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Effects of agonists of peroxisome proliferator-activated receptor γ on proteoglycan degradation and matrix metalloproteinase production in rat cartilage *in vitro*

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Summary

Objective: To examine the effects of agonists of peroxisome proliferator-activated receptor (PPAR) γ on proteoglycan degradation induced by interleukin (IL)-1β or tumor necrosis factor (TNF)α in cartilage *in vitro*.

Design: Proteoglycan degradation was measured as release of radioactivity from rat cartilage explants previously labeled with $^{35}SO_4^{2-}$. Western blots were used to examine tissue levels of aggrecan neoepitopes NITEGE and VDIPEN, generated by aggrecanases and matrix metalloproteinases (MMP), respectively. Production of MMP-2, -3 and -9 by cultured rat chondrocytes was measured by zymography and by fluorimetric assay.

Results: IL-1β-induced proteoglycan degradation was likely due to aggrecanase, since it was associated with a strong increase of NITEGE signal. MMP-dependent VDIPEN signal increased only after further incubation with pro-MMP activator APMA. PPAR agonists 15d-PGJ_2 and GI262570 (10 μM) inhibited IL-1β- and TNFα-induced proteoglycan degradation measured both before and after addition of APMA. The agonists also inhibited cytokine-induced MMP production by isolated chondrocytes.

Conclusion: This study shows that PPAR γ agonists inhibit cytokine-induced proteoglycan degradation mediated by both aggrecanase and MMP. This effect is associated with inhibition of production of MMP-3 and -9. These results support the interest for PPAR γ agonists as candidate inhibitors of pathological cartilage degradation. © 2002 OsteoArthritis Research Society International. Published by Elsevier Science Ltd. All rights reserved.

Key words: PPAR, MMP, Aggrecanase, IL-1, TNF.

Introduction

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors, which belong to the superfamily that includes nuclear receptors for steroids, retinoids and thyroid hormone[1]. After binding with their ligands, PPARs heterodimerize with 9-*cis* retinoic acid receptor (RXR), and modulate transcription of target genes through interaction with the PPAR response element (PPRE) in their promoters. Three types of PPARs are known (α, β or δ, and γ), with different tissue distribution, ligand specificity and biological effects. Collectively, PPARs play a major role in lipid and glucose metabolism and adipocyte differentiation, but their function is not restricted to energetic metabolism, since they are also involved in the inflammatory process and affect proliferation, differentiated activities and survival of various cell types[1–3]. PPAR α and γ were recently detected in isolated rat[4] articular chondrocytes, and the presence of PPAR γ was confirmed in human chondrocytes either cultured[5] or still embedded in cartilage[6].

Osteoarthritis (OA) is characterized by progressive erosion of articular cartilage, and inflammatory cytokines, such as tumor necrosis factor (TNF) α and interleukin-1 (IL-1) β, probably play an important role in this process, since they stimulate degradation and inhibit synthesis of the two main organic components of cartilage matrix, collagen II and aggrecan[7]. In addition, they activate production of inflammatory mediators such as prostaglandin E_2, nitric oxide (NO), and radical oxygen species. IL-1β and TNFα concentrations increase in the osteoarthritic joint, and inhibition of IL-1 activity ameliorates cartilage damage in animal models of disease[8,9]. In rat and human chondrocytes, IL-1β decreased PPAR γ expression at mRNA and protein level[4,5], which suggested that the activity of inflammatory cytokines could depend on inhibition of PPAR γ pathway. In agreement with this hypothesis, PPAR γ agonists prevented IL-1β effects on synthesis of NO, proteoglycan and matrix metalloproteinase (MMP)-13[4–6]. Against this background, we wondered if PPAR γ agonists could have a general anticatabolic action in articular cartilage, and in particular if they could inhibit proteoglycan degradation. Aggrecan, the most abundant cartilage proteoglycan, is cleaved by two families of metalloproteinases, namely matrix metalloproteinases (MMP) and aggrecanases[10]. Chondrocytes express various MMPs, including collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysin (MMP-3), and membrane-type MMP (MMP-14)[11]. Two aggrecanases have been so far identified[12,13], and found to belong to the ADAM-TS family (a disintegrin and a metalloproteinase with thrombospondin domain). Aggrecanase-1 and -2 (ADAM-TS-4 and -5/-11, respectively) specifically degrade proteoglycans, differently from

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MMPs, which also cleave collagens and other cartilage components. Both aggrecanases and MMPs are synthesized as proenzymes. However, while aggrecanases are activated intracellularly through cleavage at a furin-sensitive site[12,13], soluble MMPs can be processed to the mature form only after secretion, by plasmin, membrane-type MMPs, or already-activated MMPs[14]. Aggrecanase activity can be distinguished from that of MMPs on the basis of different cleavage sites in the G1–G2 interglobular domain of aggrecan[15]. While MMPs cleave at residues N_{341}–F_{342}, aggrecanases act at E_{373}–A_{374}.

The aim of this *in vitro* study was to determine if PPAR γ agonists could inhibit proteoglycan degradation induced by the inflammatory cytokines IL-1β and TNFα in cultures of cartilage explants. We also examined in more detail the effects of the agonists on aggrecan degradation by MMPs and aggrecanases. To this end, we used antibodies against C-terminal neoepitopes of aggrecan that are generated by aggrecanases and MMPs, $NITEGE_{373}$ and $VDIPEN_{341}$ respectively. These antibodies were used to examine the content of aggrecanase- and MMP-generated neoepitopes in IL-1β-stimulated cartilage fragments. The effects of two PPAR γ agonists were examined, the natural ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and the synthetic, tyrosine-based agonist GI262570, originally designed as an antidiabetic drug[16].

Materials and methods

REAGENTS

Dulbecco's minimal essential medium/Ham's F12 medium 50/50 mixture (DMEM/F12) with or without phenol red, fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS), phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}, dispase, and 100×concentrated stock solution of 10^4 IU/ml penicillin and 10 mg/ml streptomycin (PS), were from GIBCO BRL (Cergy-Pontoise, France). 15d-PGJ_2 and prostaglandin A_2 (PGA_2) were from Cayman (Ann Arbor, MI). Recombinant rat IL-1β, p-aminophenyl mercuric acetate (APMA), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), ethylenediamino tetraacetic acid (EDTA), chondroitinase ABC, keratanase and papain were from Sigma (Saint Quentin Fallavier, France). Recombinant human TNFα was from R&D (Abingdon, U.K.). Collagenase type I was from Worthington (Lakewood, NJ). Antisera against NITEGE and VDIPEN were kindly provided by Professor Michael T. Bayliss and Dr Joanne Flannelly (Royal Veterinary College, London). AG-3340 was provided by Dr. Guillaume de Nanteuil at the Division of Medicinal Chemistry of IdRS. GI262570 was provided by Professor Daniel Lesieur, University of Lille, France. PPAR γ agonists and AG-3340 were dissolved at 2×10^{-2} M in DMSO, then diluted to working concentrations in culture medium supplemented with 1% FBS previously heated at 80°C for 20 min (FBS80°C) according to Parson *et al.*[17].

CARTILAGE ISOLATION

Male Wistar rat (120–150 g, Charles River, Cléon, France) were anesthesized under isoflurane and nitrogen protoxide and killed by exsanguination. Ethical guidelines for experimental investigation in animals were followed, and the experimental protocol was used after acceptance by the IdRS ethics committee on animal experimentation. Articular cartilage was isolated from hips, knees and shoulders, and used for either chondrocyte isolation, neoepitope detection, or proteoglycan degradation assays.

CHONDROCYTE CULTURE

Cartilage fragments were finely minced, then digested for 5 h at 37°C by a mixture of 3 mg/ml dispase and 2 mg/ml collagenase type I[18]. Isolated chondrocytes were seeded in 24-well plates at the density of 1.2×10^5 cells/well/0.5 ml of DMEM/F12 medium supplemented with 10% FBS and 1% PS. Medium was renewed three times per week, and after 12 days of culture, confluent cells were washed once in HBSS, then re-fed with DMEM/F12 medium without phenol red, supplemented with 1% $FBS_{80°C}$ and 1% PS. After 24 h, medium was aspirated and cells were treated with 0.25 ml/well of the same type of medium, containing or not containing a PPAR γ agonists. All groups were matched for agonist vehicle concentration (DMSO). After 4 h, either IL-1β, TNFα or vehicle was added and after a further 24 h conditioned media were collected for MMP assay.

MMP ZIMOGRAPHY

MMP content of conditioned media was analysed by zymography. 40 µl of sample was mixed with 10 µl of 0.5 M Tris/HCl, pH 6.8, 10% SDS, 40% glycerol, 0.1% bromophenol blue. For detection of MMP-2 and -9, 10 µl of sample mixture was loaded on a 10% polyacrylamide gel containing gelatin (Invitrogen, Paisley, U.K.). For MMP-3 detection, 20 µl was loaded on a pre-stained 4–16% polyacrylamide gel containing casein (Invitrogen). After electrophoresis, gels were incubated in zymogram renaturing buffer (Invitrogen) for 30 minutes, then in zymogram developing-buffer (Invitrogen) for 20 h at 37°C. Gelatin-containing gels were stained for 30 min by GelCode® blue stain reagent (Pierce, Rockford, IL). Casein and gelatin gels were rinsed with distilled water, then air-dried by the DryEase™ system (Invitrogen). Gel images were captured by an Agfa Arcus II scanner (Ista, Paris, France) controlled by a Kodak 1D image analysis software (Rochester, NY, U.S.A.).

MMP FLUORIMETRIC ASSAY

Pro-MMPs in chondrocyte conditioned media were activated by addition of 2 mM APMA and incubation at 37°C for 1 h. 20 µl of activated media was then added to 70 µl of zymogram developing buffer (Invitrogen) and 10 µl of 0.2 mM Mca-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys-(Dnp)-NH_2 (Bachem, Voisins les Bretonneux, France), a fluorogenic substrate that is cleaved by MMP-2, -3 and -9 at comparable rates[19]. After a 3-h incubation at 37°C, substrate cleavage was measured by a cytofluor™ 2350 from Millipore (St Quentin Yvelines, France) at absorption and emission wavelengths of 340 and 440 nm, respectively.

NITEGE AND VDIPEN DETECTION

Cartilage fragments were transferred to six-well plates at a ratio of about 40 mg of tissue/5 ml of DMEM/F12 media plus 1% $FBS_{80°C}$ and 1% PS, containing the agonists or their vehicle. After 4 h, IL-1β or vehicle was added. After 2 or 3 days of culture, the media was discarded, and the

tissue washed three times with 5 ml/well of PBS, blotted dry, weighed and extracted in 3 ml of 4 M guanidinium chloride, 5 mM EDTA, 5 mM Na acetate, pH 7.2 at 4°C for 3 days. The extract was exchanged against 5 mM EDTA PBS and concentrated using Ultrafree and Centricon devices (Millipore, Saint Quentin Yvelines, France) with a MW cut-off of 10 kD. The extracts were adjusted to the same volume/cartilage-mass ratio of 7 μl/mg. The concentrates were deglycosylated by 0.1 U/ml of chondroitinase ABC and keratanase at 37°C for 18 h, reduced by NuPAGE reducing agent (Invitrogen) at 70°C for 10 min, and separated by sodium dodecylsulfate polyacrylamide gel electrophoresis[20] on 4–12% gels (Invitrogen) in parallel with Rainbow molecular weight markers from BioRad (Ivry sur Seine, France), and transferred onto Hybond ECL membranes (Amersham Pharmacia, Saclay, France), according to Tsang *et al.*[21], using a Xcell™ system from Invitrogen. Membranes were saturated with 1% BSA, 0.1% Tween (Sigma) PBS for 1 h at ambient temperature, washed in 0.1% Tween PBS, then probed with either anti NITEGE or anti VDIPEN antisera (1/1000) in 1% BSA PBS for 1 h. After three washes, membranes were probed with 1/2000 peroxidase-labeled anti rabbit IgG (Sigma) in 1% BSA PBS for 1 h. After four washes, signals were visualized using an ECL detection kit from Amersham Pharmacia. Blot images were captured by an Agfa Arcus II scanner controlled by a Kodak 1D image analysis software.

CARTILAGE DEGRADATION

Explants from the patellar surface of femurs from four rats were labeled with 3.7 MBq (100 μCi) of $^{35}SO_4^{2-}$ (Amersham, Les Ulis, France) in 25 ml of DMEM/F12 media supplemented with 10% FBS and 1% PS. After 3 days, the unincorporated radioactivity was removed by six media changes over 24 h using DMEM/F12 supplemented with 1% $FBS_{80°C}$ and 1% PS. Each fragment was then transferred to 96-well plates into 0.25 ml/well of the same type of media, containing or not containing a PPAR γ agonist. All groups were matched for concentration of agonist vehicle (DMSO). After 4 h, either IL-1β, TNFα or vehicle was added. Each group was made of eight fragments. After 2 or 3 more days, the fragments were collected and digested in 0.5 ml of 0.6 mg/ml papain, 1 mM EDTA, 0.25 mg/ml DTT 20 mM sodium phosphate, pH 6.8 at 56°C for 16 h. Radioactivity in the culture media and in the tissue digest was measured by liquid scintillation using a β-counter (Beckman, Gagny, France). Proteoglycan degradation in each fragment was expressed as the percentage of released radioactivity by the formula: degradation=media radioactivity/(media radioactivity+ tissue radioactivity)×100.

STATISTICS

Control and treated groups were statistically compared by analysis of variance followed by Dunnett's test. Significance was noted as follows: ***P<0.001; **P<0.01; *P<0.05. Shown data are averages±standard error of the mean (S.E.M.).

Results

We first determined the conditions under which proteoglycans are degraded in rat cartilage cultures by either aggrecanases or MMPs. Incubation with IL-1β for 2 days stimulated proteoglycan degradation, measured as release of $^{35}SO_4^{2-}$-labeled material [Fig. 1(a)]. Western blots of tissue extracts showed that this treatment strongly increased aggrecanase-generated NITEGE, without any change of MMP-generated VDIPEN [Fig. 1(b)]. Absence of VDIPEN increase suggested that the MMPs produced in response to IL-1β remained in the form of proenzymes. In fact, when 2-day treatment with IL-1β was followed by pro-MMP activation with APMA for one extra day, VDIPEN signal was strongly increased [Fig. 1(d)]. This was associated with an augmented release of $^{35}SO_4^{2-}$-labeled material [Fig. 1(c)]. NITEGE signal instead disappeared [Fig. 1(d)], in agreement with the fact that MMPs can further cleave at position N_{341}–F_{342} aggrecan fragments already carrying the neo C terminus E_{373} generated by aggrecanase[22]. These results suggest that proteoglycan degradation was carried out by aggrecanases in the presence of IL-1β alone, and by MMPs when IL-1β stimulation was followed by pro-enzyme activation using APMA. We reasoned that, if these two treatment procedures indeed activate two different sets of enzymes, then proteoglycan degradation observed in the two situations will be differently affected by inhibitors selective for either aggrecanases or MMPs. To confirm this, we used AG-3340 (prinomastat), a large-spectrum MMP inhibitor with K_i values between 3×10^{-11} and 3×10^{-10} M for MMP-2, -3, -9, -13 and -14[23].

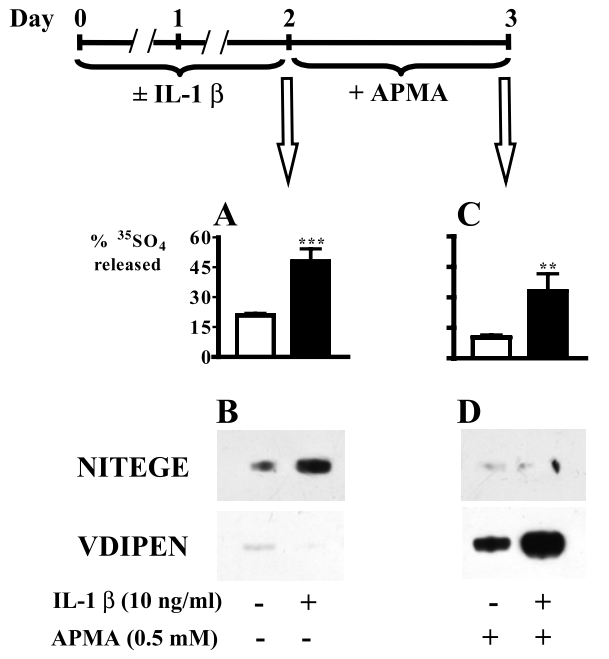

Fig. 1. Effects of IL-1 alone (a) and (b) or IL-1 followed by APMA (c) and (d) on proteoglycan degradation (a) and (c) and aggrecan neoepitope levels (b) and (d) in cultured cartilage explants. A synoptic plan of the experiment is shown at the top of the figure. In (a) and (b), cartilage explants were cultured in control medium or IL-1 for 2 days. Shown in (a) is degradation from day 0 to 2. In (c) and (d), explants were further incubated for 1 day in the presence of the MMP-activator APMA (0.5 mM). Shown in (c) is degradation from day 2 to 3. Asterisks indicate a significant difference between control and IL-1, without or with APMA: ***P<0.001; **P<0.01. Data are averages±S.E.M.; N=8. Proteoglycan degradation was measured as % release of radiolabeled material. Neoepitope levels were assessed by western blot using anti-NITEGE and anti VDIPEN antibodies.

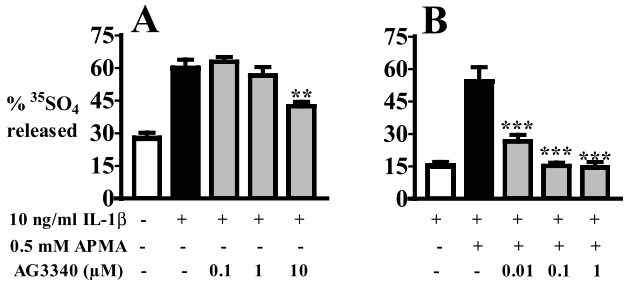

Fig. 2. Effect of AG3340 on proteoglycan degradation induced by IL-1 (a) or APMA following IL-1 (b). In (a) cartilage explants were cultured from day 0 to day 2 in control medium or IL-1±AG3340; shown is degradation between days 0 and 2. In (b) cartilage explants were stimulated from day 0 to day 2 with IL-1, then transferred to control medium or APMA±AG3340 for 1 further day; shown is degradation between days 2 and 3. Proteoglycan degradation was measured as % release of radiolabeled material. All groups were matched for concentration of AG3340 vehicle (DMSO). Asterisks indicate a significant difference between IL-1 vs IL-1 plus AG3340, without (a) or with (b) APMA: ***P<0.001; **P<0.01. Data are averages±S.E.M.; N=8.

This compound also inhibits aggrecanase, but less effectively, since a concentration around 1 μM is needed for a 50% reduction in activity of purified enzyme[24]. Figure 2(a) shows that a concentration of AG-3340 as high as 10 μM was needed to partially inhibit proteoglycan degradation induced by IL-1β alone. However, after pro-MMP activation by APMA, lower concentrations of the inhibitor (from 0.01 to 0.1 μM) were sufficient to block the release of radiolabeled material [Fig. 2(b)]. These results are in agreement with the hypothesis that sequential treatment with IL-1, followed by APMA, causes proteoglycan degradation dependent on aggrecanase first, and then MMPs.

We then examined the effects of PPAR γ agonists 15d-PGJ_2 and GI262570. From literature data, these two compounds displace the radiolabeled ligand BRL49653 (rosiglitazone) from PPAR γ with K_i values of 2.5×10^{-6} M and 1.2×10^{-9} M, respectively[25,26]. In studies of proteoglycan degradation, addition of 15d-PGJ_2 at concentrations up to 10 μM, inhibited IL-1β effect in a dose-dependent way [Fig. 3(a)]. A slightly lower effect was obtained with GI262570 [Fig. 3(b)], despite its stronger affinity for PPAR γ. Analysis of tissue extracts by western blot confirmed that 10 μM 15d-PGJ2 or GI262570 prevented the increase of NITEGE signal induced by IL-1β [Fig. 3(c)]. Next tested were the effects of the two agonists on MMP-mediated degradation. 15d-PGJ_2 significantly inhibited the release of radiolabeled material at 10 μM [Fig. 4(a)], while the effect of GI262570 at the same concentration did not reach statistical treshold [Fig. 4(b)]. By Western blot, the increase of VDIPEN signal induced by IL-1β and APMA was prevented by addition of either agonist at 10 μM [Fig. 4(c)]. The specificity of the antidegradative effect of 15d-PGJ_2 was then examined by comparison with PGA_2, which despite a closely related structure, lacks any affinity for PPAR γ[27]. Both in the case of aggrecanase- [Fig. 5(a)] and MMP-mediated degradation [Fig. 5(b)], only 15d-PGJ_2 was effective, PGA_2 being totally inactive at concentrations up to 10 μM. We then studied the effects of the two PPAR γ agonists on the production of MMP-2, -3 and -9 in primary cultures of rat chondrocytes. Analysis of conditioned media by gelatin and casein zymographies showed that IL-1β stimulated production of MMP-9 and MMP-3, while production of MMP-2 was constitutive. Addition of 15dPGJ_2

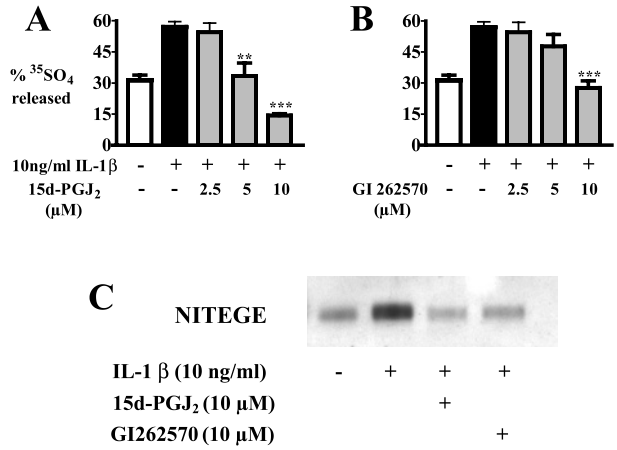

Fig. 3. Effects of 15d-PGJ_2 (a) and GI262570 (b) on aggrecanase-dependent proteoglycan degradation. c: effect of 15d-PGJ_2 and GI262570 on NITEGE signal. Cartilage explants were treated for 2 days in control medium or IL-1±agonist. All groups were matched for concentration of agonist vehicle (DMSO). Proteoglycan degradation was measured as % release of radiolabeled material between days 0 and 2. Neoepitope levels were assessed by western blot using anti-NITEGE antibodies. Asterisks indicate a significant difference between IL-1 vs IL-1 plus agonist: ***P<0.001; **P<0.01. Data are averages±S.E.M.; N=8.

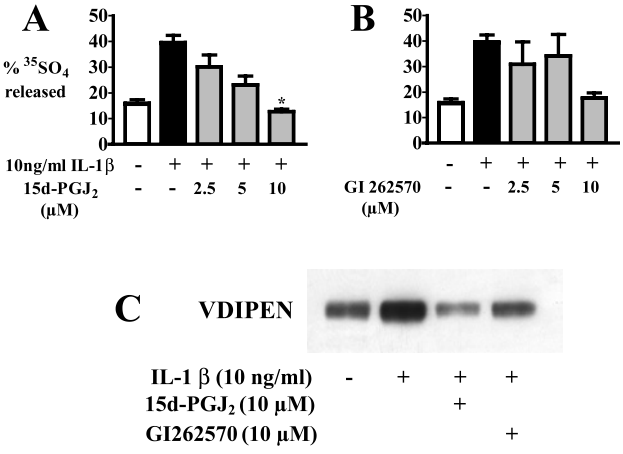

Fig. 4. Effects of 15d-PGJ_2 (a) and GI262570 (b) on MMP-dependent, proteoglycan degradation. C: effect of 15d-PGJ_2 and GI262570 on VDIPEN signal. Cartilage explants were treated from day 0 to day 2 in control medium or IL-1±agonist, then for 1 extra day in 0.5 M APMA. All groups were matched for concentration of agonist vehicle (DMSO). Shown is degradation in the presence of APMA, between days 2 and 3. Proteoglycan degradation was measured as % release of radiolabeled material. Neoepitope levels were assessed by western blot using anti VDIPEN antibodies. Asterisks indicate a significant difference between IL-1 vs IL-1 plus agonist: *P<0.05. Data are averages±S.E.M.; N=8.

inhibited the production of MMP-3 and -9 only at the highest concentration used, 10 μM [Fig. 6(a)]. The effect of GI262570 was weaker, in agreement with the results of degradation assays in cartilage explants [Fig. 6(b)]. No inhibition could be observed at lower concentrations of either agonist, and MMP-3 signal tended even to increase at 1 and 2.5 μM. The different intensity of the effects of the two agonists was confirmed by fluorimetric assay of MMP activity, using a substrate cleaved by stromelysin and gelatinases[19]. Also by this assay, the effect of 15d-PGJ_2

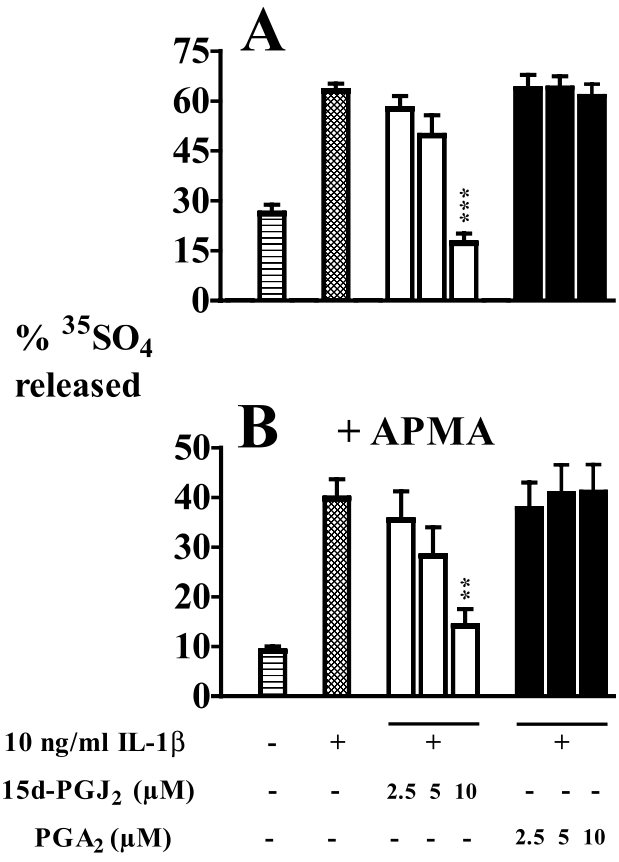

Fig. 5. Effects of 15d-PGJ_2 and PGA_2 on aggrecanase-mediated (a) and MMP-mediated (b) proteoglycan degradation. Cartilage explants were treated from day 0 to day 2 in control medium or IL-1±15d-PGJ_2 or PGA_2, then further incubated for 1 day in the presence of 0.5 M APMA. All groups were matched for concentration of agonist vehicle (DMSO). Shown in (a) is degradation between days 0 and 2; shown in (b) is degradation between days 2 and 3. Proteoglycan degradation was measured as % release of radiolabeled material. Asterisks indicate a significant difference between IL-1 vs IL-1 plus agonist, without (a) or with (b) APMA: ***P<0.001; **P<0.01. Data are averages±S.E.M.; N=8.

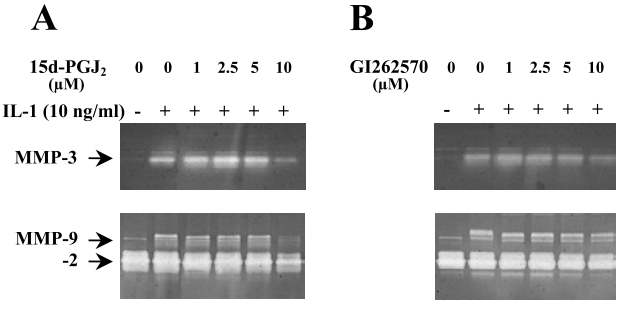

Fig. 6. Effects of 15d-PGJ_2 (a) and GI262570 (b) on IL-1-induced production of MMP-2, -3 and -9 by isolated chondrocytes. Chondrocytes were cultured for 1 day in the absence or presence of IL-1±agonists. All groups were matched for concentration of agonist vehicle (DMSO). Conditioned media were then analysed by casein (MMP-3) and gelatin (MMP-2, -9) zymography.

[Fig. 7(a)] was stronger than that of GI262570 [Fig. 7(b)]. An increase of the signal was observed, but only with GI262570, at the lowest concentration used [Fig. 7(b)]. The inhibitory effects of the agonists were not associated with

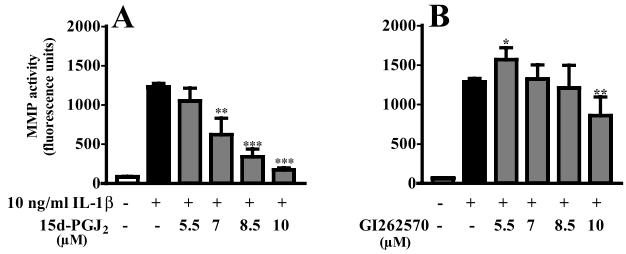

Fig. 7. Effects of 15d-PGJ_2 (a) and GI262570 (b) on on IL-1-induced production of MMPs by isolated chondrocytes. Chondrocytes were cultured for 1 day in the absence or presence of IL-1±agonists. All groups were matched for concentration of agonist vehicle (DMSO). MMP activity in conditioned media was then assayed by fluorimetric assay. Asterisks indicate a significant difference between IL-1 vs IL-1 plus agonist: ***P<0.001; **P<0.01; *P<0.05. Data are averages±S.E.M.; N=4.

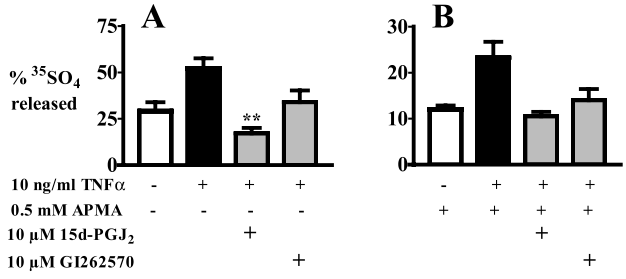

Fig. 8. Effects of 15d-PGJ_2 and GI262570 on proteoglycan degradation induced by TNFα. Cartilage explants were treated from day 0 to day 2 (a) in control medium or TNFα±agonist, then further incubated for 1 day (b) in the presence of 0.5 M APMA. All groups were matched for concentration of agonist vehicle (DMSO). Shown in (a) is degradation from day 0 to day 2, and in (b) degradation from day 2 to day 3. Proteoglycan degradation was measured as % release of radiolabeled material. Asterisks indicate a significant difference between TNFα vs TNFα plus agonist, without (a) or with (b) APMA: **P<0.01. Data are averages±S.E.M.; N=8.

cell toxicity, since LDH release did not increase in the presence of either agonist at concentrations up to 10 μM (data not shown). It was finally examined if PPAR γ agonists could inhibit the catabolic effect of another inflammatory cytokine, namely TNFα. At the concentration of 10 μM, 15d-PGJ_2, but not GI262570, significantly inhibited proteoglycan degradation induced by TNFα alone [Fig. 8(a)]. The agonists also tended to inhibit degradation caused by TNFα followed by APMA, but their effects did not attain statistical significance [Fig. 8(b)]. They also inhibited the release of MMP-3 caused by TNFα in isolated rat chondrocytes [Fig. 9(a)]. The effects of the agonist on gelatinase production were not examined, since TNFα, differently from IL-1β, did not stimulate the release of MMP-9 (data not shown). Inhibition of MMP production by 15d-PGJ_2 and GI262570 was confirmed by fluorimetric assay [Fig. 9(b)].

Discussion

This study shows that PPAR γ agonists 15d-PGJ_2 and GI262570 inhibit IL-1β-induced proteoglycan degradation mediated by both MMPs and aggrecanases in cultured explants of rat cartilage. This effect was probably due to inhibition of enzyme production, since PPAR γ agonists also decreased, at the highest concentration used of 10 μM, the release, by isolated chondrocytes, of MMP-3 and -9, two of the metalloproteases responsible for

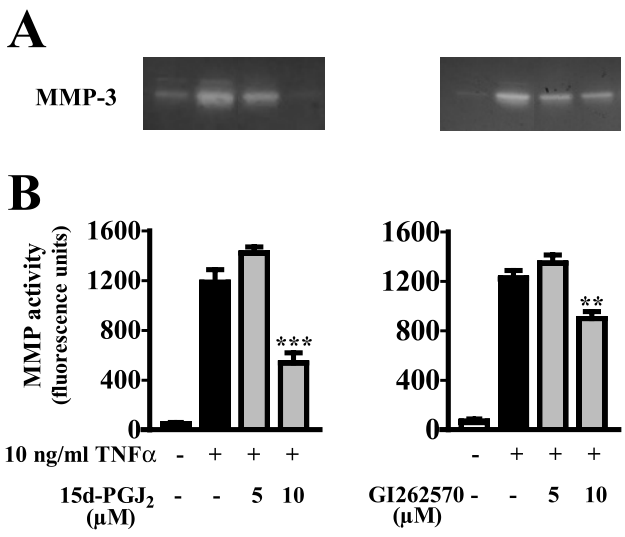

Fig. 9. Effects of 15d-PGJ_2 and GI262570 on TNF-induced MMP production. MMP levels in conditioned media were assayed by casein zymography (a) and fluorimetric assay (b). Chondrocytes were cultured in the absence or presence of TNF±agonists for 1 day. All groups were matched for agonist vehicle concentration (DMSO). Asterisks indicate a significant difference between TNF vs TNF plus agonist: ***P<0.001; **P<0.01. Data are averages±S.E.M.; N=4.

proteoglycan degradation in cartilage. This hypothesis is in agreement with the finding by Fahmi *et al*.[6], that PPAR γ agonists inhibit MMP-13 production induced by IL-1β in human chondrocytes. Moreover, inhibition of MMP-9 was not specific to chondrocytes, since this effect had also been observed in macrophages[28] and in a monocytic cell line[29]. However, the effect of the two agonists on MMP-3 production seemed to be biphasic, with an increase of enzyme activity at concentrations around 1 μM. This type of response is probably specific to MMP-3, since it was not observed either on MMP-9 by us, or on MMP-13 by Fahmi *et al.*[6]. 15d-PGJ_2 was active also against the effects of TNFα, as shown by inhibition of matrix degradation and MMP production. Inhibition of TNFα action by GI262570 was weaker than that produced by 15d-PGJ_2, and did not reach statistical significance. Throughout our study, active concentrations of 15d-PGJ_2 and GI262570 were comprised between 1 and 10 μM, the same narrow range as that necessary to inhibit other cytokine effects such as stimulation of NO and MMP-13 production, and inhibition of proteoglycan synthesis[4–6]. Higher concentrations could not be used, since they quickly resulted in shrinking and detachment of chondrocytes from culture dishes. This phenomenon was not further analysed, and even if its toxic nature cannot be excluded, it suggested induction of apoptosis, an effect of PPAR γ agonists already described in other cell types, among which synoviocytes, endothelial cells and fibroblasts[30–32]. Work is in progress to see if antiapoptotic agents, such as caspase inhibitors, can prevent at least some of the effects of PPAR γ agonists in our system. In all the experiments performed, 15d-PGJ_2 was slightly more active than GI262570, despite a lower affinity for PPAR γ[25,26]. This finding, although difficult to explain, mirrors others similarly obtained on chondrocytes, in which 15d-PGJ_2 was more active than PPAR γ agonists troglitazone[4,5] and BRL49653[6], despite lower receptor affinity and transactivating activity[27,33]. In a different cell system, GI262570 stimulated differentiation of precursor cells into adipocytes at concentrations in the nanomolar range[26]. The need for higher concentrations of synthetic agonists to exert their effects in chondrocytes, compared with other cell types, could be due to less efficient membrane passage and/or transactivation of the target genes. It could be otherwise explained by the hypothesis by Boyault *et al*., that the effects of the agonists on cartilage are independent of PPAR γ[5]. We showed nevertheless that the effects of 15d-PGJ_2 were not aspecific, since the structurally related compound PGA_2, which has a K_i value higher than 1000 μM, compared with 4 μM for 15d-PGJ_2 in the same displacement assay[27], did not inhibit proteoglycan degradation in cartilage explants (Fig. 5), or NO production by isolated chondrocytes (data not shown). Even if our results do not prove that the observed anticatabolic effects were actually due to interaction of the agonist with PPAR γ, 15d-PGJ_2 concentrations that proved active in our hands are necessary for, and compatible with, the functioning of this agonist through the PPAR-PPRE system, since Fahmi *et al*., showed that concentrations of 15d-PGJ_2 between 1 and 10 μM dose-dependently increased the transcription of a PPRE-driven reporter gene transfected into human chondrocytes[6]. The mechanism by which PPAR γ activation causes inhibition of cytokine effects in cartilage has not been determined yet. However, it was shown that PPAR γ agonists interfere with the functioning of two transcription factors that mediate the effects of inflammatory cytokines in different cell types among which chondrocytes. PPAR γ agonists blocked the binding of AP-1 and NF_kB to DNA sequences of their response elements[5,6]. Similarly to what was found in macrophages[28], this could be due to direct protein to protein interaction of PPAR γ with these transcription factors, independently of binding with PPRE. Availability of specific and powerful antagonists of PPAR γ should allow to determine the actual involvement of the receptor in the effects of 15d-PGJ_2 on chondrocytes. In conclusion, our results, although not conclusive about the involved mechanism, support the hypothesis that PPAR γ can play an important role in negative control of IL-1β and TNFα transduction pathways, and confirm the interest for PPAR γ agonists as candidate inhibitors of cartilage loss in diseases characterized by increased degradation of joint tissues, such as rheumatoid arthritis and OA.

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