent. Indeed, IL-1Ra secretion was increased 7-fold, whereas IL-1 secretion was increased at least 47-fold, leading to an increased ratio of IL-1 to IL-1Ra as compared with basal conditions. PPAR agonists failed to affect IL-1 secretion in resting cells, whereas again, 15-deoxy-PGJ<sub>2</sub> reduced the basal secretion of IL-1Ra (Table 1). In IL-1-stimulated cells, rosiglitazone and 15-deoxy-PGJ<sub>2</sub> reduced IL-1 secretion to a similar extent, but confirmed their opposite effects on IL-1Ra secretion, with slight stimulation by rosiglitazone and strong inhibition by 15-deoxy-PGJ<sub>2</sub> (Table 1). As a consequence, rosiglitazone lowered the ratio of IL-1 to IL-1Ra toward basal values, whereas 15-deoxy-PGJ<sub>2</sub> aggravated the imbalancing effect of IL-1 on this ratio.

**Effects of PPAR modulation on rosiglitazone-induced IL-1Ra secretion.** In the presence of the specific PPAR antagonist GW-9662 (10  $\mu$ M), the stimulating effect of rosiglitazone on IL-1-induced IL-1Ra secretion was not appreciably changed (Figure 2A). Overexpression of PPAR was ineffective in modulating both basal and IL-1-stimulated IL-1Ra secretion as compared with cells transfected by an empty vector (Figure 2B). In cells overexpressing PPAR, the stimulating effect of rosiglitazone on IL-1Ra secretion was similar to that in controls (Figure 2B). In synoviocytes transfected with a dominant-negative form of PPAR 24 hours before IL-1 exposure, the stimulating effect of rosiglitazone on IL-1Ra secretion was not appreciably modified (Figure 2B).

Control experiments showed that PPRE-Luc activity was enhanced by rosiglitazone in PPAR-overexpressing cells, thus demonstrating that overexpression was effective. As shown in Figure 2C, inhibition of PPAR by either the specific antagonist GW-9662 (10

$\mu$ M) or the dominant-negative form of PPAR were both effective in our model. Under these experimental conditions, the ability of rosiglitazone to decrease IL-

1-induced nitrite levels in culture supernatants was significantly reduced by transfection with the dominant-negative form of PPAR while being triggered by transfection with its wild-type form (data not shown).

**Effects of NF- $\kappa$ B inhibition on rosiglitazone-induced IL-1Ra secretion.** In synoviocytes transfected with a dominant-negative form of NF- $\kappa$ B 24 hours before IL-1 challenge, the stimulating effect of IL-1 on IL-1Ra secretion was abolished (Figure 3A). However, the potentiating effect of rosiglitazone on the IL-1Ra level was essentially unaffected by inhibition of NF- $\kappa$ B activation (Figure 3A). Control experiments confirmed that dominant-negative NF- $\kappa$ B was able to reverse the modulation of NF- $\kappa$ B activation by IL-1 in synoviocytes transfected with NF- $\kappa$ B-Luc (Figure 3B).

**Pattern of expression of PPAR isotypes in IL-1-stimulated synoviocytes.** Real-time PCR (Figure 4A) and Western blot (Figure 4B) analyses of PPARs demonstrated that all isotypes were constitutively expressed in rat synovial fibroblasts. In IL-1-stimulated cells, PPAR $\alpha$  and PPAR $\gamma$  expression remained essentially unchanged, whereas PPAR $\delta$  decreased dramatically, both at the mRNA and protein levels (Figures 4A and B).

**Contribution of PPAR $\delta$  to rosiglitazone-induced IL-1Ra secretion.** In synoviocytes transfected with a dominant-negative form of PPAR $\delta$ , the stimulating effect of rosiglitazone was abolished, which is contrary to the results of control experiments with a dominant-negative form of PPAR (Figure 5A). In both cases, the effect of IL-1 on IL-1Ra secretion was maintained. The PPAR $\delta$  agonist GW-501516 (100 pM) reproduced the stimulating effect of rosiglitazone on IL-1Ra secretion in IL-1-stimulated cells, without modifying its basal level.

## DISCUSSION

Several recent studies have highlighted the anti-inflammatory properties of PPAR agonists, either 15-deoxy-PGJ<sub>2</sub> or TZD, in experimental models of acute digestive (23) or periarticular (22) inflammation, as well as chronic polyarthritis

(21,24). Interestingly, it was recently demonstrated in the carrageenan-induced pleurisy model that rosiglitazone reduced pleural exudate volume and mononuclear cell infiltration (22), as was previously shown for 15-deoxy-PGJ<sub>2</sub> (34), which was therefore proposed to promote the resolution of inflammation (35). Within joints, several types of cells could support the antiinflammatory potency of PPAR agonists, since this PPAR isotype is expressed constitutively in synoviocytes (24), macrophages (19), and chondrocytes (36). In addition, each cell type has pathophysiologic relevance to joint inflammation by their production of mediators of inflammation or matrix-degrading enzymes and can therefore be considered a pharmacologic target for PPAR agonists (37). Consistent with this idea, PPAR ligands have been shown to decrease the expression of TNF in activated synoviocytes (28) and macrophages (19) and the production of various matrix metalloproteinases in chondrocytes (25,38) and synoviocytes (39). Synoviocytes may play a crucial role because of their ability to proliferate under inflammatory conditions (40) and to cooperate with both macrophages in the synovial pannus and chondrocytes at the synovium–cartilage junction (41). In addition, the increased expression of proinflammatory cytokines within the rheumatoid synovium (42) is accompanied by an imbalance between IL-1 and its natural antagonist IL-1Ra in synoviocytes (13), further explaining the major contribution of IL-1 to cartilage destruction. This led us to study the potency of PPAR agonists to restore the balance between IL-1 and IL-1Ra in synovial fibroblasts.

In the present study, we confirmed that synoviocytes stimulated with IL-1 produced increased amounts of IL-1Ra (43,44), as has also been reported for mononuclear phagocytes (45) and articular chondrocytes (44). However, this potent negative-feedback loop was not sufficient by itself to counterregulate the effects of IL-1, since IL-1 also stimulated the production of IL-1 and to a greater extent, as illustrated by the increased ratio of IL-1 to IL-1Ra in stimulated cells. Thus, our experimental model reproduces part of the well-described cytokine imbalance reported in RA (13) and other inflammatory situations (46).

Under the conditions used in the present study, the synthetic PPAR agonist rosiglitazone and the natural PPAR agonist 15-deoxy-PGJ<sub>2</sub> showed opposite effects on IL-1Ra secretion, although both agonists

reduced nitric oxide production. Such differences between natural and synthetic PPAR ligands is not surprising, since it has been reported in other cell types, including chondrocytes (47–49). In synoviocytes, rosiglitazone partly restored the imbalance between IL-1 and IL-1Ra, whereas 15-deoxy-PGJ<sub>2</sub> triggered the deleterious effects of IL-1 because of its ability to decrease the production of IL-1Ra more efficiently than that of IL-1. This result is in contrast with the work of Meier et al (29), who demonstrated that in THP-1 cells stimulated with PMA, both rosiglitazone and 15-deoxy-PGJ<sub>2</sub>

stimulated the production of IL-1Ra, whereas only

15-deoxy-PGJ<sub>2</sub> reduced the production of IL-1. Such a discrepancy could be ascribed to differences in cell types (monocytic versus mesenchymal), differences in the nature of the inflammatory stimulus (cytokine versus tumor inducer), or both, since biologic responses to PPAR agonists are thought to be largely cell-specific (20).

In synoviocytes, 15-deoxy-PGJ<sub>2</sub> and rosiglitazone are also distinguished by their ability to affect IL-1Ra production in resting or activated cells. Indeed, we found that 15-deoxy-PGJ<sub>2</sub> was active independently of cell stimulation, whereas rosiglitazone required challenge with IL-1 in order to be effective. This result confirmed that the induction of IL-1Ra secretion by rosiglitazone depended upon prior cell stress (29), although it may be supported by the modulation of IL-1–specific signaling pathways. Finally, the inhibitory effect of 15-deoxy-PGJ<sub>2</sub> on IL-1Ra secretion (Figure

1B) paralleled its inhibitory potency on IL-1Ra mRNA (Figure 1A), whereas rosiglitazone was essentially active with regard to IL-1Ra production. Although not supported by previous studies on the stabilization of mRNA by PPAR activators (50,51), some posttranscriptional/translational regulation of IL-1Ra by rosiglitazone cannot be excluded, since it was recently shown to contribute to the control of human immunodeficiency virus type 1 replication by PPAR agonists (52). This result confirms the apparent discrepancy between the reported levels of mRNA and protein in inflamed synovium for at least 2 IL-1Ra isoforms (14).

Modulation of the function of PPAR was performed to investigate whether the effects of rosiglitazone on IL-1–induced IL-1Ra secretion was attributable to activation of this isotype. Overexpression of either the functional or dominant-negative forms of PPAR, as well as use of the specific antagonist GW-

9662, did not alter the potency of rosiglitazone to stimulate IL-1Ra secretion. Although not investigated in the present study, PPAR-independent effects have been reported largely for 15-deoxy-PGJ<sub>2</sub>, with direct

links to inhibition of NF- $\kappa$ B transactivation (53) or

modulation of oxidative stress (54). However, rosiglitazone is one ligand that has a high affinity for PPAR (55), which was shown to modulate PPAR-dependent genes in experimental diabetes (56) and atherosclerosis (57). Therefore, we hypothesized that rosiglitazone would modulate alternate molecular targets when used at the (micromolar) concentration necessary to enhance IL-1Ra secretion.

To examine this hypothesis, we first checked for

the possible modulation of the NF- $\kappa$ B pathway. As has been reported in studies of IL-1–stimulated synoviocytes (58) and PMA-stimulated THP-1 cells (59), we demonstrated that activation of the NF- $\kappa$ B pathway contributed to the stimulating effect of IL-1 on IL-1Ra, since transient transfection with dominant-negative NF- $\kappa$ B abolished IL-1–induced IL-1Ra secretion. However, dominant-negative NF- $\kappa$ B failed to modify the rosiglitazone-induced enhancement of IL-1Ra secretion in synoviocytes, which demonstrates that this pathway was not primarily involved despite its requirement for

cellular activation. Based on previous studies on the human IL-1Ra promoter, one can suggest that rosiglitazone could have affected alternate IL-1-sensitive trans-acting factors such as CCAAT/enhancer binding proteins or activator protein 1 (60,61).

Second, we searched for the possible contribution of PPAR  $\gamma$ , since high-dose rosiglitazone was shown to activate the PPAR-responsive promoter in cells expressing PPAR  $\gamma$  but not PPAR  $\alpha$  (19), whereas it inhibited inflammatory genes through activation of the PPAR  $\alpha$  isotype in macrophages (62). This hypothesis was further supported by the inability of other PPAR (troglitazone) and PPAR (Wy-14,643) agonists to affect IL-1-induced IL-1Ra production. We demonstrated that PPAR  $\gamma$  was expressed constitutively in synovial fibroblasts, both at the mRNA and protein levels, and that its expression was not changed by cellular activation. In contrast, we showed that PPAR  $\alpha$  level decreased dramatically in activated synoviocytes, thus confirming that this isotype was regulated negatively by inflammatory stimuli in articular cells (28). Such low levels of PPAR in inflammatory conditions would likely favor the binding of rosiglitazone to PPAR  $\gamma$ , despite its low affinity for this isotype (55). Consistent with a PPAR  $\gamma$ -dependent mechanism, we demonstrated that induction of IL-1Ra secretion by rosiglitazone was abolished by transfection with a dominant-negative form of PPAR  $\gamma$ . We showed further that a low concentration of GW-501516, a highly selective PPAR  $\gamma$  agonist (27,63,64), reproduced the stimulating effect of high-dose rosiglitazone on IL-1Ra secretion. Taken together, these data demonstrate that rosiglitazone enhanced IL-1Ra secretion in a PPAR  $\gamma$ -dependent manner and that this likely occurred because its relative affinity for PPAR isotypes was counterbalanced by the pattern of expression of PPAR isotypes in response to IL-1 stimulation.

In conclusion, findings of the present study show that the PPAR  $\alpha$  agonists rosiglitazone and 15-deoxy-PGJ<sub>2</sub> had opposite effects on IL-1Ra production by IL-1-stimulated rat synovial fibroblasts. Enhancement of IL-1Ra secretion by rosiglitazone tended to normalize the imbalance between IL-1 and IL-1Ra in activated cells, suggesting that it could contribute to the anti-inflammatory properties of this molecule in experimental polyarthritis. However, stimulation of IL-1Ra by rosiglitazone was supported neither by activation of PPAR nor by modulation of the NF- $\kappa$ B pathway, but by activation of PPAR  $\gamma$ . At dosages required to enhance IL-1Ra secretion in synovial fibroblasts, the cellular response to rosiglitazone was likely influenced by the pattern of expression of PPAR isotypes, which changed dramatically in response to IL-1 challenge. Although further studies are required to elucidate the molecular events that contribute to the control of IL-1Ra by PPAR  $\gamma$ , our data suggest that activation of PPAR  $\gamma$  may open new perspectives for the modulation of inflammatory genes in articular cells.

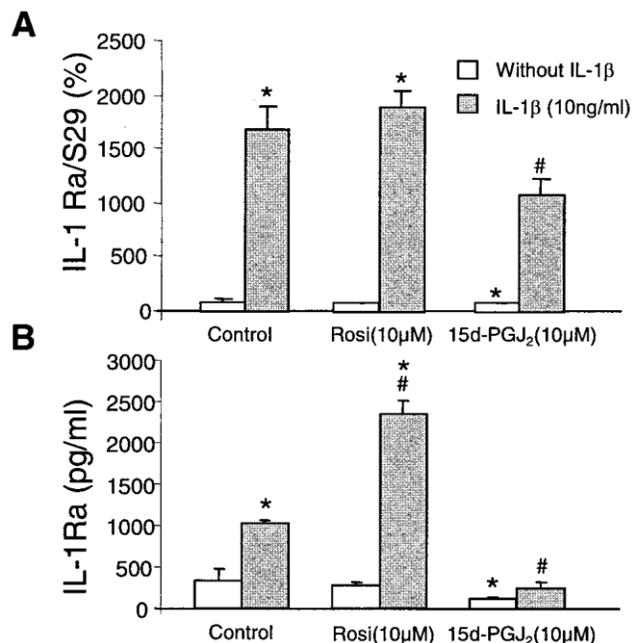
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**Figure 1.** Modulation of interleukin-1 receptor antagonist (IL-1Ra) expression by peroxisome proliferator-activated receptor agonists in rat synovial fibroblasts. Synoviocytes were incubated with 10 M 15-deoxy-<sup>12,14</sup>-prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), 10 M rosiglitazone (Rosi), or 0.1% DMSO in the presence or absence of 10 ng/ml of IL-1. **A**, Messenger RNA levels at 12 hours, as demonstrated by real-time polymerase chain reaction analysis of total RNA. Data represent the ratio of IL-1Ra mRNA to S29 mRNA and are expressed as the percentage relative arbitrary units, where control represents 100%. **B**, Protein secreted into culture supernatants at 24 hours, as measured by enzyme-linked immunosorbent assay. Values are the mean SD (n = 3 experiments). \* P < 0.05 versus control; # P < 0.05 versus IL-1 treatment.





**Table 1.** Secretion of IL-1 and IL-1Ra by IL-1 –stimulated synoviocytes\*

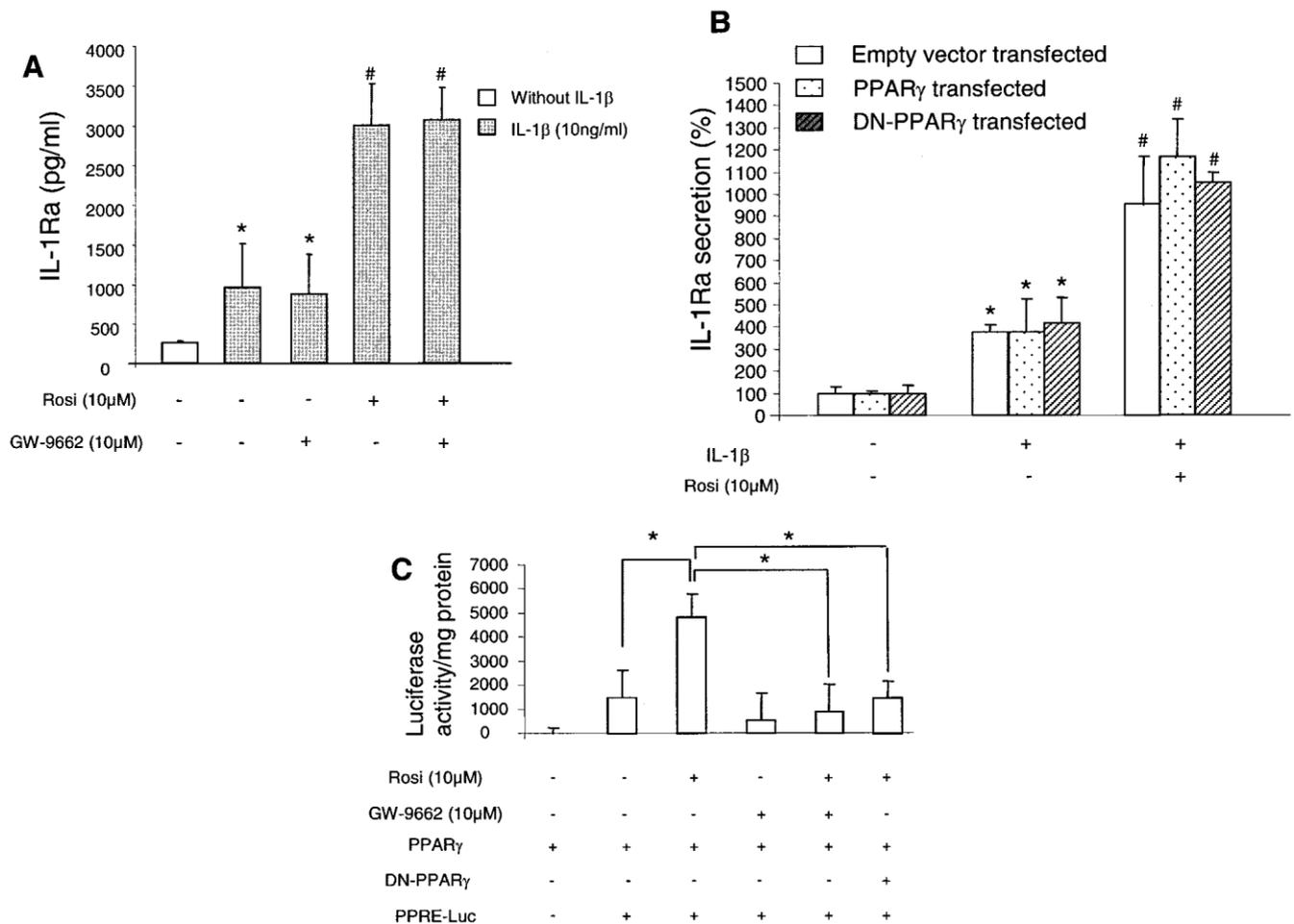
IL-1 secretion, pg/ml	IL-1Ra secretion, pg/ml	IL-1:IL-1Ra ratio
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Control	5	38	12	0.14	
IL-1, 10 mg/ml	237	18†	249	64†	0.95
Rosiglitazone, 10 M					
Alone	5		32	15	
Plus IL-1	0.16				
	128	23†‡	309	90†	
	0.41				
15-deoxy-PGJ <sub>2</sub> , 10 M					
Alone	5		18	5†	
Plus IL-1	0.28				
	137	7†‡	113	64†‡	
	1.21				

\* Values are the mean ± SD of 3 experiments. IL-1 = interleukin-1; IL-1Ra = interleukin-1 receptor antagonist; 15-deoxy-PGJ<sub>2</sub> = 15-deoxy-<sup>12,14</sup>-prostaglandin J<sub>2</sub>.

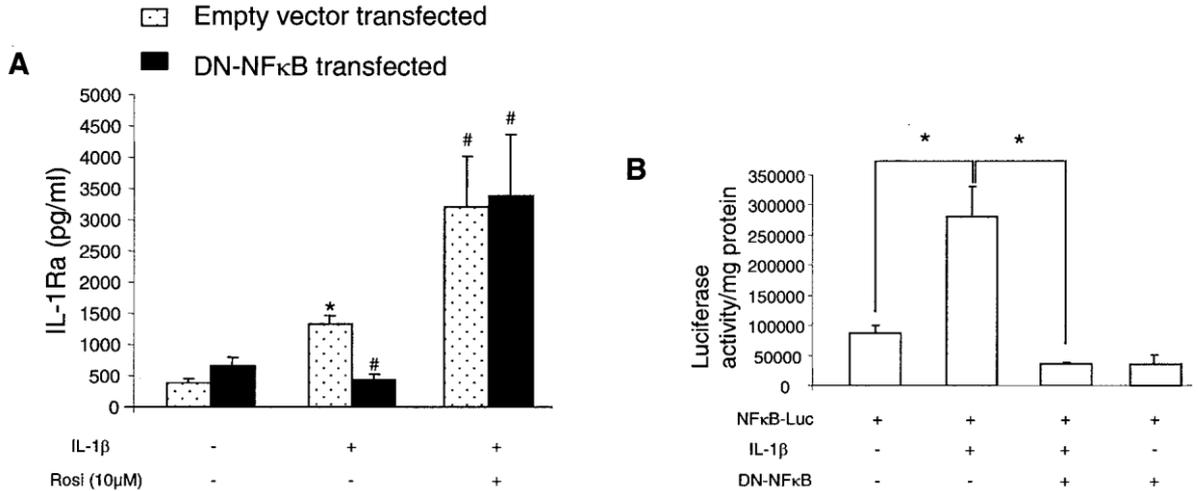
† *P* < 0.05 versus control.

‡ *P* < 0.05 versus IL-1 treatment.

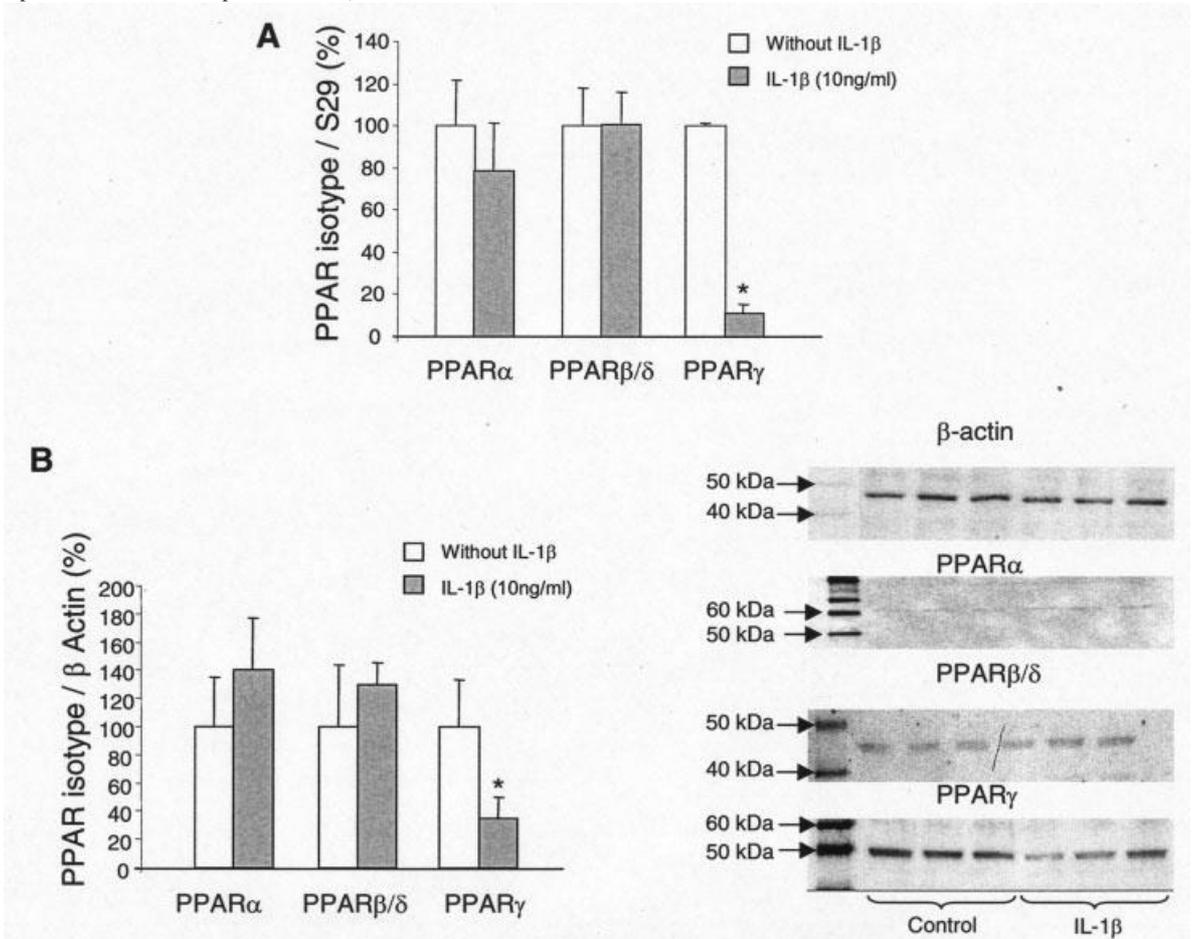


**Figure 2.** Effects of peroxisome proliferator-activated receptor (PPAR) modulation on rosiglitazone (Rosi)-induced interleukin-1 receptor antagonist (IL-1Ra) secretion by synovial fibroblasts. **A**, Effect of the PPAR-specific antagonist GW-9662 on IL-1Ra secretion into culture supernatants, as measured by enzyme-linked immunosorbent assay (ELISA). Cells were stimulated for 24 hours with 10 ng/ml of IL-1 in the presence or absence of 10 M PPAR ligands. # *P* < 0.05 versus IL-1 treatment. **B**, Effect of PPAR wild-type and dominant-negative (DN) transfection on rosiglitazone-induced IL-1Ra secretion by synovial fibroblasts. Transfected cells were stimulated for 24 hours with 10 ng/ml of IL-1 in the presence or absence of 10 M rosiglitazone. Data represent the level of IL-1Ra secretion into culture supernatants, as measured by ELISA. \* *P* < 0.05 versus control; # *P* < 0.05 versus IL-1 treatment. **C**, Peroxisome proliferator-activated receptor response element (PPRE) luciferase (Luc) activity in cells exposed to rosiglitazone. \* *P* < 0.05. Values are the mean ± SD (n = 3 experiments in **A** and **B**; n = 6

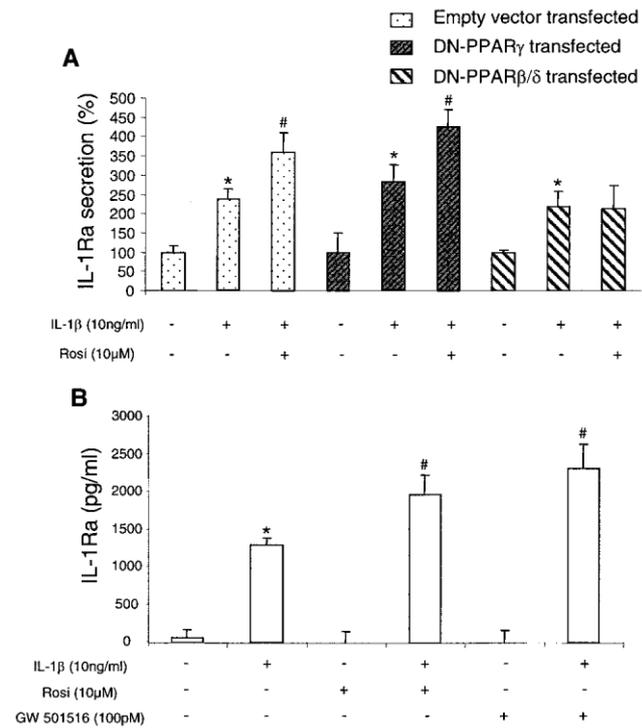
experiments in C).



**Figure 3.** Effects of NF- $\kappa$ B inhibition on rosiglitazone-induced interleukin-1 receptor antagonist (IL-1Ra) secretion by synovial fibroblasts. Cells were stimulated for 24 hours with 10 ng/ml of interleukin-1 (IL-1) in the presence or absence of 10  $\mu$ M rosiglitazone (Rosi). **A**, Effect of dominant-negative (DN) NF- $\kappa$ B on IL-1Ra secretion into culture supernatants, as measured by enzyme-linked immunosorbent assay. \*  $P$  0.05 versus control; #  $P$  0.05 versus IL-1 treatment. **B**, NF- $\kappa$ B luciferase (Luc) activity in cells exposed to IL-1. \*  $P$  0.05. Values are the mean  $\pm$  SD (n = 3 experiments in **A**; n = 6 experiments in **B**).



**Figure 4.** Effects of interleukin-1 (IL-1; 10 ng/ml) on the expression of peroxisome proliferator-activated receptor (PPAR) isotypes in rat synovial fibroblasts. **A**, Messenger RNA level at 6 hours (shown as the results of real-time polymerase chain reaction of total RNA). Data represent the ratio of PPAR mRNA to S29 mRNA and are expressed as the percentage relative arbitrary units, where control represents 100%. \*  $P$  0.05 versus control. **B**, Protein level at 12 hours, as determined by Western blot analysis. Data represent the ratio of PPAR protein to  $\beta$ -actin protein and are expressed as relative arbitrary units, where control represents 100%. \*  $P$  0.05. Controls experiments using resting cells were performed for each experiment. Values are the mean  $\pm$  SD (n = 3 experiments).



**Figure 5.** Contribution of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) to rosiglitazone-induced interleukin-1 receptor antagonist (IL-1Ra) secretion by synovial fibroblasts. Cells were stimulated for 24 hours with 10 ng/ml of IL-1 in the presence or absence of 10  $\mu$ M rosiglitazone (Rosi). **A**, Effect of dominant-negative (DN) PPAR $\gamma$  on IL-1Ra secretion into culture supernatants, as measured by enzyme-linked immunosorbent assay (ELISA). Data obtained with dominant-negative PPAR $\gamma$  are shown for comparison. Values are the mean  $\pm$  SD percentage, where control represents 100% (n = 3 experiments). \*  $P$  < 0.05 versus control; #  $P$  < 0.05 versus IL-1 treatment. **B**, Effect of the PPAR $\gamma$  agonist GW-501516 (100 pM) on IL-1Ra secretion into culture supernatants, as measured by ELISA. Data obtained with rosiglitazone are shown for comparison. Values are the mean  $\pm$  SD (n = 3 experiments). \*  $P$  < 0.05 versus control; #  $P$  < 0.05 versus IL-1 treatment.