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Agonists of peroxisome proliferators-activated receptors (PPAR) α, β/δ or γ reduce transforming growth factor (TGF)-β-induced proteoglycans' production in chondrocytes

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Summary

Objective: To investigate the potency of selective agonists of peroxisome proliferators-activated receptors' (PPAR) isotypes (α, β/δ or γ) to modulate the stimulating effect of transforming growth factor-β1 (TGF-β1) on proteoglycans' (PGs) synthesis in chondrocytes.

Method: Rat chondrocytes embedded in alginate beads and cultured under low serum conditions were exposed to TGF-β1 (10 ng/ml), alone or in combination with the following agonists: Wy14643 for PPARα, GW501516 for PPARβ/δ, rosiglitazone (ROSI) for PPARγ, in the presence or absence of PPAR antagonists (GW6471 for PPARα, GW9662 for PPARγ). PGs' synthesis was evaluated by radiolabelled sulphate incorporation and glycosaminoglycans' (GAGs) content by Alcian blue staining of beads and colorimetric 1.9 dimethyl-methylene blue assay after beads' solubilization. Phosphorylation of Extracellular Signal-related Kinase1/2 (ERK1/2), Smad2/3 and p38-MAPK was assessed by Western Blot and production of prostaglandin E_2 (PGE_2) by Enzyme immuno-assay (EIA). Levels of mRNA for PPAR target genes [acyl-CoA oxidase (ACO) for PPARα; mitochondrial carnitin palmitoyl transferase-1 (CPT-1) for PPARβ/δ and adiponectin for PPARγ], aggrecan, TGF-β1 and genes controlling GAGs' side chains' synthesis were quantified by real time polymerase chain reaction and normalized over RP29 housekeeping gene.

Results: ACO was selectively up-regulated by 100 μM of Wy14643, CPT-1 by 100 nM of GW501516 and adiponectin by 10 μM of ROSI without cell toxicity. TGF-β1 increased PGs' synthesis by four-fold, GAGs' content and deposition by 3.5-fold and six-fold, respectively, while inducing aggrecan expression around 10-fold without modifying mRNA levels of GAGs' controlling enzymes. PPAR agonists inhibited the stimulating effect of TGF-β1 by 24–44% on PGs' synthesis and over 75% on aggrecan, GAGs' content and deposition with the following rank order of potency: ROSI > GW501516 ≥ Wy14643. TGF-β1-induced phosphorylation of Smad2/3 and ERK1/2 was reduced by ROSI over GW501516 but not by Wy14643 whereas stimulated PGE_2 production was inhibited by Wy14643 over GW501516 but not by ROSI. The effect of PPAR agonists on PPAR target genes and TGF-β1-induced aggrecan expression was reversed selectively by PPAR antagonists.

Conclusion: In chondrocytes' beads, PPAR agonists reduced the stimulating effect of TGF-β1 on PGs by inhibiting TGF-β1-induced aggrecan expression in an isotype-selective manner. Thus, PPAR agonists could be deleterious in situation of cartilage repair although being protective in situation of cartilage degradation.

Key words: PPAR, TGF-β1, Chondrocytes, Proteoglycans, Smads, ERK1/2, PGE_2, Alginate system.

Introduction

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that plays an important role in immunomodulation, inflammation and tissue repair[1]. In articular cartilage, TGF-β is a potent stimulator of chondrocytes' proliferation and differentiation[2], stimulates synthesis of cartilage-specific collagen type II or aggrecan[3] and modulates matrix metalloproteases' (MMPs) activities by increasing synthesis of their natural tissue inhibitors[4]. Therefore, TGF-β is thought to play a crucial role in the maintenance and repair of articular cartilage, all the more that it counteracts the suppressive effect of inflammatory cytokines on proteoglycans' (PGs) synthesis[5], promotes the replenishment of PGs in arthritic conditions[6] and stimulates PG synthesis more actively in OA than in healthy cartilage[7]. Despite its beneficial effects on the turnover of matrix components, TGF-β may have a pathological role in OA. Indeed, intra-articular administration[8] or joint overexpression[9] of TGF-β is inflammatory and fibrotic with appearance of chondrogenesis in the synovial lining. As a common finding, repeated injection[10] or sustained expression[9] leads to the synovial-layer-dependent formation of osteophytes. Finally, TGF-β can induce the expression of aggrecanase-1 in human synovial fibroblasts[11] and chondrocytes[12] with the subsequent release of matrix-derived neo-epitope, demonstrating its contribution to cartilage breakdown. Thus, this growth factor has a complex pathophysiological role in joint diseases depending variably on cartilage protection, bone remodelling and synovial inflammation.

Peroxisome proliferators-activated receptors (PPAR) are ligand-activated nuclear transcription factors belonging to the nuclear hormone receptor superfamily[13]. PPAR bind,

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as a heterodimer with retinoid X receptor, to peroxisome proliferator-response elements (PPRE) located in the promoter of numerous genes involved in glucose and lipid homeostasis, and regulate their expression upon activation by agonists. PPAR consist of three mammalian subtypes, PPARα, PPARβ/δ and PPARγ, that are encoded by separate genes and differ in their tissue distribution[14] and ligand specificity[15]. As a major component of their specificity for one receptor isotype, PPAR ligands differ greatly in their binding affinity between endogenous compounds and synthetic agonists. Thus, arachidonic acid metabolites, as leukotriene B_4 for PPARα, prostaglandin I_2 for PPARβ/δ or 15-deoxy-prostaglandin J_2 (15d-PGJ_2) for PPARγ[15] are much less potent than pyrixinic acid (Wy14643) or fibrates for PPARα, GW501516 for PPARβ/δ or thiazolidinediones for PPARγ[16]. Albeit there is some overlapping, activation of PPAR isotypes is associated with major functions including oxidation of fatty acids in liver (mainly PPARα) and skeletal muscle (mainly PPARβ/δ) or differentiation of adipocytes in adipose tissue (mainly PPARγ), opening insight to the treatment of metabolic syndrome and cardiovascular diseases[17]. PPAR are also implicated in the control of the inflammatory response[18] and of epithelial repair pathways, with a key contribution of PPARβ/δ and PPARγ to skin wound healing and liver or kidney fibrosis, respectively[19]. PPAR are expressed in articular cells[20] and their activation is associated with a reduced production of inflammatory cytokines and less activation of MMPs[21] in cell culture systems or experimental arthropathy[22]. Although most studies were done with the endogenous PPARγ agonist 15d-PGJ_2 or with thiazolidinediones, recent studies demonstrate that agonists of other PPAR isotypes may also have pathological relevance to joint cells. Thus, interleukin-1 (IL-1)β-induced expression of IL-1Ra was promoted by PPARα agonists in rabbit chondrocytes[23] while being potentiated by activation of PPARβ/δ in rat synovial fibroblasts[24]. PPAR agonists may act therefore through activation of one or other PPAR isotype in joint cells.

Beside their classical cytokines' suppressive and metalloproteases' regulatory properties, PPAR agonists can inhibit the synthesis of extracellular matrix components in several cell types. Collagen synthesis is inhibited by thiazolidinediones in mesangial cells from diabetic rats[25] and by Wy14643 in hepatic stellate cells from rats with cholestatic liver fibrosis[26]. Similarly, fibronectin synthesis is inhibited by pioglitazone in human kidney fibroblasts[27] whereas expression of decorin induced by non-esterified fatty acids is reduced by darglitazone, another thiazolidinedione, in human smooth muscle cells[28]. In these cell types, PPAR agonists suppress also the stimulatory effect of TGF-β on collagens[29–31] and fibronectin[32] synthesis. Such inhibition of extracellular matrix synthesis by PPAR agonists contributes to their preventive effect on fibrosis in skin[30], lung[31], kidney[33] or liver[26]. Thus, activation of PPAR is associated with a reduced synthesis of matrix components, which has therapeutical relevance in clinical situations of extracellular matrix overload but may be deleterious in diseases characterized by extracellular matrix depletion, such as advanced OA.

When considering the anti-fibrotic effect associated with PPAR activation in many organs and the stimulatory effect of TGF-β on extracellular matrix synthesis in cartilage, we investigated the ability of synthetic agonists of the three PPAR isotypes (α, β/δ or γ) to affect TGF-β1-induced changes in PGs' synthesis in rat chondrocytes embedded in alginate beads. Using a panel of PPAR target genes and control experiments with isotype-selective antagonists, we demonstrated that Wy14643, GW501516 and rosiglitazone (ROSI) induced preferentially PPARα, PPARβ/δ and PPARγ-dependent responses, respectively. In our culture system, we showed that the stimulating effect of TGF-β1 on PG synthesis and deposition was reduced by all PPAR agonists, with major changes for ROSI. Genes encoding enzymes involved in glycosaminoglycans' (GAGs) side chains' synthesis were poorly affected by TGF-β1 and PPAR agonists, suggesting that PGs' depletion by PPAR agonists was supported mainly by inhibition of core protein expression. We demonstrated further that PPAR agonists affected differently several signalling pathways known to control aggrecan gene expression, with early TGF-β1-induced phosphorylation of Smad2/3 and ERK1/2 being reduced by ROSI over GW501516 but not by Wy14643 and late TGF-β1-induced prostaglandin E_2 (PGE_2) production being inhibited by Wy14643 over GW501516 but not by ROSI. We demonstrated finally that the inhibitory effects of PPAR agonists occurred in an isotype-selective manner since inhibition of TGF-β1-induced expression of aggrecan was reversed by a PPARα antagonist for Wy14643 and a PPARγ antagonist for ROSI, without consistent effect of either antagonist on GW501516.

Materials and methods

CHONDROCYTE ISOLATION AND CULTURE IN ALGINATE

Normal articular cartilage was obtained from femoral heads of Wistar male rats (150–175 g; Charles River, L'Arbresle, France) killed under dissociative anaesthesia [ketamine (Mérial, Lyon, France) and acepromazine (Sanofi Santé Animale, Libourne, France)] in accordance with national animal care guidelines, after approval by our internal ethics committee. Chondrocytes were isolated by sequential enzymatic digestion of head caps using 0.15% pronase and 0.2% collagenase B (Roche Molecular Biochemicals, Meylan, France), then washed two times in phosphate buffered saline (PBS) and cultured to confluence in 75 cm^2 flasks at 37°C in a humidified atmosphere containing 5% CO_2. The medium used was Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-glutamine (2 mM), penicillin–streptomycin (50 μg/ml), amphotericin B (0.5 μg/ml) and 10% heat-inactivated foetal calf serum (FCS) (InVitrogen, Cergy Pontoise, France). After two passages, chondrocytes were encapsulated in alginate beads as follows: cells were suspended in a 1.2% sterile solution of medium viscosity alginate (Sigma–Aldrich, Saint Quentin-Fallavier, France) at a concentration of 5×10^6 cells/ml and dispensed drop-wise into a 102 mM $CaCl_2$ solution via a 22-gauge needle attached to a syringe. After instantaneous gelation, beads were allowed to further their polymerization for 15 min in $CaCl_2$ solution before three washes in sterile 0.15 M NaCl solution followed by two washes in complete culture medium. The beads were maintained in complete culture medium for 7 days at 37°C in a humidified atmosphere of 5% CO_2 before experiments.

STUDY DESIGN

After 7 days of culture, beads were transferred into culture medium containing 1% FCS and were maintained under low FCS conditions throughout the experiments.

In a first experiment, synthetic PPAR agonists were added to chondrocytes' beads to check for their selectivity profile using isotypes-specific PPAR target genes: acyl-CoA oxidase (ACO) for PPARα, mitochondrial carnitin

palmitoyl transferase-1 (CPT-1) for PPARβ/δ, and adiponectin for PPARγ. The compounds used were ROSI (1–10 μM; Cayman Chemical, Ann Arbor, MI) as representative of PPARγ agonists, Wy14643 (Wy, 30–100 μM; Calbiochem, Meudon, France) as representative of PPARα agonists and GW501516 (GW, 0.1–100 nM; Alexis Biochemicals, Paris, France) as representative of PPARβ/δ agonists. The dependency on PPAR isotype was controlled in the presence of specific antagonists GW6471 (10 μM; Tocris Bioscience, Paris, France) for PPARα and GW9662 (10 μM; Cayman Chemical) for PPARγ. PPAR ligands were dissolved in Dimethyl-Sulfoxide, which was also added as vehicle to control cells at a final concentration of less than 0.2% (v/v).

In a second set of experiments, the effect of selected concentrations of PPAR agonists [ROSI (10 μM), Wy14643 (100 μM) or GW501516 (100 nM)] was studied on TGF-β1-induced (10 ng/ml; Peprotech Tebu, Le Perrey en Yvelines, France) changes in PGs' metabolism and signalling events. In all experiments, agonists were added 2 h before TGF-β1. PGs-related responses were studied 48 h (gene expression) or 72 h (matrix metabolism) after addition of TGF-β1. After preliminary kinetic studies, phosphorylation of Smad2/3 or ERK1/2 and production of PGE_2 were studied 1 h and 24 h after addition of TGF-β1, respectively.

In a third set of experiments, the contribution of each PPAR isotype to agonists-induced changes in PGs' metabolism, namely TGF-β1-induced expression of aggrecan and TGF-β1, was controlled with the specific antagonists GW6471 and GW9662 added at the same time as PPAR agonists.

ASSAY FOR CHONDROCYTE VIABILITY

Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich) into formazan. Briefly, cells were incubated for 48 h at 37°C in the presence or absence of TGF-β1 and/or PPAR agonists (added 2 h before TGF-β1 when co-incubated) in low FCS (1%) culture medium. Chondrocytes' beads were incubated further with MTT (1 mg ml^{-1} final concentration) for 4 h at 37°C. After removal of culture medium, beads were solubilized in citrate–Ethylenediaminetetraacetate (EDTA) buffer (55 mM/50 mM; pH 6.8) and chondrocytes' viability was assessed after addition of lysing buffer (20% w/v of sodium dodecyl sulphate [SDS] in a 50% aqueous solution of dimethyl formamide (DMF), pH 4.7). After 24 h of incubation at 37°C, solubilization of formazan crystals was quantified by measurement of absorbance at 580 nm on a Multiskan® microplate reader (Labsystems, Montigny-le-Bretonneux, France).

REGIONAL LOCALIZATION OF GAGS IN BEADS

After stimulation with TGF-β1, in the presence or absence of PPAR agonists, alginate beads were fixed for 4 h at 20°C in a 4% paraformaldehyde solution (pH 7.4) (Sigma–Aldrich) containing 100 mM sodium cacodylate and 10 mM $CaCl_2$, then washed overnight in 100 mM sodium cacodylate buffer containing 50 mM $BaCl_2$. Samples were dehydrated in graded solutions of ethanol, then embedded in paraffin and cut into sections (5 μm thickness), before staining with Alcian blue (Sigma–Aldrich) (1% w/v in acetic acid 3%) for the visualization of PGs' deposition. Sections ($n = 5$ beads per condition) were observed on three different areas at a magnification 100× with a photonic microscope (Nikon type 104, Tokyo, Japan) and staining intensity was analysed using NIH Image software (NIH/ImageJ, http://rsb.info.nih.gov).

ASSAY FOR GAGS IN FURTHER REMOVED MATRIX (FRM)

After stimulation with TGF-β1, in the presence or absence of PPAR agonists, alginate beads (50 per condition) were dissolved in citrate–EDTA buffer (55 mM/50 mM; pH 6.8) for 10 min at 37°C. The suspension was centrifuged for 15 s at 10,000 rpm to separate the cells surrounded by "cell-associated-matrix" (CM, the pellet) from components originating predominantly from the "interterritorial matrix" further removed from the cells (FRM; alginate supernatants) as described[34]. Alginate supernatants were further incubated for 2 h at 60°C in papain buffer [NaH_2PO_4 (0.2 M), EDTA (0.001 M), dithiotreitol (0.001 M) and papain (5 U/ml)] to prevent non-specific interactions of 1.9-dimethyl-methylene blue (DMB, Sigma–Aldrich) with proteins, and enzymatic digestion was stopped by addition of sodium mono-iodoacetate (Merck, Nogent-sur-Marne, France) at a final concentration of 10 mM. Then, GAGs were extracted from supernatants with an equal volume of 8 M guanidine–HCl (GuHCl, Sigma–Aldrich) in 0.05 M sodium acetate buffer pH 5.8 for 48 h at 60°C, followed by centrifugation at 10,000 rpm for 20 min at 4°C. The supernatants were discarded and the pellets were washed successively five times with PBS 1× (for optimal reduction of alginate's interference with GAGs) then with ethanol 70% (for eliminating the interference of GuHCl with GAGs). The washed pellets containing GAGs were dried for 20 min at room temperature before solubilization in 500 μl Tris/HCl buffer (50 mM, pH 8). The metachromatic reaction of GAGs with DMB was performed by adding 40 μl of each diluted sample ($1/40^{eme}$) to 960 μl of DMB solution [32 mg of DMB, 5 ml ethanol 70° and 4 ml formic acid solubilized in 1 L formate buffer 0.4% (w/v)][35]. The rapid measurement of absorbance at 525 nm on a UV1601® spectrophotometer (Shimadzu, ScienceTech, Paris, France) was used to calculate the amounts of GAGs in the samples using shark chondroitin-6-sulphate (Sigma–Aldrich) as a standard and cell free alginate beads as a blank.

CHONDROCYTES' PGS' SYNTHESIS

After stimulation with TGF-β1, in the presence or absence of PPAR agonists, alginate beads (five beads per well) were incubated in complete low FCS DMEM medium containing 10 μCi/ml of sodium sulphate $^{35}S[Na_2SO_4]$ for 4 h at 37°C in a 5% CO_2 humidified atmosphere. Alginate beads were washed five times with 0.15 M NaCl and solubilized overnight in citrate–EDTA buffer (55 mM/50 mM; pH 6.8). The $^{35}SO_4$-labelled PGs' content was assessed on aliquots of digested samples by liquid scintillation counting (Packard, Rungis, France) with an LKB 1214 counter (Wallac, Perkin Elmer, Courtabœuf, Paris Cedex, France).

ASSAY FOR PGE_2

PGE_2 levels were assessed in the same culture supernatants 24 h after addition of TGF-β1, using a commercially available enzyme immunoassay kit (Assay Designs Inc, Ann Arbor, MI, USA), according to the manufacturer's instructions. This assay showed no significant cross-reactivity with other prostaglandins and the limit of detection was 39.1 pg/ml.

WESTERN BLOTTING FOR SMAD2/3, ERK1/2 AND P38-MAPK

After stimulation with TGF-β1, in the presence or absence of PPAR agonists, alginate beads were dissolved in citrate−EDTA buffer (55 mM/50 mM; pH 6.8) for 10 min at 37°C, then centrifuged at 10,000 rpm for 15 s. The supernatants were discarded and the pellets were washed five times with PBS 1× before suspension in 200 μl of Laemmli buffer 1× (Sigma−Aldrich). Total DNA was sheared by sonication and pelleted by centrifugation at 3000 rpm for 10 min. The supernatants were further collected and 20 μl of each protein sample was analysed by SDS-polyacrylamide gel electrophoresis (10% acrylamide) and electroblotted onto polyvinylidene difluoride membrane. After one night at 4°C in 5% (w/v) blocking buffer, membranes (Immobilon; Waters, St. Quentin-en-Yvelines, France) were blotted 2 h at room temperature with primary antibodies against phospho or non-phospho Smad2/3, p44/p42-MAPK (ERK1/2) or p38-MAPK (Cell Signaling, Beverly, MA), and against β-actin (Sigma). All antibodies were diluted 1:1000. After three washings with Tris buffered saline (TBS)-Tween [(pH 7.6, 50 mM Tris containing 150 mM NaCl and 0.1% Tween20 (v/v)], blots were incubated for 1 h at room temperature with horseradish peroxydase-conjugated secondary antibodies (Cell Signaling) diluted at 1:2000 in 1% (w/v) blocking buffer. After four washings with TBS-Tween, specific proteins were detected by chemiluminescence with the Phototope Detection system (Cell Signaling) according to the manufacturer's instructions. Densitometric scans were analysed using NIH Image software (NIH/ImageJ, http://rsb.info.nih.gov).

RNA EXTRACTION AND REAL TIME POLYMERASE CHAIN REACTION (PCR)

After stimulation with TGF-β1, in the presence or absence of PPAR agonists, alginate beads were dissolved in citrate−EDTA buffer (55 mM/50 mM; pH 6.8) for 10 min at 37°C, then centrifuged at 10,000 rpm for 15 s. The supernatants were discarded and the pellets were washed five times with PBS before extraction of total RNA using an RNeasy minikit (Qiagen, Valencia, CA). Total RNA (500 ng) were reverse transcribed in a volume of 20 μl containing 4 μl of 5× First Strand Buffer, 20 μM hexanucleotides random primers, 1 μl of Recombinant Ribonuclease Inhibitor (RNA-se OUT 40 U/μl), 2 μl of dNTP (5 mM), 2 μl of dithiotreitol (0.1 M) and 1 μl of Moloney murine leukaemia virus (M-MLV; 200 U/μl) (Invitrogen) for 1 h 30 min at 37°C and 5 min at 95°C. To quantify aggrecan, xylosyl transferase-1, chondroitin-4-sulphate-transferase, chondroitin-6-sulphate-transferase, TGF-β1, adiponectin, ACO, CPT-1 mRNAs expression, real time PCR was performed using the Lightcycler® Technology (Roche) and the SYBRgreen master mix system® (Qiagen, Courtabœuf, France). The sequences of the primers used and corresponding products' lengths are summarized in Table I. After amplification, a melting curve was plotted to determine the melting temperature of each PCR product; their sizes were checked on a 2% agarose gel stained with ethidium bromide (0.5 μg/ml). Each run included positive and negative controls. The transcript level of the housekeeping gene RP29 was determined in parallel for each sample and data are expressed as the normalized ratio of mRNA level of each gene of interest over the RP29 gene.

STATISTICAL ANALYSIS

Data are expressed as mean ± standard error of the mean (S.E.M.) of three to ten values obtained from one to three separate experiments. Comparisons were made by ANOVA, followed by Fisher's protected least significant difference test using StatView® 5.0 software (SAS Institute, Cary, NC). Statistically significant differences from the control are indicated as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, from TGF-β1-treated cells as #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$, and from "TGF-β1 + PPAR agonists" or "PPAR agonists"-treated cells as §, $P < 0.05$; §§, $P < 0.01$; §§§, $P < 0.001$.

Results

SELECTIVITY OF SYNTHETIC PPAR AGONISTS FOR PPAR TARGET GENES

Preliminary experiment using the MTT assay showed no loss of viability of rat chondrocytes exposed to PPAR agonists and/or antagonists in the concentration range tested (data no shown). In our experimental conditions, ACO mRNA level was increased moderately (around 1.4-fold) by Wy14643 (100 μM), while being reduced strongly by ROSI (1 or 10 μM) and GW501516 (only 100 nM) (Fig. 1). CPT-1 mRNA level was up-regulated by five-fold by GW501516 (100 nM) while being induced around two-fold by Wy14643 and ROSI (Table II). Adiponectin mRNA level was increased by ROSI in dose-related manner (3.9-fold at 10 μM), while being reduced marginally by GW501516 (100 pM) or the highest concentration of Wy14643 (100 μM) (Fig. 1). These data supported that, in our experimental conditions, Wy14643 behaved as a selective PPARα agonist at 100 μM, GW501516 as a selective PPARβ/δ agonist at 100 nM and ROSI as a selective PPARγ agonist at 10 μM.

Table I
Primers used for real time PCR and corresponding products' lengths

Genes	Primer sequence, forward	Primer sequence, reverse	Tm (°C)	PS (pb)
RP29	5′-AAG ATG GGT CAC CAG CAG CTC TAC TG-3′	5′-AGA CGC GGC AAG AGC GAG AA-3′	59	67
Aggrecan	5′-ACA CCC CTA CCC TTG CTT CT-3′	5′-AAA GTG TCC AAG GCA TCC AC-3′	59	124
Xyl-transferase 1	5′-GTC GCA CCT CTG ACC TTC TC-3′	5′-CCA GGC TTC TAG TGC TGT CC-3′	61	201
C4S-transferase	5′-CAT GCC ACA TCC ACT ACG AC-3′	5′-AAC TCC GTG GTC ATC TCG TC-3′	60	151
C6S-transferase	5′-CCT CGG AGC AGT TTG AGA AG-3′	5′-GTT GGT GAG TGA GGC GGT AT-3′	60	131
TGF-β1	5′-CTG CTG ACC CCC ACT GAT A-3′	5′-TGA AGC GAA AGC CCT GTA TT-3′	57	103
ACO	5′CCAATCACGCAATAGTTCTGG-3′	5′-CGC TGT ATC GTA TGG CGA T-3′	58	362
Adiponectin	5′-AAT CCT GCC CAG TCA TGA AAG -3′	5′-TCT CCA GGA GTG CCA TCT CT-3′	58	433
CPT-1	5′-ATG ACG GCT ATG GTG TCT TCC-3′	5′-GTG AGG CCA AAC AAG GTG AT-3′	58	154

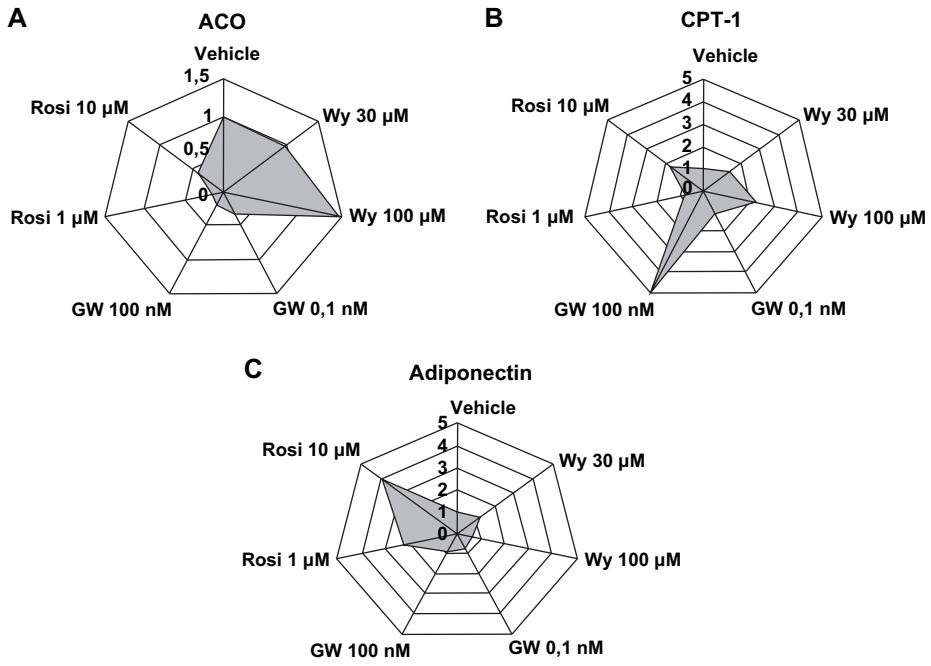

Fig. 1. Selectivity of synthetic PPAR agonists for PPAR target genes in chondrocytes. Rat chondrocytes, embedded in alginate beads (5×10^4 cells/bead) were exposed or not to Wy14643 (10−30 μM, PPARα agonist), GW501516 (0.1−100 nM, PPARβ/δ agonist) or ROSI (1−10 μM, PPARγ agonist) for 48 h. Data expressed as mean levels of mRNA of specific target genes normalized to RP29 ($n = 4$–8): (A) ACO for PPARα; (B) CPT-1 for PPARβ/δ; (C) adiponectin for PPARγ.

REVERSION OF PPAR TARGET GENES' EXPRESSION BY PPAR ANTAGONISTS

The dependency on PPAR isotype was controlled with specific antagonists. Experiment showed that GW6471 suppressed selectively the inducing effect of Wy14643 on ACO expression, although it was itself inhibitory (−60%) on ACO expression and stimulatory (1.6-fold) on adiponectin expression (Fig. 2). Moreover, GW9662 reduced ROSI-induced expression of adiponectin below control level, although it reduced partly (−60%) the stimulating effect of GW501516 on CPT-1 mRNA level (Fig. 2). In basal conditions, GW9662 stimulated ACO expression by two-fold while reducing adiponectin expression by −45% (Fig. 2). These results were consistent with GW6471 acting as a PPARα antagonist with a marginal potency to activate PPARγ, and GW9662 as a PPARγ antagonist with a marginal potency to activate PPARα. These data also confirmed the isotype selectivity of 100 μM of Wy14643, 100 nM of GW501516 and 10 μM of ROSI and led us to select these concentrations for subsequent experiments.

EFFECTS OF SELECTIVE PPAR AGONISTS ON TGF-β1-INDUCED CHANGES IN PG METABOLISM

Preliminary experiments in the MTT assay were consistent with a non-significant proliferating effect of TGF-β1 (10 ng/ml) on chondrocytes embedded in beads (data not shown). In these conditions, TGF-β1 stimulated the overall content in GAGs by 3.5-fold [Fig. 3(A)] and their deposition in the interterritorial matrix area by 6.2-fold without

Table II

*Effects of selective PPAR agonists on TGF-β1-induced expression of genes involved in PG synthesis in chondrocytes. Rat chondrocytes, embedded in alginate beads (5×10^4 cells/bead), were exposed or not to Wy14643 (100 μM), GW501516 (100 nM) or ROSI (10 μM) for 2 h before stimulation with TGF-β1 (10 ng/ml), alone or in combination with PPAR agonists, for 48 h. Normalized mRNA levels of aggrecan, xylosyl transferase-1 and chondroitin-(4 or 6)-sulphate-transferases quantified by real time PCR (n = 4). Data are expressed as mean ± S.E.M. Statistically significant differences from the control are indicated as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ and from TGF-β1-treated cells as #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$*

PPAR agonists	Genes/RP29	Aggrecan	Xylosyl transferase-1	Chondroitin-4-sulphate-transferase	Chondroitin-6-sulphate-transferase
None	w/o TGF-β1	1.00 ± 0.13	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.11
	+ TGF-β1	12.4 ± 2.56***	1.44 ± 0.08*	0.76 ± 0.15	0.68 ± 0.08
Wy14643	w/o TGF-β1	0.45 ± 0.20*	1.72 ± 0.18*	2.73 ± 0.14**	0.51 ± 0.02**
	+ TGF-b1	3.44 ± 1.10###	1.89 ± 0.62	1.41 ± 0.27#	0.66 ± 0.24
GW501516	w/o TGF-β1	0.70 ± 0.11	1.30 ± 0.15	2.60 ± 0.10**	1.30 ± 0.15
	+ TGF-β1	3.40 ± 0.73###	1.23 ± 0.17	1.64 ± 0.21#	1.25 ± 0.18#
Rosiglitazone	w/o TGF-β1	0.48 ± 0.07**	4.14 ± 1.05**	3.00 ± 0.53**	1.48 ± 0.18**
	+ TGF-β1	2.40 ± 0.52###	3.07 ± 0.82##	1.14 ± 0.02#	1.14 ± 0.06#

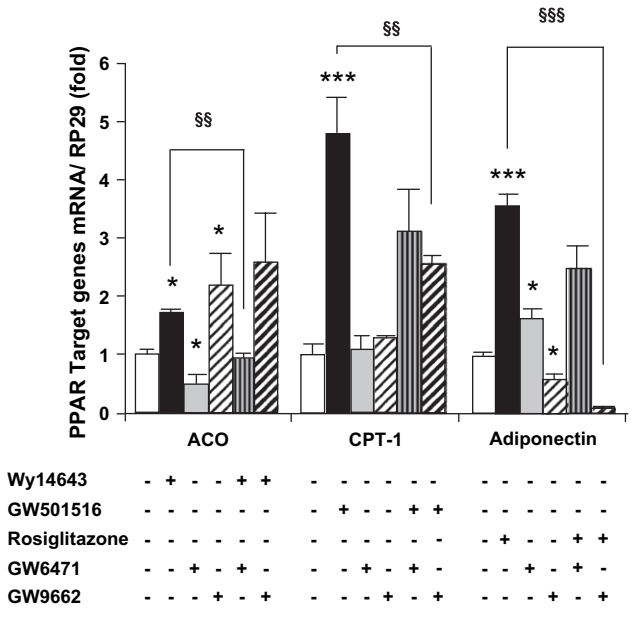

Fig. 2. Reversion of PPAR target genes' expression by selective antagonists in chondrocytes. Rat chondrocytes, embedded in alginate beads (5×10^4 cells/bead), were exposed or not to Wy14643 (100 μM), GW501516 (100 nM), or ROSI (10 μM), in the presence or absence of PPARα (GW6471, 10 μM) or PPARγ (GW9662, 10 μM) antagonists for 48 h. Data are expressed as mean ± S.E.M. of normalized mRNA levels for ACO, CPT-1 or adiponectin quantified by real time PCR ($n = 4$). Statistically significant differences from the control are indicated as *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$, and from "PPAR agonists"-treated cells as §, $P < 0.05$; §§, $P < 0.01$; §§§, $P < 0.001$.

modifying their pericellular localization [Fig. 3(B)]. Besides, PGs' synthesis was increased by approximately four-fold [Fig. 3(C)] and the mRNA level of aggrecan was increased by 12.5-fold without marked changes in the expression level of xylosyl transferase-1, chondroitin-4 and -6 transferases (Table II).

In the presence of PPAR agonists, the MTT assay was not significantly reduced except for the highest dose of ROSI (data not shown). The stimulating effect of TGF-β1 on GAGs' content was suppressed from 90% for GW501516 to below basal level for ROSI [Fig. 3(A)] and their deposition in interterritorial matrix was reduced dramatically (from −73% for Wy14643 to −95% for ROSI) [Fig. 3(B)]. The enhancing effect of TGF-β1 on PG synthesis was also reduced by PPAR agonists (from −24% for Wy14643 to −44% for ROSI), but the basal rate of radiolabelled sulphate incorporation was slightly increased by Wy14643 while being slightly reduced by ROSI [Fig. 3(C)]. TGF-β1-induced expression of aggrecan was inhibited from −76% for Wy14643 to −83% for ROSI, but all PPAR agonists tended to decrease the basal level of aggrecan (Table II). In contrast, PPAR agonists increased moderately the basal levels of mRNA for xylosyl transferase-1, chondroitin-4 and -6 sulphate transferases except for Wy14643 on chondroitin-6 sulphate transferase, and a reproducible decrease was observed only for chondroitin-4 transferase in the presence of TGF-β1 (Table II). For most TGF-β1-induced responses, the maximal inhibitory effect was observed with ROSI [Fig. 3(A–C)], a finding consistent with its ability to decrease mitochondrial activity in the MTT assay.

EFFECTS OF SELECTIVE PPAR AGONISTS ON TGF-β1-INDUCED SIGNALLING PATHWAYS

Time course effect of Smad2/3, ERK1/2 and p38-MAPK phosphorylation by TGF-β1

In our experimental system, TGF-β1 induced a fast phosphorylation of Smad2/3 which was maximal around 1 h, whereas no significant variation was observed on p38-MAPK [Fig. 4(A)]. TGF-β1 promoted also the phosphorylation of p44- and p42-MAPK although the kinetics revealed an early (1–2 h) and a late (24 h) activation of this pathway [Fig. 4(A)]. This led us to study the effects of PPAR agonists on signalling events 1 h after the addition of TGF-β1 and to consider only Smads and ERK pathways.

Effects of agonists on TGF-β1-induced phosphorylation of Smad2/3 and ERK1/2

In chondrocytes' beads, TGF-β1-induced phosphorylation of Smad2/3 (4.5-fold) was reduced by −50% to −75% by GW501516 and ROSI, respectively, whereas Wy14643 was ineffective [Fig. 4(B)]. At the same time, TGF-β1-induced phosphorylation of p44-MAPK (2.5-fold) was completely suppressed by GW501516 and ROSI while being reduced slightly by Wy14643 [Fig. 4(C)]. Similar results were observed on TGF-β1-induced phosphorylation of p42-MAPK (3.5-fold) with a −70% to −75% decrease obtained in the presence of GW501516 and ROSI, respectively [Fig. 4(C)].

EFFECTS OF SELECTIVE PPAR AGONISTS ON TGF-β1-INDUCED PGE_2 PRODUCTION

In our experimental conditions, TGF-β1 stimulated PGE_2 production by 3.9-fold at 24 h. This stimulating effect was abrogated by Wy14643 and inhibited by −68% by GW501516, whereas ROSI was marginally inhibitory (Fig. 5). Control experiment showed that the basal production of PGE_2 was decreased (−59%) by Wy14643 but stimulated (1.9-fold) by ROSI.

REVERSION OF THE ANTI-ANABOLIC POTENCY OF PPAR AGONISTS BY SELECTIVE ANTAGONISTS

Effects on aggrecan mRNA expression

TGF-β1-induced increase in aggrecan mRNA level (ninefold increase) was neither affected by the PPARα antagonist GW6471 (10-fold increase) nor by the PPARγ antagonist GW9662 (11-fold increase) [Fig. 6(A)]. As observed previously, TGF-β1-induced aggrecan expression was reduced by all PPAR agonists (from −77% for Wy14643 to below basal level for ROSI). In the presence of GW6471, the inhibitory potency of Wy14643 was relieved whereas those of GW501516 and ROSI remained unchanged [Fig. 6(A)]. Corollary, the inhibitory potency of ROSI was relieved in the presence of GW9662, whereas those of Wy14643 and GW501516 remained unaffected or poorly affected, respectively [Fig. 6(A)]. Control experiment showed that GW6471 and GW9662 did not modify the basal level of aggrecan mRNAs whereas most PPAR agonists were inhibitory (data not shown).

Effects on TGF-β1 mRNA expression

The stimulating effect of TGF-β1 on the mRNA level of TGF-β1 (6.3-fold increase) was neither affected by the

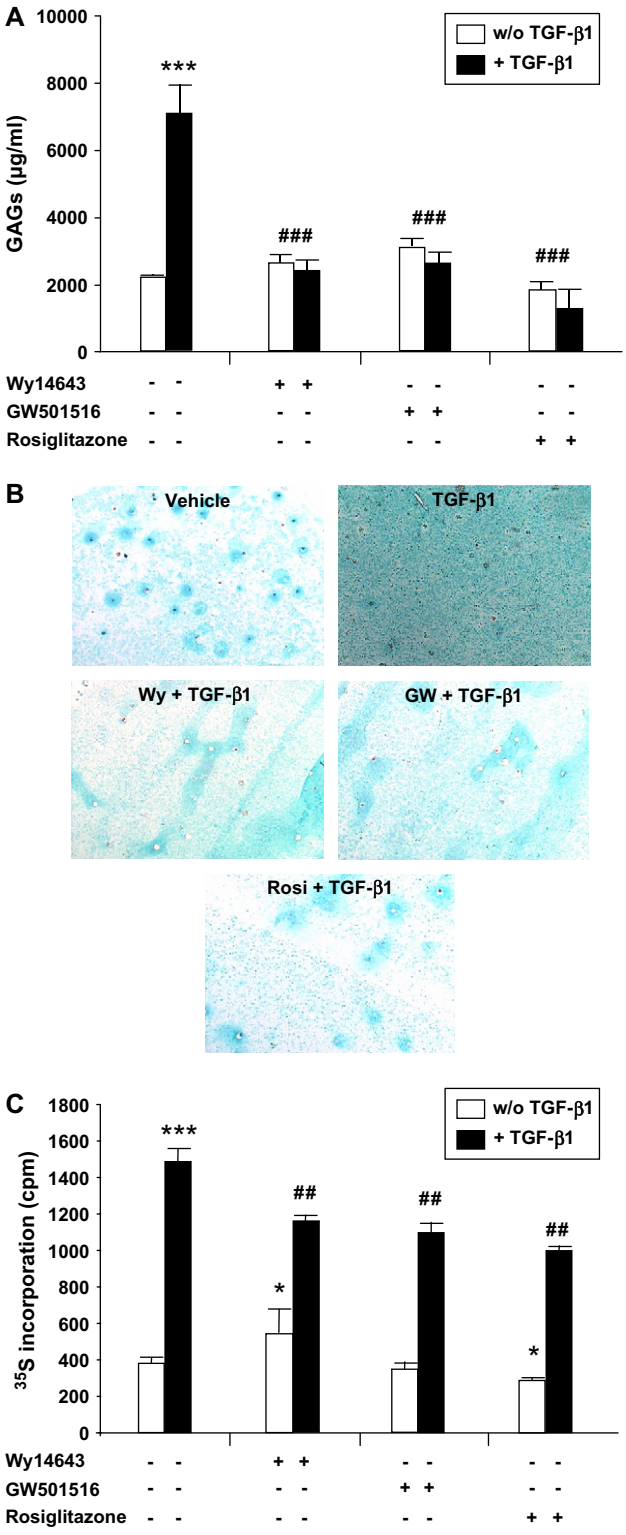

Fig. 3. Effects of selective PPAR agonists on TGF-β1-induced changes in PG metabolism in chondrocytes. Chondrocytes, embedded in alginate beads (5×10^4 cells/bead), were exposed or not to Wy14643 (100 μM), GW501516 (100 nM) or ROSI (10 μM) for 2 h before stimulation with TGF-β1 (10 ng/ml), alone or in combination with PPAR agonists, for 72 h. (A) GAG content after solubilization of alginate beads ($n = 10$); (B) representative sections of Alcian blue staining of beads ($n = 5$); (C) PG synthesis by incorporation of radiolabelled sulphate ($n = 5$). Data are expressed as mean ± S.E.M. Statistically significant differences from the control are indicated as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ and from TGF-β1-treated cells as #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$.

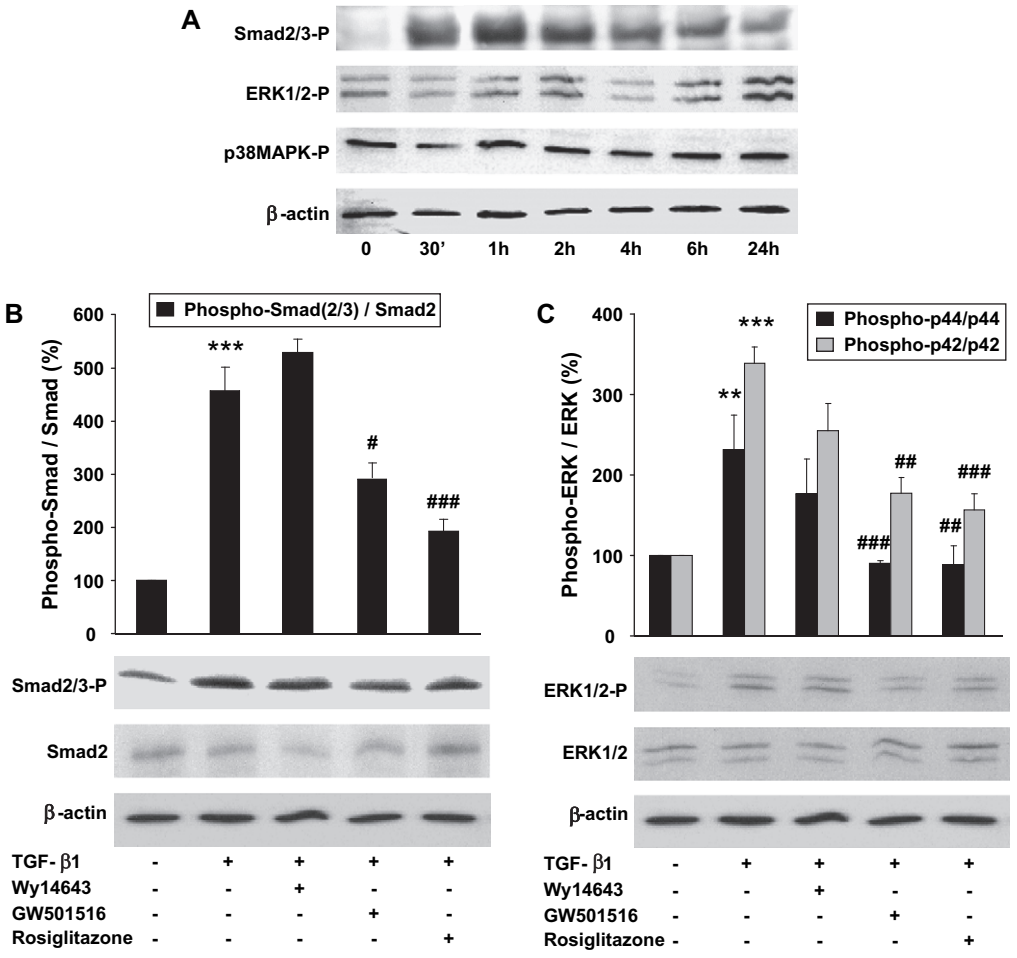

Fig. 4. Effects of selective PPAR agonists on TGF-β1-induced signalling pathways in chondrocytes. Rat chondrocytes, embedded in alginate beads (5×10^4 cells/bead), were exposed or not to Wy14643 (100 μM), GW501516 (100 nM) or ROSI (10 μM) for 2 h before stimulation with TGF-β1 (10 ng/ml), alone or in combination with PPAR agonists, for 1 h. Phosphorylation of Smad, ERK1/2 and p38-MAPK were evaluated by Western Blot ($n = 3$). (A) Time course effect of Smad2/3, ERK1/2 and p38-MAPK phosphorylation in response to TGF-β1; (B) effect of selective PPAR agonists on TGF-β1-induced phosphorylation of Smad2/3; (C) effect of selective PPAR agonists on TGF-β1-induced phosphorylation of ERK1/2. Data are expressed as mean ± S.E.M. Statistically significant differences from the control are indicated as *$P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ and from TGF-β1-treated cells as #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$.

PPARα antagonist GW6471 (4.5-fold increase) nor by the PPARγ antagonist GW9662 (5.3-fold increase) [Fig. 6(B)]. As observed previously, TGF-β1-induced TGF-β1 expression was reduced by all PPAR agonists (from −59% for Wy14643 to below basal level for ROSI). In the presence of GW6471, the inhibitory potency of Wy14643 was relieved whereas those of GW501516 and ROSI remained unchanged [Fig. 6(B)]. Corollary, the inhibitory potency of ROSI was relieved in the presence of GW9662, whereas those of Wy14643 and GW501516 remained unaffected or poorly affected, respectively [Fig. 6(B)]. Control experiment showed that GW6471 and GW9662 did not modify the basal level of TGF-β1 mRNAs whereas ROSI was solely inhibitory (data not shown).

Discussion

In the present work, we investigated the potency of selective PPAR agonists to impair anabolic effect of TGF-β1 on PGs in chondrocytes. Therefore, experimental conditions were selected to reproduce the tridimensional environment of articular cartilage and to obtain an optimal response to TGF-β1. Alginate was chosen as culture system to maintain chondrocytes' phenotype[36] and produce aggrecan with a compartmentalization thought to reproduce the pericellular and territorial matrix areas and interterritorial compartment of articular cartilage, respectively[34]. Chondrocytes' beads were used at a time suitable to overcome early cell death[37] and to assume a reproducible synthesis of extracellular matrix components in response to growth factors[34] without extensive cell proliferation[38]. The latter point is of primary importance since PPAR agonists have well-known antiproliferative effects in other cell types[39]. We showed that, except for ROSI after 72 h of incubation, none of the agonists impaired the MTT assay supporting that our data were rather representative of changes in cell metabolism than in cell proliferation. Preliminary dose-ranging studies confirmed that optimal biosynthetic responses of rat cells were observed for 10 ng/ml of TGF-β1 in low (1%) FCS culture medium, as reported for bovine chondrocytes[38] or mouse cartilage[40]. In these experimental conditions, TGF-β1 stimulated [^{35}S]PGs and overall GAG content to a similar extent while favouring their deposition in the

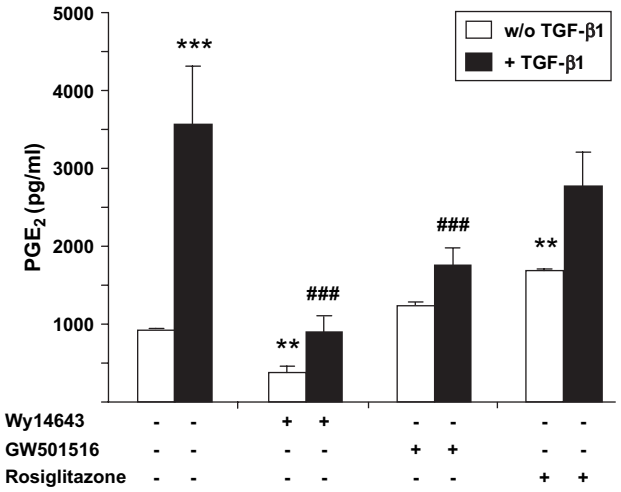

Fig. 5. Effects of selective PPAR agonists on TGF-β1-induced PGE_2 production in chondrocytes. Rat chondrocytes, embedded in alginate beads (5×10^4 cells/bead), were exposed or not to Wy14643 (100 μM), GW501516 (100 nM) or ROSI (10 μM) for 2 h before stimulation with TGF-β1 (10 ng/ml), alone or in combination with PPAR agonists, for 24 h. PGE_2 level was measured by EIA assays in culture supernatants ($n = 4$). Data are expressed as mean ± S.E.M. Statistically significant differences from the control are indicated as $^*P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ and from TGF-β1-treated cells as #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$.

interterritorial matrix of beads. Maximal changes were observed on aggrecan gene expression, which is a well-known target gene of TGF-β1[41], whereas marginal changes were noted for genes involved in the formation of the GAGs' side chains. Thus, it is likely that PGs found in the beads' matrix were essentially neosynthesized. Thus, the anabolic effect of TGF-β1 on PGs' metabolism was driven mainly by the transcriptional activation of the aggrecan gene in rat beads although increased C4S/C6S ratios were reported in human[42] TGF-β1-stimulated chondrocytes.

As many PPAR independent effects were reported for 15-deoxy-$\Delta^{12,14}$prostaglandin J_2 (15d-PGJ_2)[43], a natural agonist with a low binding affinity to PPAR gamma, we used only synthetic PPAR agonist with a high binding affinity for one PPAR isotype[16] and confirmed their selectivity using a panel of PPAR target genes: ACO as representative for PPARα[44], mitochondrial CPT-1 as representative for PPARβ/δ[45] and adiponectin as representative for PPARγ[46]. In our culture system, the contribution of each PPAR isotype was further controlled by reversion experiments with the selective PPARα antagonists GW6471[47] and the selective PPARγ antagonist GW9662[48]. Such pharmacological approach was developed as a convenient surrogate to classical gene reporter or dominant negative strategies[49,50] which were poorly adapted to our culture system. Indeed, efficiency of non-viral transfection was low in primary rat chondrocytes compared to other species[69] and transgene expression was shown to decrease gradually with time in the alginate system[51].

As expected, ACO expression was increased moderately by Wy14643 whereas adiponectin expression was stimulated more robustly by ROSI in rat chondrocytes. Expression of CPT-1 was induced by all PPAR agonists but with a maximal response for GW501516. This result agreed well with less restrictive dependency of CPT-1 towards PPAR isotypes[52] and the influence of tissue environment on the response of a given target gene to PPAR agonists[53].

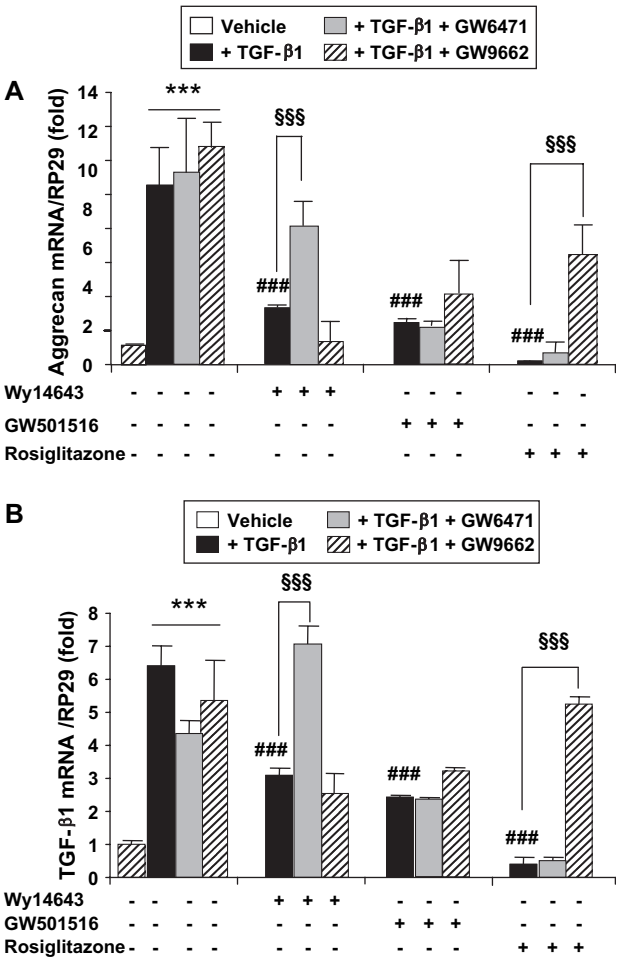

Fig. 6. Reversion of anti-anabolic potency of PPAR agonists by selective antagonists in chondrocytes. Rat chondrocytes, embedded in alginate beads (5×10^4 cells/bead), were exposed or not for 2 h with Wy14643 (100 μM), GW501516 (100 nM), or ROSI (10 μM), in the presence or absence of PPARα (GW6471, 10 μM) or PPARγ (GW9662, 10 μM) antagonists, before stimulation with 10 ng/ml TGF-β1, alone or in combination with PPAR ligands, for 48 h. Data are expressed as mean ± S.E.M. of normalized mRNA levels quantified by real time PCR ($n = 4$). (A) Aggrecan expression; (B) TGF-β1 expression. Statistically significant differences from the control are indicated as *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$, from TGF-β1-treated cells as #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ and from "TGF-β1 + PPAR agonists"-treated cells as §, $P < 0.05$; §§, $P < 0.01$; §§§, $P < 0.001$.

The concentrations which were selected from this inducing profile on PPAR target genes were in the range of those used previously to study the effect of Wy14643[49], GW501516[24], or ROSI[54] in articular cells. However, they were far above those required for the transactivation of chimeric murine or human PPAR receptors in cell-based transfection assays[55] and PPAR-dependent activities were also reported at lower concentrations in rabbit chondrocytes[23,50]. These higher concentrations are supported likely by species differences in the response of PPAR to a given agonist[56] and by the need to activate the whole cellular machinery after crossing of the alginate and extracellular matrix network[34].

Reversion experiments demonstrated that GW6471[47] antagonized selectively the expression of ACO induced by Wy14643 and GW9662[48] the expression of adiponectin

induced by ROSI. In TGF-β1-stimulated cells, GW6471 reversed also the inhibitory effect of Wy14643 on aggrecan and TGF-β1 expression whereas GW9662 reversed the inhibitory effect of ROSI on these parameters. These data were consistent with Wy14643 and ROSI controlling PPARα- and PPARγ-dependent responses, respectively, both in the PPAR and aggrecan systems. The effect of GW501516 was suppressed partly by GW9662, as well on induction of CPT-1 as on the inhibition of TGF-β1-induced aggrecan expression. This could reflect a weak antagonism of PPARβ/δ in chondrocytes since GW9662 was shown to interfere possibly with isotypes other than γ in PPAR reporter cell lines[57]. Nonetheless, this effect remained negligible compared to its covalent inhibition of PPARγ[48] which reproduced the effect of a dominant negative strategy in synovial fibroblast[49] while antagonizing the transcriptional activity of ROSI in SW-1353 chondrosarcoma cells[58]. Although one cannot exclude that agonists may act on other intracellular pathways than PPAR, our data strongly supported that Wy14643, GW501516 and ROSI were responsible for PPARα, β/δ and γ-dependent responses in rat chondrocytes, respectively.

In the present work, we showed for the first time that the stimulating effect of TGF-β1 on GAG content and deposition was reduced dramatically by PPAR agonists whereas the production of newly synthesized PGs was reduced by at least 25%. At the molecular level, TGF-β1-induced expression of aggrecan was inhibited by more than 75% by all PPAR agonists without marked changes on genes involved in GAGs' side chains' synthesis. This effect was seen independently of the selectivity for a given PPAR isotype, although with the following rank order of potency: ROSI $>$ GW501516 $\geq$ Wy14643. The higher potency of ROSI was not supported by a higher level of PPARγ since we confirmed that all PPAR isotypes were expressed at the mRNA level in rat chondrocytes, with PPARβ/δ being the most abundant (data not shown). However, in contrast to our previous reports of a selective decrease of the γ isotype in response to pro-inflammatory cytokines[20,24], we observed that the expression of all PPAR isotypes was reduced significantly in response to TGF-β1 (data not shown). Thus, the ability of selective PPAR agonists to bind to a given isotype was comparable in resting and TGF-β1-stimulated chondrocytes. The inhibitory effect of ROSI on chondrocyte PGs was in line with the ability of PPARγ agonists to suppress decorin expression in human arteries[28] as well as TGF-β1-induced expression of other extracellular matrix components in mesangial cells[36], skin fibroblast[30] or in pulmonary myofibroblasts[31]. In contrast, no anti-fibrotic potency has been reported for PPARβ/δ agonists whereas PPARα agonists were unable to suppress TGF-β1-induced collagen synthesis in pulmonary myofibroblasts[31].

To clarify the contribution of either PPAR isotype to an anti-TGF-β1 effect, we investigated the ability of agonists to impair TGF-β1-induced signalling events thought to regulate aggrecan expression in chondrocytes[59]. Interestingly, we demonstrated that early TGF-β1-induced phosphorylation of Smad2/3 was reduced by ROSI and GW501516 but not by Wy14643. As overexpression of Smad7, a well-known inhibitory Smad, suppressed the stimulating effect of TGF-β1 on PG synthesis in a murine chondrocyte cell line[60], it is likely that inhibition of the Smad pathway contributed to the inhibitory potency of ROSI and GW501516. However, further studies are required to investigate if these agonists were able to interact physically with Smad proteins as PPAR–ligands complexes[61] or to regulate aggrecan expression at the level of Smad-dependent promoter activity induced by TGF-β[30]. We also demonstrated that the early phosphorylation of ERK1/2 was reduced by ROSI and GW501516 but not by Wy4643. Although its contribution was reported primarily in TGF-β-induced cell growth[62,63], activation of ERK1/2 was shown to regulate TGF-β-induced aggrecan gene expression[59] and to interplay with other signalling pathways in chondrocytes[59,63]. Interestingly, inhibition of ERK1/2 and Smad2/3 phosphorylation by ROSI and GW501516 was complementary and may reduce TGF-β-induced expression of aggrecan gene with a different time course[59]. In contrast, we showed that TGF-β1 failed to activate p38-MAPK in our culture system, a finding consistent with previous kinase assays on chondrocytes' monolayers[62] which did not support its major contribution to PGs' synthesis. This result is at variance with the blunting effect of p38-MAPK inhibitors on TGF-β1-induced PGs' synthesis in human chondrocytes[64], but species dependent differences were reported for the contribution of p38-MAPK pathway in cartilage[65].

We demonstrated finally that the stimulating effect of TGF-β1 on PGE_2 synthesis was reduced by Wy14643 and GW501516 but not by ROSI in chondrocytes' beads. TGF-β was reported to stimulate PGE_2 production in growth plate chondrocytes by activation of several enzymes of the arachidonic acid cascade[66–68]. Effect of PGE_2 on chondrocytes' functions depends strongly on differentiation and corresponding expression of EP receptors[69], with EP-1 and EP-2 being shown to promote cell growth and PGs' synthesis in growth plate-derived chondrocytes[67] and EP-2 to account for the growth of mature articular chondrocytes[70]. TGF-β-dependent PG production was shown to involve a protein kinase C (PKC) signalling pathway[66,67], which was sensitive to EP-2-dependent activation of Protein kinase A (PKA) by PGE_2 in growth plate chondrocytes[67] whereas crosstalk between ERK, PKA and PKC was reported in mature cells[63]. Although further studies are required to elucidate the direct modulation of each signalling pathway by PPAR agonists, our data suggested that inhibition of TGF-β1-induced PGE_2 synthesis by Wy14643 and GW501516 contributed to their inhibitory effect on PG synthesis. Taken together, our data were in favour of PPAR agonists being able to reduce TGF-β1-induced responses by distinct mechanisms ranging from inhibition of PGE_2 for Wy14643 to inhibition of ERK1/2 and Smad2/3 phosphorylation for ROSI, with GW501516 being effective on both. Such differences agreed well with the recent demonstration that clofibrate, a PPARα agonist, and ROSI, a PPARγ agonist, were able to reduce IL-1-induced MMP-1 expression in rabbit chondrocytes by promoting sIL-1Ra production[23] or interacting on a composite PPRE/AP1 site in the MMP-1 promoter[50], respectively.

From a physiopathological point of view, the inhibitory potency of PPAR agonists on TGF-β1-induced PG synthesis could theoretically be viewed as a reduction of the repair capacity of articular cartilage. However, TGF-β has also a key role in extracellular matrix remodelling by regulating the balance between MMPs and their natural inhibitors[4]. Thus, TGF-β was shown to stimulate MMP-13 depending on the physiologic state of OA chondrocytes[71] and to favour MMP-9[72] or aggrecanase production[12] while limiting the catabolic potency of pro-inflammatory cytokines[73]. PPARα or γ agonists were demonstrated to down-regulate the cytokine-induced expression of several MMPs[21,23,50] or aggrecanase[74] whereas n-3 polyunsaturated fatty acids, which are putative PPAR agonists[56], prevented the IL-1-induced release of PG metabolites from cartilage explants[75]. Our data suggest that PPAR agonists could also interfere with TGF-β1-induced degradation of PG. As a

consequence, due to their dual cytokines- and TGF-β-suppressive properties, PPAR agonists could protect cartilage PGs variably depending on the imbalance between these factors and/or the level of inflammation[76]. This could explain part of the reduction of cartilage lesions that was reported for ROSI[5] or fenofibrate[77] in experimental polyarthritis and for pioglitazone in the menisectomy model of OA[22], since a decreased expression of pro-inflammatory cytokines was reported. Although, this must be confirmed *in vivo*, our data strongly suggest that PPAR agonists could be deleterious in situation of cartilage repair while being protective in situation of cartilage degradation.

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