15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 inhibits IL-1β-induced IKK enzymatic activity and IκBα degradation in rat chondrocytes through a PPARγ-independent pathway

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Abstract Peroxisome proliferator-activated receptor γ (PPARγ) ligands have been shown to inhibit the effects of proinflammatory cytokines such as interleukin-1β (IL-1β). This cytokine plays a key role in articular pathophysiologies by inducing the production of inflammatory mediators such as nitric oxide (NO) and prostaglandin E_2 (PGE_2). We previously demonstrated that 15d-PGJ_2 was more potent than troglitazone to counteract IL-1β effects on chondrocytes. Here, we studied the action of 15d-PGJ_2 on intracellular targets in nuclear factor-κB (NF-κB) signalling pathway in IL-1β treated rat chondrocytes. We found that 15d-PGJ_2 decreased inhibitor κBα (IκBα) degradation but not its phosphorylation by specifically inhibiting IκB kinase β (IKKβ), but not IKKα, enzymatic activity. We further evaluated the involvement of PPARγ in the anti-inflammatory action of its ligands. In chondrocytes overexpressing functional PPARγ protein, 15d-PGJ_2 pre-treatment inhibited inducible NO synthase and COX-2 mRNA expression, nitrite and PGE_2 production, p65 translocation and NF-κB activation. Troglitazone or rosiglitazone pre-treatment had no effect. 15d-PGJ_2 exhibited the same effect in chondrocytes overexpressing mutated PPARγ protein. These results suggest that 15d-PGJ_2 exerts its anti-inflammatory effect in rat chondrocytes by a PPARγ-independent mechanism, which can be conferred to a partial inhibition of IκBα degradation.

Keywords: Nuclear factor-κB; Cartilage; Inhibitor κBα; Peroxisome proliferator-activated receptor γ; 15-deoxy-^{12,14}-prostaglandin J_2

1. Introduction

Articular joint degradation is a common feature of rheumatoid arthritis and osteoarthritis. Proinflammatory cytokines, such as interleukin-1 β (IL-1β), have been shown to inhibit the synthesis of cartilage matrix proteins and to promote their degradation by activating metalloproteases [1,2]. In addition, IL-1β induces the production of inflammatory mediators, such as nitric oxide (NO), prostaglandins and other reactive oxygen species [3]. Therefore, the modulation of IL-1β effects and/or its production is known to be an important key to counteract the cartilage degradation.

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated nuclear transcription factor belonging to the nuclear receptor superfamily. PPARγ heterodimerizes with retinoid X receptor and binds to peroxisome proliferator response element located in the promoter of PPARγ target genes. Agonists of PPARγ include synthetic compounds, such as thiazolidinediones, and natural compounds such as fatty acids and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2). PPARγ ligands are best characterized as regulators of adipocyte differentiation, fatty acids storage and glucose homeostasis [4–6]. Moreover, it has been suggested that PPARγ ligands have a role in the inflammation control by inhibiting the transcriptional induction of genes such as TNF-α, inducible NO synthase (iNOS) and gelatinase B in murine macrophages [7,8]. Interestingly, other studies showed that these ligands could inhibit the production of TNFα, IL-1 and IL-6 by activated human monocytes [9].

In addition, in IL-1β stimulated chondrocyte cultures, we and others recently showed that 15d-PGJ_2 modulated NO and prostaglandin production, as well as expression of proinflammatory genes [10–13]. These PPARγ ligands inhibit gene expression in part by antagonizing the activities of transcription factors such as nuclear factor-κB (NF-κB) and activating protein-1 (AP-1) [9,14]. Other works suggested PPARγ independent mechanisms for the anti-inflammatory effect of 15d-PGJ_2 by interacting with NF-κB signalling pathway [15–17].

Two main mechanisms were described in several cell lines to explain the 15d-PGJ_2 inhibitory effects. Firstly, by interacting with a cysteine residue in the DNA-binding domain of the NF-κB subunit p65 [18]. Secondly, 15d-PGJ_2 was shown to block IκB kinase (IKK) activity, possibly through covalent

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Abbreviations: AP-1, activating protein-1; COX-2, cyclooxygenase-2; DMSO, dimethylsulfoxide; EDTA, ethylene diaminetetraacetic; EGCG, epigallocatechin-3-gallate; EGTA, ethylene glycol-*O*,*O*′-bis-[2-amino-ethyl]-*N*,*N*,*N*′,*N*′,-tetraacetic acid; HRP, horseradish peroxidase; IL-1β, interleukin-1β; IκBα, inhibitor κBα; IKK, IκB kinase; iNOS, inducible NO synthase; NF-κB, nuclear factor-κB; NO, nitric oxide; PBS, phosphate-buffered saline; PPARγ, peroxisome-proliferator activated receptor γ; PGE_2, prostaglandin E_2; Rosi, rosiglitazone; TBST, Tris Buffered saline Tween; Tro, troglitazone; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2

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modifications of critical cysteine residues in IKKβ [15,16]. However, controversy still exists about the identification of intracellular targets involved in the mechanism of action of 15d-PGJ_2. Moreover, the involvement of PPARγ appears to be strongly dependent on the cell type and very few data exist in chondrocytes.

Following our previous published work [10], the aim of the present study was to identify intracellular targets of 15d-PGJ_2 in the NF-κB transcriptional pathway in rat chondrocytes. The involvement of the nuclear receptor in 15d-PGJ_2 action was thereafter evaluated in chondrocytes transfected with a dominant-negative PPARγ vector and by comparing the action of three PPARγ ligands.

Our results showed that this cyclopentenone specifically decreased IKKβ activity without affecting IKKα. This action leads to the inhibition of inhibitor κBα (IκBα) degradation without any modification of its phosphorylation state. Finally, we found that 15d-PGJ_2 significantly inhibited IL-1β effects in chondrocytes transfected either with a PPARγ-expression vector or with a dominant-negative PPARγ vector. This strongly suggests that 15d-PGJ_2 exerts its anti-inflammatory action in chondrocytes by a PPARγ-independent mechanism.

2. Materials and methods

2.1. Isolation and culture of rat chondrocytes

Chondrocytes were isolated from femoral head caps of Wistar male rats by sequential digestion with pronase and collagenase B (Roche, Meylan, France) as described previously [19]. The cells were cultured to confluence in 75-cm^2 flasks at 37 °C in a humidified atmosphere containing 5% CO_2. The complete medium used was Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and heat-inactivated fetal calf serum (10%, Invitrogen, Cergy Pontoise, France). Chondrocytes were used from the first to the third passage.

2.2. Plasmids and transient transfection experiments

The pcDNA3.1 PPARγ expression vector was generously provided by Pr Johanne Martel-Pelletier (University of Montreal, Hopital Notre Dame, Montreal, Canada). The pSG5 dominant negative PPARγ vector was a gift from Pr Marie Thérèse Corvol (Unité INSERM 530, Université de Paris 5, Paris, France).

Twenty-four hours before transfection, chondrocytes were seeded in 6-well plates at 5×10^5 cells /well and grown to 60–80% of confluency. Cells were transfected with 500 ng of a PPARγ expression vector or with 500 ng of dominant negative PPARγ vector. The transfection was performed using 10 μl of polyethylenimine reagent (Euromedex, Souffelweyersheim, France) in 1 ml of complete medium. Two hours later, the reagent containing medium was replaced by complete medium. After a 36 h incubation, cells were treated with IL-1β in the presence or absence of PPARγ ligands.

2.3. Recombinant rat IL-1β treatments in the presence of PPARγ ligands

Chondrocytes were incubated with a PPARγ ligand or the vehicle (0.1% dimethylsulfoxide (DMSO) in final concentration) 1 h before the addition of IL-1β (10 ng/ml, Sigma, St. Quentin Fallavier, France). Cells were treated with IL-1β for 6 h (iNOS, cyclooxygenase-2 (COX-2), RP29 mRNA analysis), for 30 min (NF-κB activation study), for 5 min (IκB and IKK phosphorylation studies) or for 24 h (NO and prostaglandin E_2 (PGE_2) production measurements) in the presence of the ligand. The PPARγ ligands tested were troglitazone (Tro), rosiglitazone (Rosi, 10 μM, Laboratoires Servier, Suresnes, France) and 15d-PGJ_2 (10 μM, Calbiochem, Meudon, France). The three ligands were found to be ineffective at 1 μM on the parameters tested, whereas they were highly toxic for the cells at 100 μM.

2.4. RNA extraction and real-time PCR analysis

Total RNA was extracted from cell cultures by a single-step guanidinium thiocyanate–phenol–chloroform method using Trizol reagent (Invitrogen). Two micrograms of total RNA was reverse transcribed using hexa-nucleotides random primers. To quantify iNOS and COX-2 mRNA expression, real-time quantitative RT-PCR was performed using Lightcycler Technology (Roche). The sequences for the primers and product length are shown in Table 1. PCR was performed with SYBRgreen master mix system (Qiagen, Courtaboeuf, France). Melting curve was performed to determine the melting temperature of the specific PCR products. Each run included positive and negative controls. To standardize iNOS and COX-2 mRNA concentrations, transcript levels of the housekeeping gene RP29 were determined in parallel for each sample.

2.5. Quantification of NO and PGE_2 production

Thirty-six hours after transfection, chondrocyte cultures were treated as described before. NO production was measured as the amount of nitrite (NO_2^-) and nitrate (NO_3^-) levels (stable NO endproducts) released into the culture medium using a spectrofluorimetric assay with 2,3-diaminonaphthalene (DAN) as reagent (Sigma), as previously described [20,21]. Nitrate was previously converted to nitrite by the action of nitrate reductase from *Aspergillus niger* (Roche).

The concentrations of PGE_2 in culture medium were determined by EIA kit (Oxford Biomedical Research, Oxford, USA) according to the manufacturer's instructions.

2.6. Nuclear proteins extraction and NF-κB transactivation analysis

Nuclear protein extracts were prepared with the TransAM nuclear extract kit according to the manufacturer's protocol (Active Motif Europe, Rixensart, Belgium). Briefly, cells were scraped into phosphate-buffered saline (PBS) with phosphatase and protease inhibitors, centrifuged, resuspended in 1× hypotonic buffer and kept on ice for 15 min. After addition of detergent, the lysates were centrifuged at $14\,000 \times g$ for 30 s. The pellets were resuspended in complete lysis buffer (20 mM HEPES, pH 7.5, 350 mM NaCl, 20% glycerol, 1% Igepal CA630, 1 mM $MgCl_2$, 0.5 mM ethylene diaminetetraacetic (EDTA), 0.1 mM ethylene glycol-*O*,*O*′-bis-[2-amino-ethyl]-*N*,*N*,*N*′,*N*′,-tetraacetic acid (EGTA), 1 mM DTT, phosphatase and protease inhibitors) and vortexed. After incubation on ice and centrifugation, the supernatants were collected and protein concentration was determined by a Bradford-based assay (Bio-rad Laboratories).

NF-κB activation was determined by the TransAM ELISA kit (Active Motif Europe). This assay based on a colorimetric reaction is

Table 1
Primers used for real-time quantitative RT-PCR and product length

Gene	Primer sequence	T_m (°C)	PS (pb)	Cycle number
INOS	Sense 5′-AATGCGGAAGGTCATGGC-3′ Antisense 5′-CGACTTTCCTGTCTCAGTAGCAAA-3′	60	65	–
COX-2	Sense 5′-TACAAGCAGTGGCAAAGGCC-3′ Antinense 5′-CAGTATTGAGGAGAACAGATGGG-3′	60	301	–
RP29	Sense 5′-AAGATGGGTCACCAGCAGCTCTACTG-3′ Antisense 5′-AGACGCGGCAAGAGCGAGAA-3′	59	67	–

PS, product size in base pair and T_m, melting temperature.

an alternative to electrophoretic mobility-shift assay. Oligonucleotide containing an NF-κB consensus binding site has been immobilized in each well of a 96-well plate. 5 μg of nuclear extract was added per well and incubated for 1 h with middle agitation. The wells were then washed three times with washing buffer (100 mM phosphate buffer, pH 7.5, 500 mM NaCl, and 1% Tween 20) and incubated with p65 antibody (1:1000 in washing buffer) for 1 h at room temperature. The wells were finally incubated for 1 h with diluted horseradish peroxidase (HRP)-conjugated antibody (1:1000 in washing buffer) before the addition of 100 μl of developing solution (3,3′,5,5′ tetramethylbenzidine substrate solution diluted in 1% of DMSO) and a 5 min incubation. The reaction was stopped by addition of 0.5 M H_2SO_4 solution. Absorbance was read on a spectrophotometer at 450 nm.

Cells were washed two times with ice-cold PBS and scrapped 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 β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin and 1 mM phenylmethyl sulphonyl fluoride). Cells were disrupted by sonication and centrifuged at 3000 rpm for 10 min. The supernatants were collected and the protein concentration was determined by a Bradford-based assay. Protein samples were analyzed by SDS/PAGE and electroblotted onto polyvinylidene fluoride membrane. After 1 h in blocking buffer (5% non-fat dried milk in TBSTween 0.1%), membranes were blotted overnight at 4 °C with antibodies against phosphorylated IκBα, phosphorylated IKKα/β (Ozyme, St. Quentin en Yvelines, France), IκBα, PPARγ and β-actin (Sigma) for total protein extracts (dilution 1:500 for all antibodies) and p65 (dilution 1:500; Santa Cruz, USA) for nuclear extracts. After 3 washings with TBS-Tween, the blot was incubated with anti-rabbit IgG conjugated with HRP (Cell signaling, St. Quentin en Yvelines, France) at 1:2000 dilution in blocking buffer for 1 h at room temperature. Proteins were detected by the Phototope chemiluminescence detection system (Cell signaling).

2.7. Statistical analysis

After comparison of data by analysis of variance, the different groups were compared using Fisher's t test. Assays were made in triplicate ($n = 3$) and P values (vs. control or IL-1β treatment) less than 0.05 were considered significant.

3. Results

3.1. Modulation of IL-1β-induced iNOS and COX-2 mRNA expression and nitrite and PGE_2 production by PPARγ ligands

Preliminary experiments using Trypan blue and MTT showed no modification of cell viability and proliferation in rat chondrocytes treated with IL-1β (10 ng/ml) and/or PPARγ

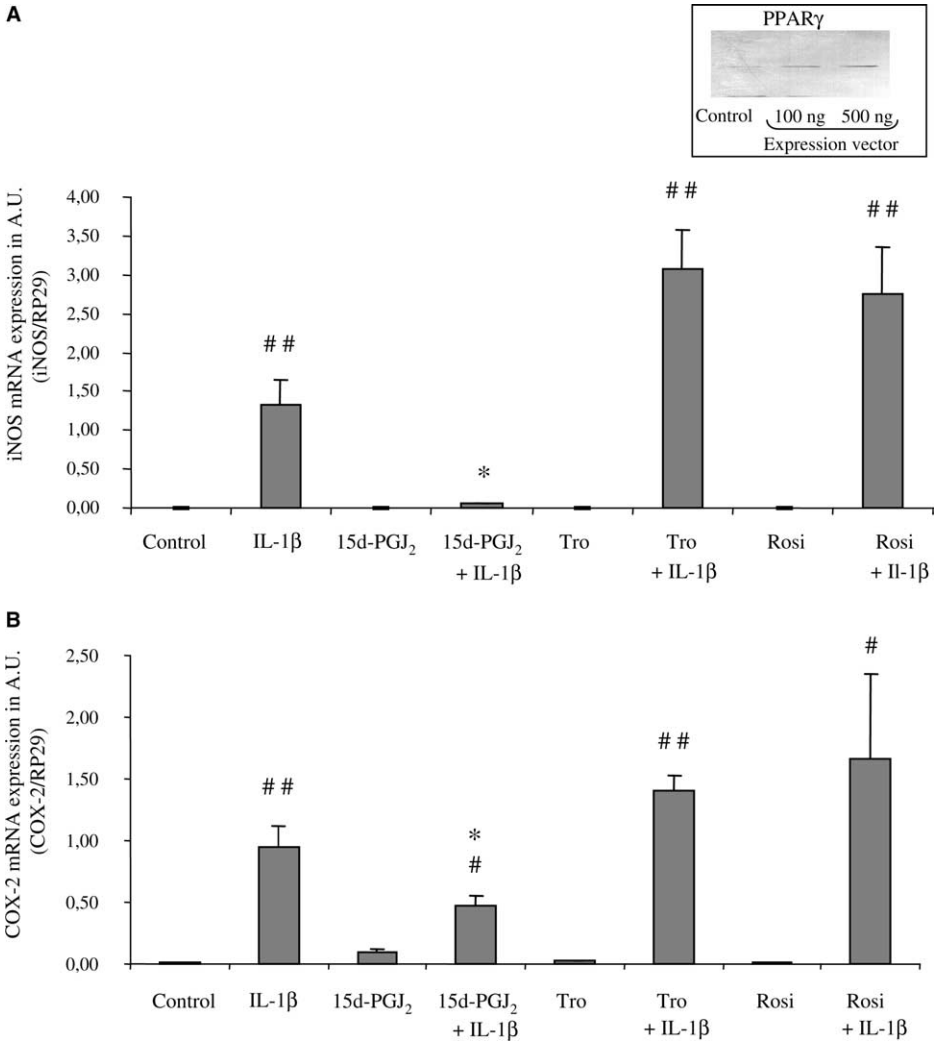

Fig. 1. Inhibition by PPARγ ligands of IL-1β induced iNOS (A) and COX-2 (B) mRNA expression in rat chondrocytes. Rat chondrocytes were transiently transfected with a PPARγ expression vector before being treated with IL-1β for 6 h in the presence or absence of PPARγ ligands (10 μM). Total RNA was extracted and was analyzed for iNOS (A) and COX-2 (B) mRNA expression by real-time quantitative RT-PCR. Results are expressed as relative arbitrary units ($n = 3$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control; $^{*}P < 0.05$ vs. IL-1β treatment).

ligand (10 μM). Chondrocytes were transfected with a PPARγ expression vector as described before. Thirty-six hours later, the cultures were pre-incubated for 1 h in the presence of PPARγ ligands before being stimulated with IL-1β (10 ng/ml) for 6 h (iNOS and COX-2 mRNA expression) or for 24 h (NO and PGE_2 production). IL-1β treatment induced a strong iNOS (Fig. 1A) and COX-2 (Fig. 1B) mRNA expression. 15d-PGJ_2 pre-incubation led to a 90% ($P < 0.05$) decrease in IL-1β induced iNOS mRNA expression and to a 52% ($P < 0.05$) decrease in IL-1β induced COX-2 mRNA expression. In contrast, Tro and Rosi pre-incubation did not modulate induction of iNOS and COX-2 mRNA by IL-1β treatment. Moreover, IL-1β treatment induced a strong production of nitrites (57 μM) and PGE_2 (2.52 ng/ml) by chondrocytes. 15d-PGJ_2 pre-incubation led to a 97% decrease ($P < 0.01$, 1.7 μM) in nitrite production and a 63% decrease ($P < 0.01$, 0.94 ng/ml) in PGE_2 production (Fig. 2A and B). These results were coherent with its effect on iNOS and COX-2 mRNA expression. In contrast to the results obtained by iNOS and COX-2 mRNA expression, Tro slightly but significantly reduced nitrite (−18%, $P < 0.05$, 47.2 μM) and PGE_2 (−24%, $P < 0.05$, 1.9 ng/ml) production induced by IL-1β. We also observed an effect of Rosi on nitrite production (−34%, $P < 0.05$, 37.4 μM). However, 15d-PGJ_2 was more efficient to counteract IL-1β induced nitrite and PGE_2 release than Tro or Rosi. This also suggests that 15d-PGJ_2 effects are PPARγ independent in rat chondrocytes.

3.2. *Effect of 15d-PGJ_2 in IL-1β treated chondrocytes overexpressing a dominant negative form of PPARγ*

Chondrocytes were transfected with a dominant negative PPARγ vector. Thirty-six hours later, cells were pre-incubated for 1 h with 15d-PGJ_2 and treated with IL-1β. 15d-PGJ_2 completely suppressed iNOS mRNA expression (Fig. 3A) and significantly inhibited (−77%, $P < 0.01$) COX-2 mRNA expression (Fig. 3B). We also found that 15d-PGJ_2 by itself induced an increase in COX-2 mRNA expression (+227%, $P < 0.05$).

In the same way, Fig. 3C and D show that 15d-PGJ_2 decreased NO production at level less than the control but was

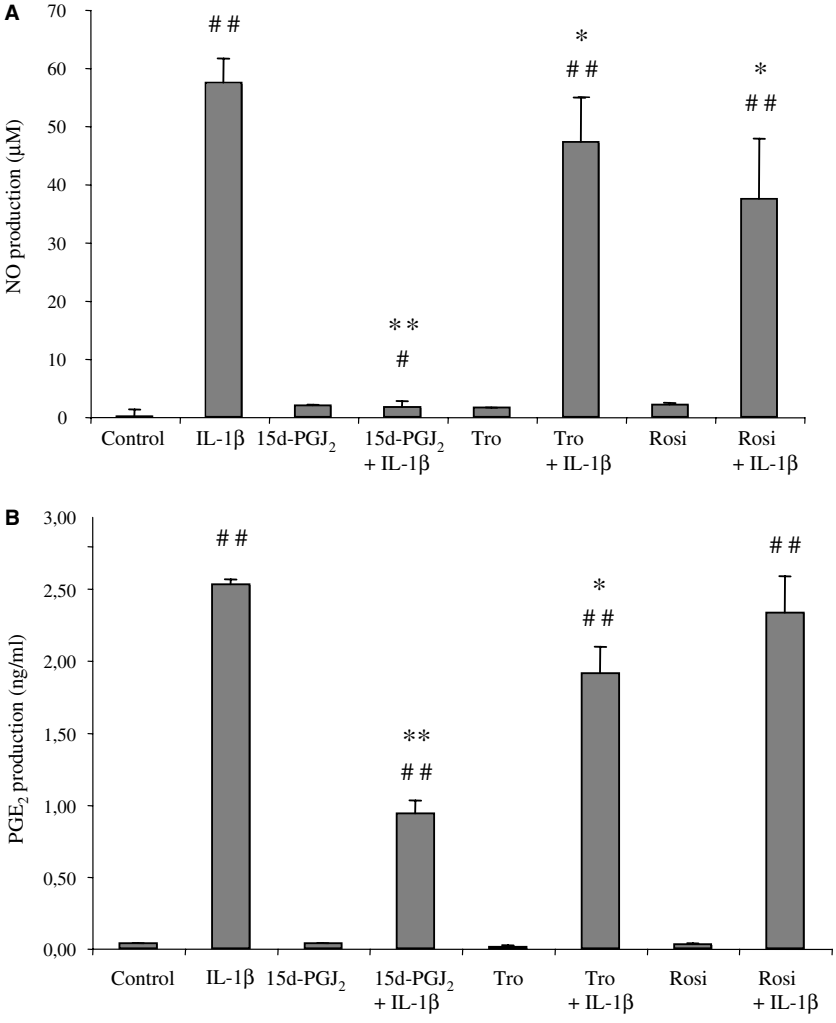

Fig. 2. Effect of PPARγ ligands on IL-1β induced NO (A) and PGE_2 (B) production in rat chondrocytes. After 36 h transfection, cells were stimulated with IL-1β (10 ng/ml) for 24 h in the presence or absence of 10 μM PPARγ ligands. NO production (A) was measured in the culture medium according to the method of Griess and PGE_2 production (B) was determined by ELISA kit. The results are expressed as concentrations ($n = 3$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control, $^{*}P < 0.05$; $^{**}P < 0.01$ vs. IL-1β treatment).

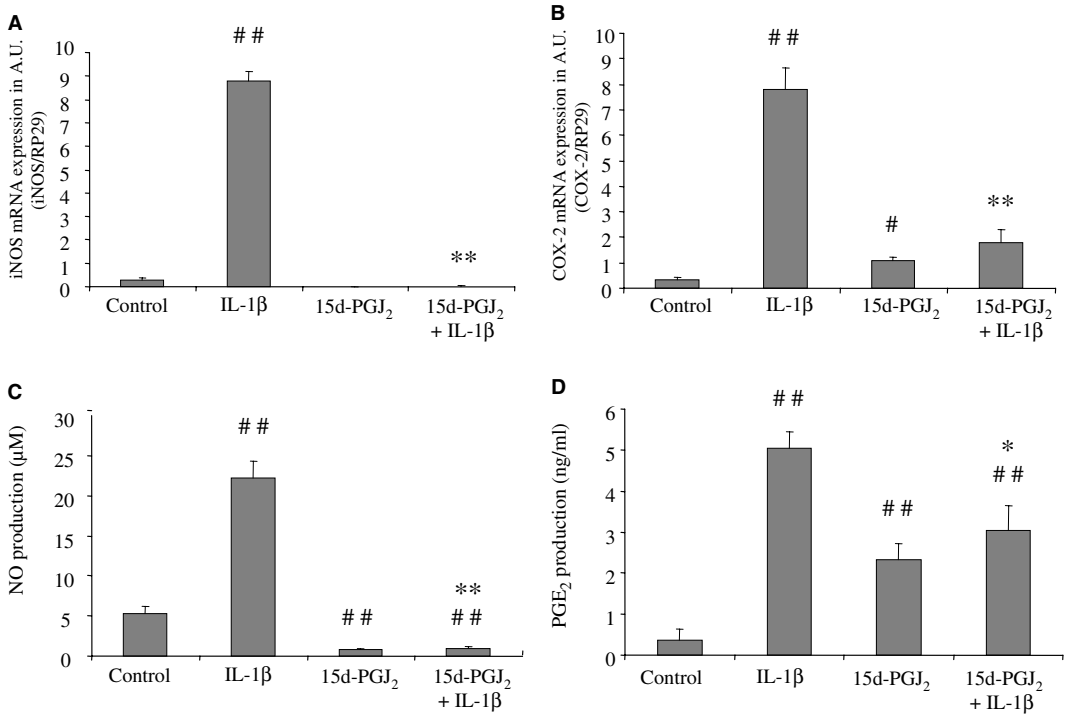

Fig. 3. Effect of an overexpression of a dominant negative form of PPARγ on the modulation by 15d-PGJ_2 of IL-1β induced iNOS (A), COX-2 (B) mRNA expression and NO (C), PGE_2 (D) production. Rat chondrocytes were transiently transfected with a dominant negative PPARγ vector before being treated with IL-1β for 6 or 24 h in the presence or absence of 15d-PGJ_2 (10 μM). Total RNA was extracted and was analyzed for iNOS (A) and COX-2 (B) mRNA expression by real-time quantitative RT-PCR. NO production (C) was measured as the amount of nitrite (NO_2^-) and nitrate (NO_3^-) levels released into the culture medium using a spectrofluorimetric assay with DAN as reagent, and PGE_2 production (D) was determined by ELISA kit ($n = 3$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control, $^{*}P < 0.05$; $^{**}P < 0.01$ vs. IL-1β treatment).

less effective to reduce PGE_2 release (−40%, $P < 0.05$). Here again, the cyclopentenone prostaglandin alone induced an increase in PGE_2 production.

3.3. Effect of PPARγ ligands on NF-κB signalling pathway: p65 translocation and DNA binding activity

We firstly examined the effects of 15d-PGJ_2, Tro and Rosi on p65 subunit translocation in nuclei of rat chondrocytes treated with IL-1β (Fig. 4A). IL-1β treatment for 30 min induced an important translocation of p65 in the nucleus. We found that 15d-PGJ_2 pre-incubation partially inhibited p65 translocation in the nucleus (−43%, $P < 0.05$). In contrast, Tro or Rosi pre-incubation did not modulate IL-1β-induced p65 translocation in the nucleus.

We thereafter studied the effect of the ligands on NF-κB activation by measuring its DNA binding activity. NF-κB was strongly activated by IL-1β (Fig. 4B). A pre-incubation with Tro or Rosi led to no change on NF-κB activation. However, 15d-PGJ_2 induced a 55% decrease ($P < 0.05$) in NF-κB DNA binding activity. No significant change in NF-κB activation and p65 translocation was observed in cells incubated with PPARγ ligands alone.

3.4. Determination of intracellular targets of 15d-PGJ_2 in NF-κB transcriptional pathway

Phosphorylation of IκBα, the inhibitory subunit of NF-κB heterodimer on Ser-32 and Ser-36, is an important step in the NF-κB activation process and is known to be mediated by the IKKα/β complex. In order to identify potential targets of 15d-PGJ_2 in the NF-κB pathway, Western blots for phosphorylated IκBα, IκBα and phosphorylated IKKα/β (Fig. 5A, B, and C, respectively) were performed. IL-1β treatment strongly induced the phosphorylation (Fig. 5A) and the degradation (Fig. 5B) of IκBα (−80%, $P < 0.05$). Protein extracts from cells pretreated with 15d-PGJ_2 showed a partial inhibition (−55%, $P < 0.05$) of IκBα degradation but did not affect IL-1β-induced IκBα phosphorylation. A pretreatment with Tro or Rosi has no effect on these parameters. Phosphorylation of IKKα/β complex was induced by IL-1β treatment as shown in Fig. 5C. If 15d-PGJ_2 was shown to completely inhibit IL-1 β-induced IKKβ phosphorylation, we also observed an increase in IKKα phosphorylation (+144%, $P < 0.05$). Troglitazone or Rosi pretreatment did not modulate IKKα/β phosphorylation induced by IL-1β.

4. Discussion

Anti-inflammatory effects of PPARγ ligands were highly described in the literature. However, most of the studies describing their mechanism of action were performed on established cell lines such as HeLa cells [22], cancer colorectal cell lines [23], microglia cells [17] or macrophages [8]. Moreover, significant effects with Tro and Rosi were obtained at high concentrations (up to 100 μM), which are highly toxic for chondrocytes. An important point of the present work is the use of chondrocyte primary cultures, because cartilage is the main tissue affected in osteoarthritis pathology.

In a previous work, we have shown that 15d-PGJ_2 was the most effective among PPARγ ligands to modulate IL-1β action

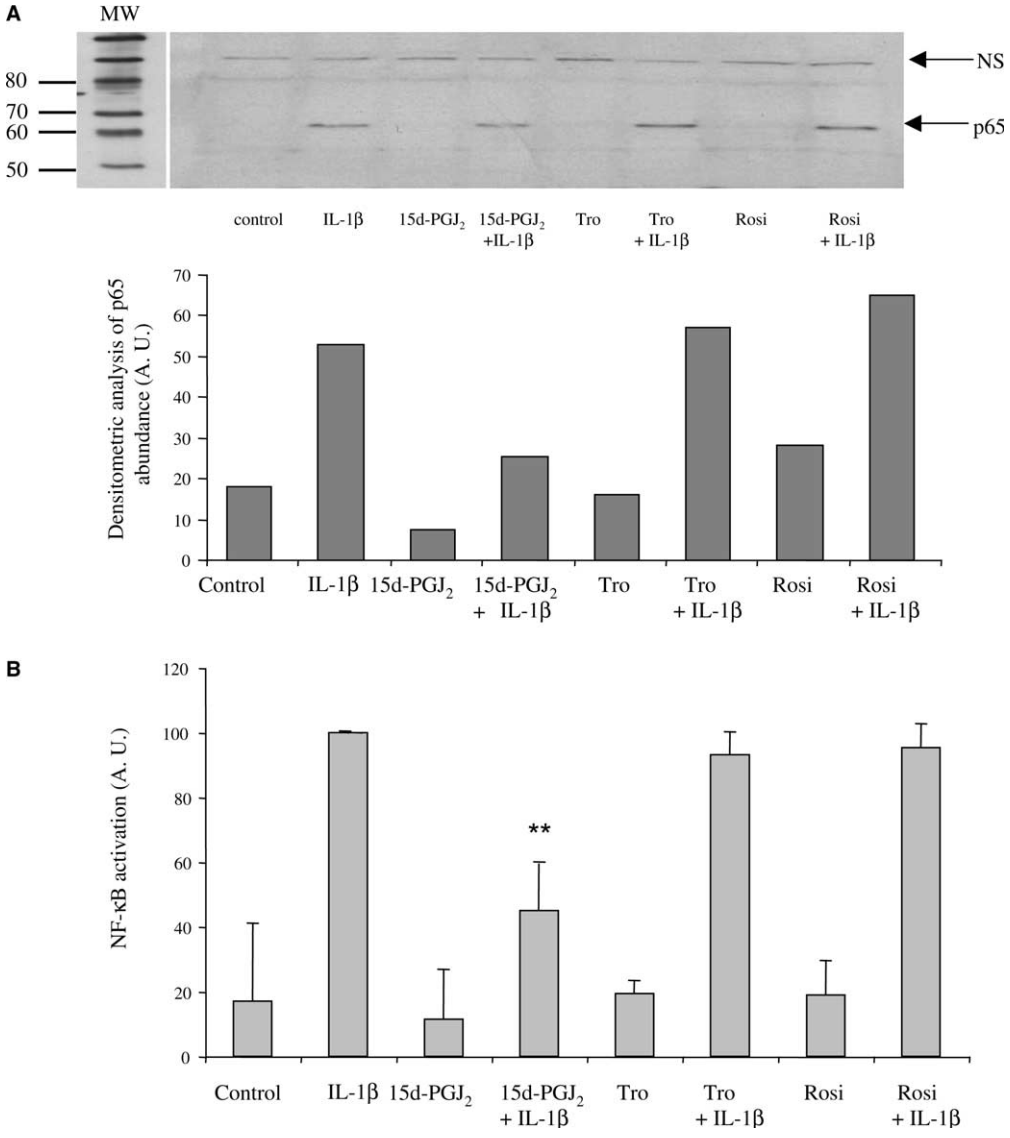

Fig. 4. Effect of PPARγ ligands on the translocation of the p65 subunit in the nucleus (A) and on NF-κB activation (B) in IL-1β treated chondrocytes. Thirty-six hours after transfection with a PPARγ expression vector, cells were pre-incubated with 10 μM PPARγ ligands for 1 h before being treated with IL-1β (10 ng/ml) for 30 min. (A) Nuclear protein extracts were prepared and 5 μg of nuclear extract was resolved on SDS/PAGE, transferred to nitrocellulose membrane and probed with an antibody against p65. The gel shown is representative of three independent experiments. (B) NF-κB activation was determined by ELISA kit (TransAm). Results are expressed as relative arbitrary units with a value of 100 for IL-1β treatment and are representatives of three different experiments.

in rat chondrocytes. A hypothesis given to explain the weak effects of synthetic ligands was that the level of PPARγ expression in chondrocytes would be too low. Since 15d-PGJ_2 affinity for its nuclear receptor is lower than Tro, this would strongly suggest a PPARγ-independent action of the cyclopentenone.

To increase PPARγ level in chondrocytes, overexpression of PPARγ in rat chondrocyte cultures was induced through transient transfection experiments with an expression vector. In transfected cultures, we observed that only 15d-PGJ_2 was able to decrease iNOS and COX-2 mRNA expression and NO and PGE_2 production induced by IL-1β. Taken together, these results are very similar to those previously obtained in non-transfected rat chondrocyte cultures. Thus, this strongly suggests a main PPARγ-independent action of 15d-PGJ_2 in rat chondrocytes.

To further demonstrate that 15d-PGJ_2 acts through a PPARγ-independent mechanism, we transfected chondrocytes with a vector expressing a dominant negative form of PPARγ. The results clearly demonstrate that 15d-PGJ_2 was still effective to counteract IL-1β, by repressing iNOS and COX-2 mRNA. In the same way, the production of inflammatory mediators was decreased. These results strengthen the hypothesis that 15d-PGJ_2 acts through a PPARγ-independent mechanism in rat chondrocytes.

NF-κB as well as AP-1 are transcriptional factors that govern the expression of many genes involved in the development of inflammatory processes [24–27] and in tissue degradation [28]. Thus, these two transcriptional pathways have been described as playing a central role in mediating the effects of IL-1β or TNFα in arthritic diseases and in cartilage degradation [29,30]. NF-κB is activated after phosphorylation and

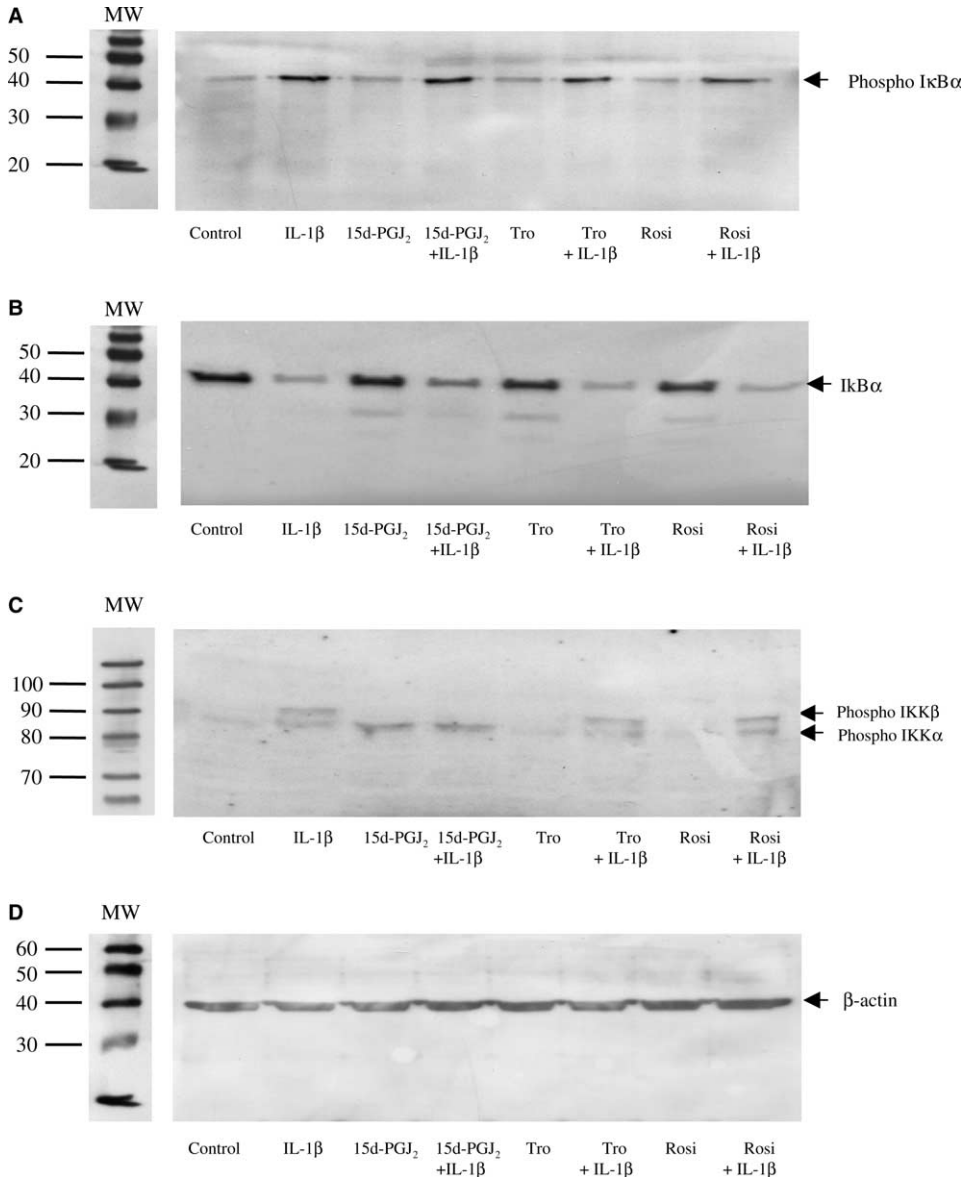

Fig. 5. 15d-PGJ_2 did not affect IL-1β-induced IκBα phosphorylation in rat chondrocytes but partially inhibited its degradation. Cells were incubated 1 h in the presence of PPARγ ligands, then with 10 ng/ml IL-1β for 5 min. Total proteins were extracted and immunoblot analysis was performed with specific antibodies. Total lysates were analyzed with antibodies against phosphorylated IκBα (A), non-phosphorylated IκBα (B), phosphorylated IKKα/β (C) and β-actin (D). The results shown are representative of three independent experiments.

degradation of the NF-κB inhibitor IκBα. IκBα is phosphorylated by IKK complex, which contains two catalytic subunits (IKKα and IKKβ) at sites that trigger its ubiquitin-dependent degradation [31].

In several recent publications, it has been demonstrated that 15d-PGJ_2 could interfere with multiple steps in NF-κB signalling pathway. In activated murine macrophages, 15d-PGJ_2 affects IKK activity due to the inhibition of the phosphorylation of serine residues [15]. In the same way, Rossi et al. [16] showed an inhibition of IKKβ phosphorylation by 15d-PGJ_2 in HeLa cells. However, to our knowledge, no data are available concerning the mechanism by which 15d-PGJ_2 acts in chondrocytes, as well as putative intracellular target(s).

In the present work, we demonstrated that 15d-PGJ_2, but not synthetic ligands, inhibited the IL-1β-induced activation of NF-κB transcriptional pathway. Interestingly, we also observed that 15d-PGJ_2 importantly inhibited IκBα degradation. However, at the same time, this inhibition was not accompanied by a decrease in phosphorylated IκBα level. This surprising result suggests that 15d-PGJ_2 could inhibit IκBα protein degradation without affecting its phosphorylation that normally should trigger its ubiquitin-dependent degradation. IκBα phosphorylation is the result of IKK complex activity. We have then studied the effects of a pre-treatment with 15d-PGJ_2 on both IKKα/β subunits enzymatic action. Our results showed that only IL-1β-induced IKKβ phosphorylation was decreased but not IKKα phosphorylation. This would mean that the phosphorylation of IκBα is the result of IKKα activity. According to the literature, IKKβ phosphorylation triggers the degradation of the inhibitory protein. Thus, we can

also suggest that the degradation of the inhibitory protein through the proteasome pathway is inhibited as a result of the absence of IKKβ phosphorylation activity. To our knowledge, this mechanism has not been described for cyclopentenone prostaglandins in the literature, especially in chondrocytes. Interestingly, a recent paper describing the effects of epigallocatechin-3-gallate (EGCG), a green tea polyphenol [32,33], reported a similar mechanism of action [34] in human chondrocytes. EGCG was shown to suppress IL-1β-induced IκBα degradation but had no inhibitory effect on the IκBα phosphorylating activity of IKK. However, the specific activities of IKKα and IKKβ subunits were not analyzed in this work.

Taken together, the results of the present work suggest a new PPARγ-independent inhibitory mechanism of NF-κB activation by 15d-PGJ_2 in rat chondrocytes. The IκBα phosphorylating activity of IKKα is not decreased, indicating that the inhibition of activation and translocation of NF-κB/p65 to the nucleus may result from inhibition of the proteasome pathway. This would then cause the stabilization and the accumulation of IκBα in the cytoplasm of rat chondrocytes. In conclusion, the anti-inflammatory effects of 15d-PGJ_2 seem to be mainly PPARγ-independent in IL-1β treated rat chondrocytes. These new findings might help to understand new aspects of the anti-inflammatory effects of cyclopentenone prostaglandins, especially in chondrocytes.

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