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Peroxisome proliferator activated receptor alpha activation decreases inflammatory and destructive responses in osteoarthritic cartilage

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SUMMARY

Objective: Peroxisome proliferator activated receptor α (PPARα) agonists are used in clinical practice as lipid-lowering drugs and are also known to exert anti-inflammatory effects on various tissues. We hypothesized that PPARα activation leads to anti-inflammatory and anti-destructive effects in human OA cartilage.

Methods: Cartilage explants obtained from six OA patients were cultured for 48 h with 10 ng/ml interleukin (IL)1β as a pro-inflammatory stimulus. 100 μM Wy-14643, a potent and selective PPARα agonist, was added to the cultures and gene expression of matrix metalloproteinase (MMP)1, MMP3, MMP13, collagen type II (COL2A1), aggrecan and PPARα in cartilage explants and the release of glycosaminoglycans (GAGs), nitric oxide (NO) and prostaglandin E$_2$ (PGE$_2$) in the culture media were analyzed and compared to the control without Wy-14643.

Results: Addition of Wy-14643 decreased mRNA expression of MMP1, MMP3 and MMP13 in cartilage explants that responded to IL1β, whereas Wy-14643 did not affect gene expression of COL2A1 and aggrecan. Wy-14643 also decreased secretion of inflammatory marker NO in the culture medium of cartilage explants responding to IL1β. Wy-14643 inhibited the release of GAGs by cartilage explants in culture media.

Conclusion: PPARα agonist Wy-14643 inhibited the inflammatory and destructive responses in human OA cartilage explants and did not have an effect on COL2A1 or aggrecan mRNA expression. These effects of PPARα agonists on osteoarthritic cartilage warrant further investigation of these drugs as a potential therapeutic strategy for osteoarthritis (OA).

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Introduction

Loss of structure of articular cartilage is an important characteristic of osteoarthritis (OA). Inflammatory responses in the joint, leading to the production of cartilage matrix enzymes and pro-inflammatory cytokines, contribute to the disruption of the balance between anabolism and catabolism in cartilage[1]. To date, no drugs are able to prevent the initiation and progression of OA[2]. Peroxisome proliferator activated receptor (PPAR)α is a class I nuclear receptor belonging to the superfamily of ligand-dependent transcription factors regulating the transcription of target genes by binding retinoid X receptor. In addition, PPARα modulates gene transcription in a DNA binding-independent manner[3,4]. Natural ligands of PPARα are polyunsaturated fatty acids or arachidonic metabolites (prostaglandins and leukotrienes). Synthetic ligands of PPARα are Wy-14643 and fibrates, including gemfibrozil, clofibrate, fenofibrate and bezafibrate[5–7]. Fibrates are clinically used as drugs that lower plasma triglyceride and cholesterol levels through the induction of β- and ω-oxidative pathways, which neutralize and degrade fatty acids[8]. In addition, anti-inflammatory effects of PPARα activation have been described[9]. These anti-inflammatory effects mainly depend on the inhibition of the nuclear translocation of nuclear factor-κB (NF-κB), which plays an important role in the regulation of inflammatory responses[10].

PPARα is present in a broad range of cells such as hepatocytes, myocardiocytes, skeletal myocytes, endothelial cells, and also in chondrocytes[11–15]. PPARα activation inhibits the production of metalloproteinases in rabbit articular chondrocytes cultured in monolayer and stimulated with interleukin (IL)1β by potentiating the IL1β induced upregulation of IL1 receptor antagonist (IL1ra)[16].

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The inhibitory effects of PPARα activation on MMP production were confirmed by Poleni et al.[17]. They also showed that PPARα might decrease proteoglycan and collagen type II (COL2A1) synthesis in rat chondrocytes embedded in alginate beads[18]. The effect of PPARα activation on inflammatory and destructive responses in human OA cartilage explants has not yet been established. The aim of this study is to investigate whether PPARα stimulation can reduce inflammatory responses in human OA cartilage and to further explore the influence of this stimulation on anabolic factors COL2A1 and aggrecan. More evidence for a beneficial role of PPARα stimulation on cartilage homeostasis is crucial in order to clarify the potential of drugs such as fibrates as a therapeutic strategy for OA.

In this study, we investigated the effect of Wy-14643 on anabolic, catabolic and inflammatory markers in osteoarthritic cartilage explants. COL2A1 and aggrecan are important molecules in the extracellular matrix, and matrix metalloproteinase (MMP)1 (collagenase 1), MMP3 (stromelysin 1), MMP13 (collagenase 3) are involved in matrix degradation in OA[19,20]. To assess the anti-inflammatory properties of Wy-14643, we analyzed nitric oxide (NO) and prostaglandin E_2 (PGE_2) production by the cartilage explants since these are known mediators of inflammation, destruction and pain[21–23]. As mentioned before, NF-κB is a target of PPARα agonists. Therefore, we also investigated whether PPARα activation inhibits the nuclear translocation of NF-κB in human OA chondrocytes.

Materials & methods

Preparation of human cartilage explants and human chondrocytes

Cartilage was obtained as anonymous waste material from six human OA patients who underwent total knee arthroplasty. The patients had the right to consent as stated by the guidelines of the Dutch Federation of Biomedical Scientific Societies (www.federa.org). This method was approved by the Local Ethical Committee (number MEC 2004-322). Explants with diameter of 6 μm were prepared using biopsy punches. Nine explants were consolidated in 3 ml Dulbecco's modified Eagle's medium (DMEM) high glucose (GibcoBRL, Gaithersburg, MD, USA), supplemented with 2% fetal calf serum (Lonza, Basel, Switzerland), 50 μg/ml gentamycin (GibcoBRL, Grand Island, NY, USA) and 1.5 μg/ml fungizone (GibcoBRL, Grand Island, NY, USA) for 24 h in six well plates.

Study design

After pre-culture the explants were washed in phosphate buffered saline and the culture medium was replaced by DMEM high glucose with Insulin-Transferrin-Selenium (Becton Dickinson Biosciences, Erembodegem, Belgium). Explants were cultured simultaneously with or without 10 ng/ml IL1β (Peprotech Rocky Hill, NJ, USA) as a pro-inflammatory stimulus and with or without 10 or 100 μM Wy-14643 (Cayman Chemical, Ann Arbor, MI, USA), a potent and selective PPARα agonist[17]. Because Wy-14643 was dissolved in Dimethyl-Sulfoxide (DMSO; Sigma, St. Louis, MO, USA), DMSO was also added to the control and 10 μM conditions in order to correct for the concentration present in 100 μM Wy-14643. After 48 h of culture, cartilage explants and culture media were snap frozen in liquid nitrogen and stored in −80°C until further use.

RNA extraction and real-time polymerase chain reaction (PCR)

For each of the six donors, three samples were analyzed per experimental condition. Each sample consisted of three explants that were homogenized with a Mikro-Dismembrator (Braun Biotech International GmbH, Melsungen, Germany) and suspended in 1.8 ml/

100 mg tissue RNA-Bee (Bioconnect, Huissen, The Netherlands), in order to obtain sufficient RNA. RNA-bee suspended RNA was precipitated with 0.2 ml chloroform. RNA was purified using an RNeasy Micro Kit (Qiagen, Hilden, Germany). 500 ng of total RNA was reverse-transcribed into cDNA using RevertAid First Strand cDNA synthesis Kit (MBI Fermentas, St. Leon-rot, Germany). By means of PrimerExpress 2.0 software (Applied Biosystems, Foster City, CA, USA), forward and reverse primers for the real-time (RT)-PCR reaction were designed to meet TaqMan requirements, and to bind the separate exons to avoid amplification of genomic DNA. The designed primer sequences were run against a genome-wide database Basic Local Alignment Search Tool (BLASTN) to ensure gene specificity of the primers and probes. The following forward and reverse oligonucleotides were used: MMP1 (Fw: CTCAATTTCACTTCTGTTTTCTG Rv: CATCTCTGTCGGCAAATTCGT probe: CACAACTGCCAAATGGGCTT-GAAGC; GenBank accession no. NM_002421), MMP3 (Fw: TTTTGGCCATCTCTTCCTTCA Rv: TGTGGATGCCTCTTGGGTATC probe: AACTTCATATGCGGCATCCACGCC; GenBank accession no. NM_002422), MMP13 (Fw: AAGGAGCATGGCGACTTCT Rv: TGGCCCAGGAGGAAAAGC probe: CCCTCTGGCCTGCGGCTCA; GenBank accession no. NM_002427), COL2A1; Fw: GGCAA-TAGCAGGTTCACGTACA Rv: CGATAACAGTCTTGCCCCACTT probe: CCGGTATGTTTCGTGCAGCCATCCT; GenBank accession no. NM_033150, aggrecan (Fw: TCGAGGACAGCGAGGCC Rv: TCGAGGGTGTAGCGTGTAGAGA probe: ATGGAACACGATGCCTTTCA-CCACGA; GenBank accession no. NM_001135) and PPARα (Fw: GACGTGCTTCCTGCTTCATAGA Rv: CCACCATCGCGACCAGAT; GenBank accession no. NM_005036). TaqMan Universal PCR Master Mix (ABI, Branchburg, NJ, USA) or qPCR™ Mastermix Plus for SYBR®Green I (Eurogentec, Nederland B.V., Maastricht, The Netherlands) was used to perform real-time (RT)-PCR in 20 μl reactions according to the manufacturer's guidelines and using an ABI PRISM 7000 with SDS software, version 1.2.3. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; fw: ATGGGGAAGGTGAAGGTCG Rv: TAAAAGCAGCCCTGGT-GACC Probe: CGCCCAATACGACCAAATCCGTTGAC GenBank accession no. NM_002046.3) was compared with two other housekeeping genes (data not shown) and was found to have good stability throughout the conditions and samples. Relative gene expression was calculated by means of the $2^{-\Delta CT}$ formula.

Assays on culture media for viability, NO, PGE_2 and glycosaminoglycans (GAGs)

The pooled culture media of three samples per condition for each patient ($N = 6$ patients) were harvested to test the effect of Wy-14643 on the viability of chondrocytes using the Cytotoxicity Detection Kit (Roche Diagnostics, IN, USA) in accordance with the manufacturer's instructions to determine the presence of lactate dehydrogenase (LDH) in culture media. The amount of NO was measured using the spectrophotometric method based on the Griess reaction (Sigma, St. Louis, MO, USA). Absorbance was measured at 540 nm. PGE_2 content of the culture media was determined using the PGE_2 Assay (RnD systems, Minneapolis, MN, USA) according to manufacturer's instructions at absorbance of 450 nm. The release of GAGs from cartilage explants into the culture medium was analyzed with the dimethylmethylene blue (DMB) assay[24]. The metachromatic reaction with DMB was monitored with a spectrophotometer and a ratio $A_{530}:A_{590}$ was used to determine the GAG amount with chondroitin sulfate C (Sigma) as standard. Analyses were performed in duplicate and the averages of these results were used for statistical analysis.

NF-κB nuclear translocation in humane chondrocytes

Chondrocytes were isolated by pronase (2 mg/ml, Sigma, St. Louis, MO, USA) digestion for 1 h and a half, followed by

overnight digestion with collagenase B (1.5 mg/ml, Roche Diagnostics, Mannheim, Germany). The obtained chondrocytes were seeded in a 48 wells plate with at a density of 20,000 cells/cm^2 and cultured in DMEM high glucose (GibcoBRL, Gaithersburg, MD, USA), supplemented with 10% fetal calf serum (Lonza, Basel, Switzerland), 50 µg/ml gentamycin (GibcoBRL, Grand Island, NY, USA) and 1.5 µg/ml fungizone (GibcoBRL, Grand Island, NY, USA). Chondrocytes were precultured during 5–7 days. A preliminary experiment showed that 1 h of IL1β incubation was the optimal moment to evaluate NF-κB activation (data not shown), as also previously reported[25]. Chondrocytes were cultured for immunohistochemical detection of NF-κB with or without 10 ng/ml IL1β and with or without 100 µM Wy-14643 during 1 h. The cells were fixated on the plate in formalin 4%. This was followed by incubation for 1 h with rabbit polyclonal NF-κB p65 antibody (1/100 Ab-276; Signalway Antibody, Leusden, The Netherlands). Finally, alkaline phosphatase labeled secondary antibody was used in combination with a freshly prepared new fuchsine substrate (Chroma, Kongen, Germany). An isotype IgG1 monoclonal antibody was used as negative control. If the nucleus stained intensively red, cells were defined as positive for the nuclear presence of NF-κB. The percentage of positive cells was determined by two blinded and independent observers by scoring three times 100 cells/well.

Data analysis

Data were analyzed with PSAW statistics 18.0 (SPSS inc. Chicago, IL, USA). A mixed linear model was used for the mRNA expression data of in total 18 samples/condition, obtained from six donors. For the pooled media analysis, we used a general linear model. Since the error terms of these models are assumed to have a normal distribution, we analyzed and confirmed the normal distribution of the residuals with the Wilk–Shapiro test. To account for the differences in IL1β response between donors, the statistical models for analyzing the effect of PPARα activation were adjusted for IL1β response. Therefore, the statistical analysis indicates whether there is a significant effect of PPARα activation when considering the IL1β response. For the histological analysis of NF-κB, a one-way analysis of variance (ANOVA) was performed with a Bonferroni *post hoc* test, after testing the residuals for normality with Wilk–Shapiro test. $P < 0.05$ was considered statistically significant.

Results

PPARα genes were expressed in human OA cartilage. Stimulation with IL1β did not have effect on PPARα gene expression (data not shown).

In order to exclude detrimental effects of Wy-14643 on viability of chondrocytes, we performed an LDH cytotoxicity assay on cartilage explants cultured in 1 µM, 10 µM, 100 µM and 1000 µM of Wy-14643. Only at the concentration of 1000 µM Wy-14643 LDH concentration in the culture media was increased, indicating a deleterious effect of Wy-14643 on chondrocyte viability. Viability was preserved at concentrations of 1, 10 and 100 µM Wy-14643 (data not shown).

We observed no effects of 10 µM Wy-14643 on MMP, COL2A1, aggrecan gene expression, GAG release, NO or PGE$_2$ production and therefore used the 100 µM concentration in our experiments.

We investigated the effect of Wy-14643 on degradation markers in OA cartilage by gene expression analysis on MMP1, MMP3 and MMP13 in OA cartilage samples stimulated with 10 ng/ml IL1β. The addition of IL1β increased mRNA expression of MMP1 in samples of five of six patients ($P = 0.01$), MMP3 in samples of five of six patients ($P = 0.08$) and MMP13 in samples of all patients ($P = 0.06$).
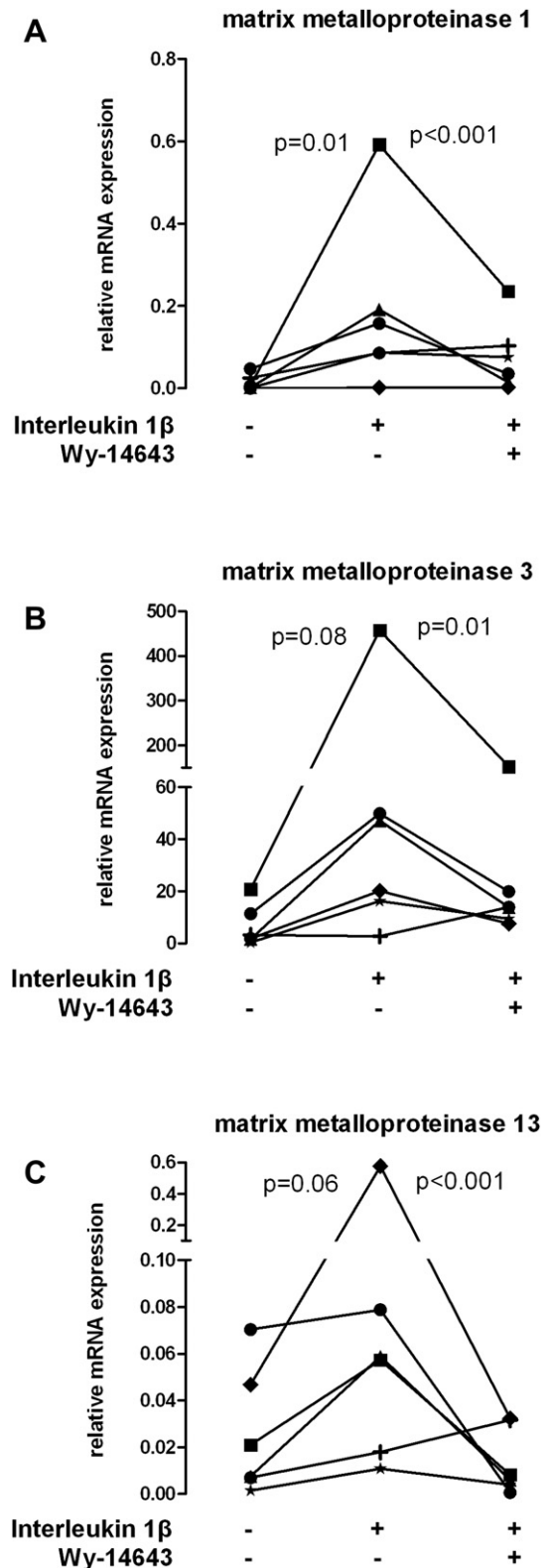

Fig. 1. Catabolic responses of cartilage explants after 48 h of culture with IL1β and with(out) PPARα agonist Wy-14643. mRNA expression of (A) MMP 1, (B) MMP3 and (C) MMP13. Gene expression is normalized to GAPDH. For each condition, $N = 18$ samples of cartilage, obtained from six donors. Averages of three samples are shown for each donor. Each symbol represents one donor.

mRNA expression of MMP1 ($P < 0.001$), MMP3 ($P = 0.01$) and MMP13 ($P < 0.001$) was significantly decreased by the addition of Wy-14643 in the samples from patients that responded to the IL1β stimulation by an increased expression of MMPs (Fig. 1). The addition of IL1β decreased mRNA expression of COL2A1 in explants of four of six patients ($P = 0.02$) and aggrecan in samples of five of six patients ($P = 0.02$). This effect was not counteracted by Wy-14643 (Fig. 2).

NO and PGE$_2$ were analyzed in culture media of the cartilage samples as markers of inflammation. IL1β increased the production of NO in five of six patients ($P = 0.10$) and PGE$_2$ production was increased in five of six patients ($P = 0.13$). Wy-14643 decreased the production of NO ($P = 0.02$) and PGE$_2$ ($P = 0.07$) in samples that responded to IL1β by an increased production of NO and PGE$_2$ (Fig. 3).

IL1β increased GAG release in the culture media in three of six patients ($P = 0.23$). Wy-14643 decreased the release of GAG, again only in samples that responded to IL1β by an increased release of GAGs ($P = 0.005$) (Fig. 3).

To investigate whether inhibition of the nuclear translocation of NF-κB is an underlying mechanism contributing to the anti-destructive and anti-inflammatory effects of PPARα activation, we examined the location of NF-κB in human chondrocytes with immunohistochemistry (Fig. 4). Here, we observed a 30% increase of positive staining cells for nuclear NF-κB when adding 10 ng/ml IL1β ($P = 0.004$). There was no effect of adding Wy-14643 to the cells that were not incubated with IL1β ($P = 1.00$), but we observed a decrease
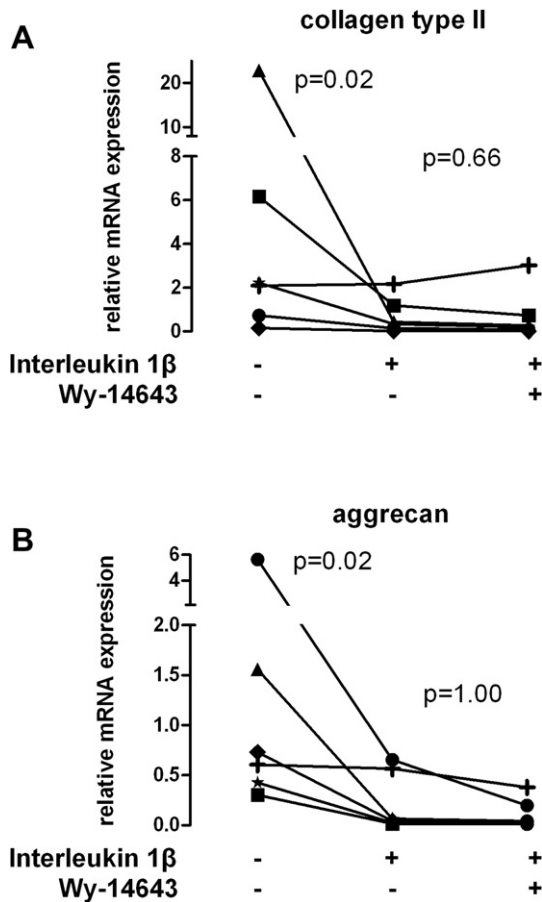

Fig. 2. Anabolic responses of cartilage explants after 48 h of culture with IL1β and with(out) PPARα agonist Wy-14643. Relative mRNA expression of (A) COL2A1 and (B) aggrecan. Gene expression is normalized to GAPDH. For each condition, $N = 18$ samples of cartilage, obtained from six donors. Averages of three samples are shown for each donor. Each symbol represents one donor.
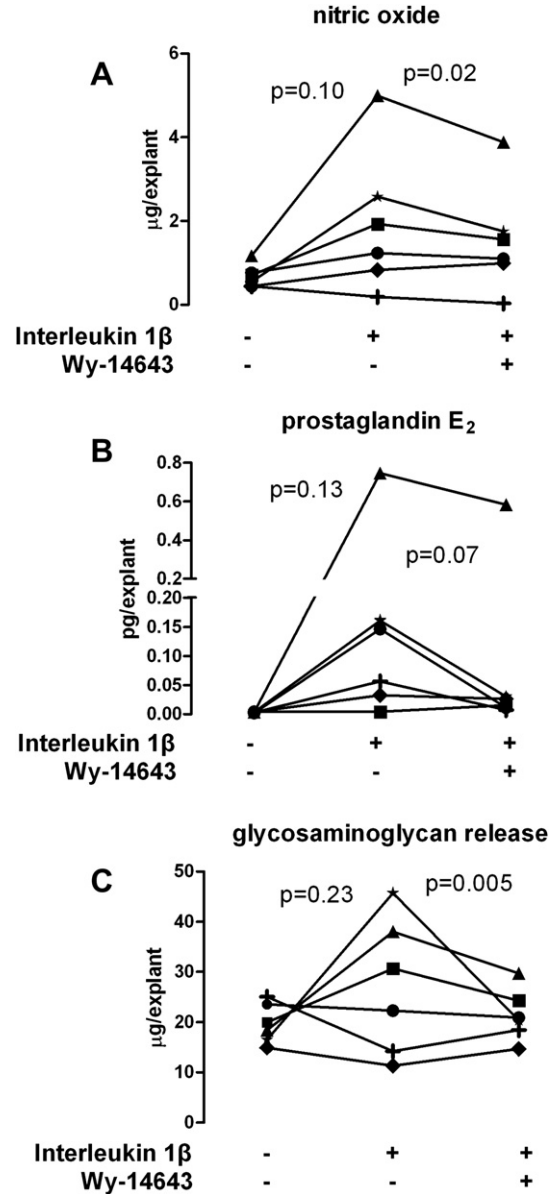

Fig. 3. Inflammatory and destructive responses of cartilage explants after 48 h of culture with IL1β and with(out) PPARα agonist Wy-14643: supernatant was analyzed for (A) NO, (B) PGE$_2$ and (C) GAG release. For each condition and for each donor pooled culture media were analyzed, resulting in $N = 6$ (donors). Each symbol represents one donor.

of 34% ($P = 0.001$) by adding 100 μM Wy-14643 to the IL1β incubated cells.

Discussion

Addition of 100 μM PPARα agonist Wy-14643 decreased gene expression of the catabolic markers MMP1, MMP3 and MMP13, and decreased release of GAG and NO in human OA cartilage explants. There was no effect of Wy-14643 on COL2A1 and aggrecan mRNA expression. Interestingly, effects of PPARα activation were only found in explants that responded to IL1β.

We show that PPARα agonists are able to counteract inflammatory and destructive responses but only in IL1β responsive human OA cartilage samples, as analyzed statistically with IL1β response as a covariable. Our results are in concordance with a study of Francois et al., that demonstrated that the addition of clofibrate, another PPARα agonist, counteracts IL1β induced MMP1, 3 and 13 production

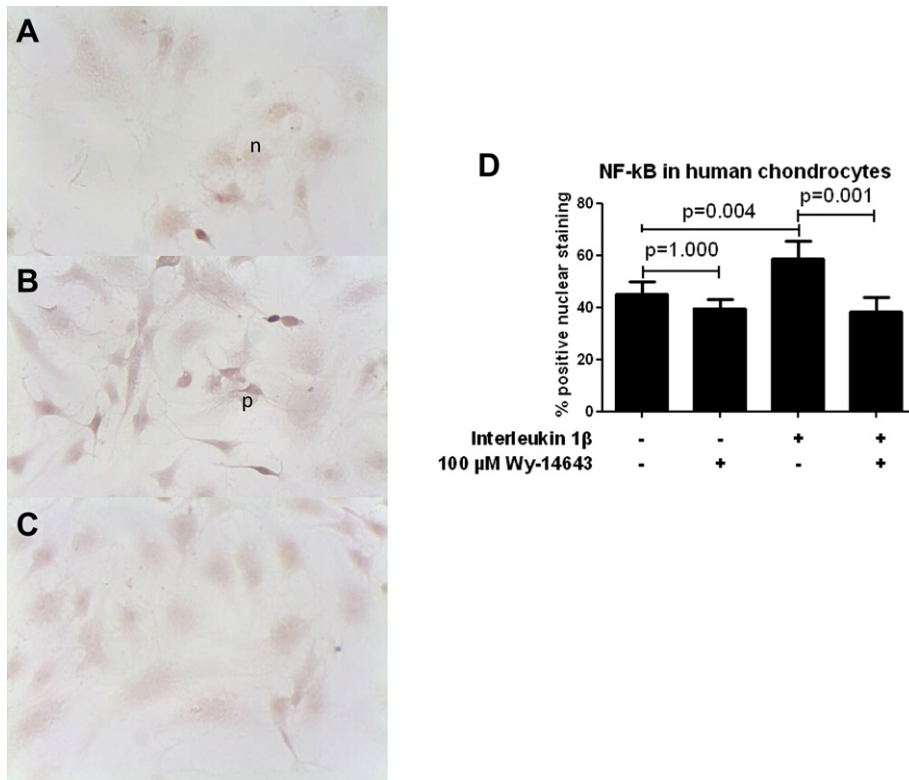**Fig. 4.** Nuclear translocation of NF-κB in human chondrocytes cultured in monolayer. Immunolocalization (magnification 200 times) of (A) NF-κB in cells cultured in medium alone, (B) with IL1β, (C) and with the combination of IL1β and 100 μM Wy-14643. $N = 3$ different cultures. P indicates a cell with positive nuclear staining, n: chondrocyte without intranuclear presence of NF-κB. Figure 4D shows the percentages of cells with a positive nuclear staining for NF-κB in the different conditions. Bars indicate standard deviation.

in rabbit articular chondrocytes and also had no effects without IL1β. Their study demonstrated that the decrease in MMP1, 3 and 13 by PPARα activation is due to the potentiation of IL1β stimulated production of IL1ra. Without PPARα activation, IL1β modestly induces the production of IL1ra, but the combination of IL1β and PPARα leads to higher IL1ra levels that inhibit IL1β stimulation[16]. In our study however, IL1ra mRNA expression (data not shown) did not correlate with the outcome parameters. Therefore, other mechanisms were considered for explaining the effect of PPARα activation. NF-κB plays a central role in the generation of inflammatory and destructive mediators in OA[10,26]. PPARα ligands have been described to inhibit the nuclear translocation of NF-κB, in part by increasing the expression of inhibitor κB-α (IκB-α)[10]. Under normal conditions, NF-κB is present in the cytoplasm in an inactive form bound to IκB-α, but inflammatory reactions induce the degradation of IκB-α and allow NF-κB to translocate to the nucleus where it orchestrates the transcription of cytokines, growth factors, chemokines, and survival genes[10]. We confirmed that a PPARα ligand partly inhibits the nuclear translocation of NF-κB in human IL1β stimulated chondrocytes, although we cannot exclude that other inflammatory pathways such as mitogen-activated protein kinase phosphorylation (extracellular signal-regulated kinase, c-Jun NH2-terminal kinase, p38) are involved[10]. Interestingly, in human colorectal epithelial cells, PPARα ligands increased cyclo-oxygenase (COX2) gene expression resulting in production of prostaglandins. This might be due to the presence of a peroxisome proliferator response element (PPRE) in the COX2 promotor region that binds to activated PPARα[27]. In our study, COX2 gene expression and PGE₂ protein expression are not increased, and even decreased by Wy-14643 in the explants that responded to the IL1β stimulation. This indicates that, in IL1β stimulated chondrocytes, the NF-κB dependant regulation of COX2

overrules the promotion of COX2 gene expression by PPRE. In contrast, the samples of patients that did not respond to IL1β may lack NF-κB activation, leading to an overweight of the binding to the PPRE and therefore showing no effect of Wy-14643 or even an increase in gene expression of COX2 gene expression and PGE₂ release in some patients.

Not all IL1β induced responses were counteracted by the PPARα agonist. IL1β decreased gene expression of COL2A1 and aggrecan in cartilage explants but this was not influenced by Wy-14643. COL2A1 and aggrecan mRNA expression in the IL1β stimulated samples were very low. To exclude the possibility that expression of these genes could not be lowered by adding Wy-14643 because the expression was already very low, we have added 100 μM Wy-14643 to unstimulated samples and analyzed the mRNA expression of COL2A1 and aggrecan. Here, 100 μM Wy-14643 also did not affect the gene expression of COL2A1 and aggrecan (data not shown) indicating that the main effect of PPARα activation is inhibiting inflammatory and destructive, but not anabolic processes in cartilage. However, there was a correlation between mRNA expression level (MMPs) and protein release (NO, PGE₂ and GAG). The samples of two patients responded modestly to IL1β at both mRNA expression and protein level, while four other patients had a great response at both levels, indicating that gene expression data can be extrapolated to the protein level.

In this study, we used human OA cartilage explants. Explants resemble the *in vivo* situation since the extracellular matrix that influences chondrocyte metabolism is still present. The explants were stimulated with 10 ng/ml IL1β to mimic inflammatory processes in an *in vitro* model for OA[28–30]. However, not all cartilage of patients responded to the IL1β stimulation. The difference in IL1β response of the human cartilage explants to IL1β in our study are

comparable with previous studies using IL1β to stimulate human explants[31]. The large variability in response may be due to differences in age, different levels in oxidative stress responsible for activating NF-κB, and zonal differences in cartilage metabolism[32–34]. We observed no effect of IL1β on PPARα mRNA expression, in accordance with the results of previous studies[11,35].

Wy-14643 was selected because it is a potent and selective PPARα agonist. It exhibits 10 times more affinity for PPARα (kd < 1 μM) than does clofibrate and fenofibrate[36–38] and is 50–500 times more selective for PPARα than other subtypes of PPAR. Wy-14643 has been used in concentrations higher than 100 μM before in other *in vitro* studies[11,18,39] and was demonstrated to remain a selective PPARα agonist in chondrocytes at concentrations of 100 μM[18] by Poleni *et al.* In our study, 10 μM Wy-14643 had no effect on any parameter in the explants cultures, although effects of even lower concentrations of less potent PPARα agonists have been observed on chondrocytes[16]. The absence of an effect for 10 μM Wy-14643 might be explained by difficulties in penetration in the extracellular matrix of the explants, since other studies were using chondrocyte cultures in monolayer. Nonetheless, our study indicates that 100 μM Wy-14643 decreases both destructive and inflammatory markers on cartilage explants, confirming the proof of principle that PPARα activation leads to a decrease of inflammatory and destructive responses. Further studies are needed to examine the effects of PPARα activation on cartilage metabolism and cartilage quality using longer term cultures and to investigate whether PPARα ligands such as fibrates, used in clinical practice, can induce similar responses in cartilage as observed in this study. N-3 polyunsaturated fatty acids are naturally occurring agonists of PPARα and have been described to exert anti-inflammatory properties on articular cartilage[40]. Since n-3 fatty acids are PPARα ligands with low affinity, we believe that high concentrations or a high total flux of fatty acids might induce the anti-inflammatory effects of fatty acids in a joint through the activation of PPARα.

Conclusion

Drug research in OA has mainly focussed on cartilage, although metabolic factors such as body mass index, plasma triglycerides, cholesterol levels and subchondral vascular pathology might play a role in the OA pathogenesis[41–46]. Therefore it would be interesting to find drugs capable of altering metabolic factors together with inhibiting destructive or inflammatory responses in cartilage. PPARα agonists may indirectly influence the OA disease process by the prevention of subchondral vascular pathology, by lowering triglycerides and cholesterol levels in plasma, and through their anti-inflammatory effects on the blood vessels[15,47,48]. Our data provide evidence that PPARα agonists, used as lipid-lowering drugs in clinical practice (e.g., fibrates), may improve cartilage metabolism in an osteoarthritic joint by lowering MMP1, 13-gene expression, GAG release and NO production. Therefore, we believe that the use of the synthetic ligands of PPARα as a potential therapeutic strategy for OA should be further explored.

Conflict of interest
All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence this work.

Author contributions

S. Clockaerts contributed to acquisition, analysis and interpretation of data, drafting and critically reviewing the manuscript, collection and assembly of the data and final approval of the submitted version.

Y.M. Bastiaansen-Jenniskens contributed to conception and design, acquisition, analysis and interpretation of data, critically reviewing the manuscript and final approval of the submitted version.

C. Feijt contributed to the study by the collection and assembly of data, providing administrative and technical support and final approval of the submitted version.

J.A.N. Verhaar contributed to the critically reviewing the manuscript and final approval of the submitted version.

J. Somville contributed to the interpretation of data, critically reviewing the manuscript and final approval of the submitted version.

L.S. De Clerck contributed to the interpretation of data, critically reviewing the manuscript and final approval of the submitted version.

G.J.V.M. Van Osch contributed to the conception and design, analysis and interpretation of the data, critical revised the article for important intellectual content and also contributed to the final approval of the article, conception and design, analysis and interpretation of the data.

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