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# Peroxisome Proliferator-Activated Receptor (PPAR)-γ Positively Controls and PPARα Negatively Controls Cyclooxygenase-2 Expression in Rat Brain Astrocytes through a Convergence on PPARβ/δ via Mutual Control of PPAR Expression Levels

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## ABSTRACT

Peroxisome proliferator-activated receptor (PPAR) transcription factors are pharmaceutical drug targets for treating diabetes, atherosclerosis, and inflammatory degenerative diseases. The possible mechanism of interaction between the three PPAR isotypes (α, β/δ, and γ) is not yet clear. However, this is important both for understanding transcription factor regulation and for the development of new drugs. The present study was designed to compare the effects of combinations of synthetic agonists of PPARα [2-[4-[2-[4-cyclohexylbutyl (cyclohexylcarbamoyl)amino]ethyl]phenyl] sulfanyl-2-methylpropanoic acid (GW7647)], PPARβ/δ [4-(3-(2-propyl-3-hydroxy-4-acetyl)phenoxy)propyloxyphenoxy acetic acid, (L-165041)], and PPARγ (rosiglitazone, ciglitazone) on inflammatory gene regulation in rat primary astrocytes. We measured cyclooxygenase-2 (COX-2) expression and prostaglandin $E_2$ synthesis in lipopolysaccharide (LPS)-stimulated cells. PPARα, PPARβ/δ, and PPARγ knockdown models served to delineate the contribution of each PPAR isotype. Thiazolidinediones enhanced the LPS-induced COX-2 expression via PPARγ-dependent pathway, whereas L-165041 and GW7647 had no influence. However, the addition of L-165041 potentiated the effect of PPARγ activation through PPARβ/δ-dependent mechanism. On the contrary, PPARα activation (GW7647) suppressed the effect of the combined L-165041/rosiglitazone application. The mechanism of the interplay arising from combined applications of PPAR agonists involves changes in PPAR expression levels. A PPARβ/δ overexpression model confirmed that PPARβ/δ expression level is the point at which PPARγ and PPARα pathways converge in control of COX-2 gene expression. Thus, we discovered that in primary astrocytes, PPARγ has a positive influence and PPARα has a negative influence on PPARβ/δ expression and activity. A positive/negative-feedback loop is formed by PPARβ/δ-dependent increase in PPARα expression level. These findings elucidate a novel principle of regulation in the signaling by synthetic PPAR agonists that involves modulating the interaction between PPARα, -β/δ, and -γ isoforms on the level of their expression.



Transcription factors peroxisome proliferator-activated receptors (PPARs) control the expression of genes related to lipid and glucose homeostasis and inflammatory responses (Michalik et al., 2006; Bensinger and Tontonoz, 2008; Michalik and Wahli, 2008). Three subtypes, PPARα, PPARβ/δ, and PPARγ, have been described previously (Michalik et al., 2006). Disturbance of PPAR pathways promotes the progression of diseases, such as obesity, type 2 diabetes, cardiovascular diseases, cancer, neurodegenerative diseases, hypertension, and chronic inflammation (Michalik et al., 2006; Heneka and Landreth, 2007; Bensinger and Tontonoz, 2008). Thus, mechanisms of PPAR-induced pathways are under intensive investigation.

Some synthetic PPAR agonists are U.S. Food and Drug Administration-approved drugs. PPARα is the target for the

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**ABBREVIATIONS:** PPAR, peroxisome proliferator-activated receptor; COX-2, cyclooxygenase-2; DMEM, Dulbecco's modified Eagle's medium; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; L-165041, 4-(3-(2-propyl-3-hydroxy-4-acetyl)phenoxy)propyloxyphenoxy acetic acid; GW9662, 2-chloro-5-nitrobenzanilide; GW7647, 2-[4-[2-[4-cyclohexylbutyl (cyclohexylcarbamoyl)amino]ethyl]phenyl]sulfanyl-2-methylpropanoic acid; FCS, fetal calf serum; RT-PCR, reverse transcription polymerase chain reaction; PCR, polymerase chain reaction; siRNA, small interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; $PGE_2$, prostaglandin $E_2$.

fibrate class of hypolipidemic drugs and PPARγ for the thiazolidinediones class of insulin-sensitizing drugs (Bensinger and Tontonoz, 2008). Synthetic PPARα and PPARγ agonists have anti-inflammatory and antiatherosclerotic activity (Heneka and Landreth, 2007; Bensinger and Tontonoz, 2008; Michalik and Wahli, 2008). PPARβ/δ agonists are considered useful for treating dyslipidemia and obesity and for the control of tissue-repair mechanisms (Bensinger and Tontonoz, 2008; Peters et al., 2008). PPARs are drug targets with overlap in their therapeutic effects. Therefore, synthesis of substances with dual-agonist action was stimulated (Balakumar et al., 2007). Compounds with combined PPARα and PPARγ activities are in clinical trials (Balakumar et al., 2007). However, such dual agonists with PPARα/PPARγ activity produce cardiovascular risks and carcinogenicity (Balakumar et al., 2007). Understanding of the combined effects of PPAR agonists is mandatory for developing novel strategies for designing multimodal PPAR drugs for the treatment of various diseases.

The idea of a combined application of agonists for different PPAR isotypes comes from the biology of PPARs. Unsaturated fatty acids and their derivatives are endogenous ligands of PPARs but are not isotype-specific (Michalik et al., 2006). After ligand binding, PPARs form heterodimers with the retinoid X receptor and subsequently bind to PPAR response elements in the regulatory region of target genes (Michalik et al., 2006; Ricote and Glass, 2007). PPAR isotypes have no DNA binding specificity (Lemay and Hwang, 2006) and may compete for one DNA site (Lemay and Hwang, 2006; Ricote and Glass, 2007). The canonical view is that the selectivity in the action of PPARs in different tissues depends on the isotype-specific tissue expression, interactions with different coregulator complexes, and the presence of different spectra of endogenous PPAR ligands (Michalik and Wahli, 2008). Nevertheless, some interplay between PPAR isoforms was suggested for the repression of the PPARγ- and PPARα-mediated activation of target gene expression after PPARβ/δ activation (Shi et al., 2002) and for PPARβ/δ-dependent PPARγ-activation (Consilvio et al., 2004). These results indicate that a functional cross-talk between PPARs might exist concerning the control of their expression levels.

To test this hypothesis, we investigated the influence of specific synthetic agonists of PPARα (GW7647), PPARβ/δ (L-165041), and PPARγ (thiazolidinediones rosiglitazone, ciglitazone) and their combinations on the expression of cyclooxygenase-2 (COX-2). To analyze the role of PPAR activation for cell functions, we chose COX-2, an important inflammation-related gene responsible for prostaglandin synthesis (Kang et al., 2007).

We used rat brain primary astrocytes stimulated by LPS, a useful cellular model. First, astrocytes are important participants of inflammatory responses in the brain, and disturbance in their activity is related to neurodegenerative diseases (Heales et al., 2004; Heneka and Landreth, 2007; Bernardo and Minghetti, 2008). Second, PPARγ and PPARα agonists modulate inflammatory responses in astrocytes (Pahan et al., 2002; Luna-Medina et al., 2005; Xu et al., 2006a). All three PPAR isoforms are present in primary astrocytes (Cristiano et al., 2005). Nevertheless, an investigation of PPARβ/Δ activation is still missing. Third, we (Strokin et al., 2007) and others (Consilvio et al., 2004; Tzeng et al., 2005) showed that astrocytes respond to proinflammatory stimulation with massive release of arachidonic acid and prostaglandin synthesis.

The role of the three PPAR isotypes in regulation of gene expression in brain is still under intense investigation. To understand the mechanism of COX-2 regulation, we determined the expression levels and activities of PPARα, PPARβ/δ, and PPARγ. Here, we used PPARα, PPARβ/δ, and PPARγ knockdown models and application of specific PPAR agonists to characterize the contribution of each PPAR isotype in regulation of COX-2 expression. In addition, using a PPARβ/δ overexpression model, we demonstrated that PPARβ/δ is the point of convergence in the control of COX-2 gene expression. In summary, thiazolidinediones increase COX-2 expression via a PPARγ-dependent increase of PPARβ/δ receptors. We found that positive influence of PPARγ and negative influence of PPARα on PPARβ/δ transcriptional activity occurs via regulation of the expression level of PPARβ/δ. Furthermore, the PPARβ/δ-activation resulted in an increase of PPARα expression level, thus forming a positive/negative feedback loop. These findings suggest a novel mechanism of signaling by PPAR synthetic agonists.

## Materials and Methods

**Materials.** The cell culture medium was from Invitrogen (Carlsbad, CA) except for fetal calf serum (FCS), penicillin, and streptomycin, which were from Biochrom (Berlin, Germany). Lipopolysaccharide (LPS) was purchased from Sigma Chemicals (Taufkirchen, Germany), and LPS was dissolved in deionized water at a final concentration of 1 mg/ml and stored at −20°C. Rosiglitazone, ciglitazone, GW7647, and GW9662 were purchased from Cayman Chemical (Tallinn, Estonia). L-165041 was purchased from Sigma Chemicals, as were PPARα, PPARβ/δ, and β-tubulin antibodies. COX-2 antibodies were purchased from Cayman Chemical. PPAR agonists and GW9662 were dissolved in dimethyl sulfoxide to produce stock solutions that were dispensed into aliquots and stored at −20°C. One fresh vial was used each day.

The cloning of the expression vector for PPARβ from rat (RnPPARβ) was carried out in the following way. Total RNA was prepared from rat brain with RNeasy Midi Kit (QIAGEN, Hilden, Germany). The cDNA synthesis was performed with 1 to 2 μg of total RNA, 20 pmol of oligo(dT)$_{12–18}$, and 1 U of Omniscript (Qiagen) for 1 h at 37°C. In an RT-PCR with 1 U of AccuPrime (Invitrogen) and the two oligonucleotides RnPPARβStart, GATCAAGCTTGGATCCCGATGGAACAGCCACAGGAGGAGACC, and RnPPARβRev, GATCGATATCTTAGTACATGTCCTTGTAGATCTC, the full-length RnPPARβ was generated. The HindIII/EcoRV fragment of this PCR product was subcloned to pBlueScriptKSII (Stratagene, La Jolla, CA). Positive clones were verified via nucleotide sequence analysis (SeqLab, Göttingen, Germany), and a BamHI/EcoRV DNA fragment coding for the full-length RnPPARβ was cloned into pcDNA3.1-A-mychis vector (Invitrogen). From the empty vector pcDNA3.1-A-mychis and pcDNA3.1-A-mychisRnPPARβ, plasmid DNA was purified using NucleoBond Xtra Midi EF purification system (Macherey-Nagel, Düren, Germany) and used for transfection experiments of rat brain astrocytes.

**Primary Cell Cultures.** Highly pure primary astrocyte cell cultures were obtained from newborn rats, as reported previously (Strokin et al., 2004). In these cultures, more than 95% of the cells were positive for glial fibrillary acidic protein, and only less than 2% were positive for CD11b, a microglia-specific marker. All experiments conformed to guidelines from Sachsen-Anhalt (Germany) on the ethical use of animals, and all efforts were made to minimize the number of animals used. In brief, brains from decapitated newborn rats were rinsed with ice-cold Puck's solution (137.0 mM NaCl, 5.4 mM KCl, 0.44 mM $KH_2PO_4$, 0.3 mM $Na_2HPO_4$, and 5.5 mM glucose,

pH 7.4). The brains were gently passed through nylon meshes of 250 and 136 μm pore width, in consecutive order. Cells were plated onto 75-cm$^2$ culture flasks at a starting density of $6 \times 10^5$ cells/ml and maintained for 5 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. After a further 2 days, the cells were washed with Hanks' solution (50 mM NaCl, 5 mM KCl, 0.2 mM $KH_2PO_4$, 0.17 mM $Na_2HPO_4$, 5.0 mM glucose, and 58.4 mM sucrose, pH 7.4). Cells were trypsinized and replated onto six-well plates. Cells ($10^6$) were seeded in each well. Two days later, cells were used for experiments.

Cells with the expression vector for PPARβ were transfected using magnet beads and magnet plate from IBA GmbH (Goettingen, Germany) according to the manufacturer's instructions. In brief, 2 h before the transfection of astrocytes in six-well plates, the medium was changed to fresh DMEM with 10% FCS with a final volume of 2 ml. The plasmid–bead (1 μg of DNA per well) complex was added to the cells, and the plate was put on the magnet plate for the 15 min at 37°C and 10% $CO_2$. Then the medium was changed to fresh DMEM with 10% FCS, and the cells were incubated for 42 h. The expression level was assessed by Western blot.

**RNA and Protein Preparation.** Before RNA and protein preparation, cells were washed three times with 5 ml of cold (10°C) phosphate-buffered saline (PBS) per well. Total RNA was isolated and DNAase-treated from cultured astrocytes with the total RNA isolation kit RNeasy (QIAGEN). The total RNA isolated by this method is nondegraded and free of protein and DNA contamination. For protein isolation, astrocytes were lysed in modified radioimmunoprecipitation assay buffer [50 mM Tris, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM $Na_3VO_4$, 1 mM NaF, and one tablet of Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Mannheim, Germany) per 50 ml]. After sonication, lysates were centrifuged at 14,000*g* (4°C) for 15 min, and the pellet was discarded. The protein concentration was determined by the Bradford method using bovine serum albumin as standard.

**Measurement of the Relative RNA Expression Level.** cDNA was generated from 250 ng of total RNA with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) in a final volume of 5 μl according to the manufacturer's protocol. Real-time PCR was performed on the iCycler (Bio-Rad) in 20-μl reaction volume using SYBR green PCR Master Mix (Bio-Rad) as described by the manufacturer. Amplification specificity of PCR products was confirmed by melting curve analysis and agarose gel electrophoresis. The sequences of PCR primers used in the present study were as follows: GAPDH: sense, 5′-CAAAATCAAGTGGGGCGATGCT-3′; antisense, 5′-ACCACCTGGTGCTCAGTGTAGC-3′; COX-2: sense, 5′-GCAGCTTCCTGATTCAAATGAG-3′; antisense, 5′-ATCATCTCTGCCTGAGTATCT-3′; PPARα: sense, 5′-TGCGGACTACCAGTACTTAG-3′; antisense, 5′-CGACACTCGATGTTCAGTGC-3′; and PPARβ/δ: sense, 5′-CTCCTGCTGACTGACAGATG-3′; antisense, 5′-TCTCCTCCTGTGGCTGTTC-3′.

The sets of primers for the target genes (COX-2, PPARα, and PPARβ/δ) have been confirmed to have amplification efficiency equal to that of the internal reference gene (GAPDH) (data not shown). The relative expression level of an individual target gene was normalized to that of GAPDH and to a control sample that was run on the same plate. Thus, the relative RNA expression level of a gene was normalized for GAPDH mRNA and expressed relative to that in control cells treated with vehicle.

**Analysis of COX-2, PPARα, PPARβ/δ, and PPARγ Expression by Western Blot.** For immunoblotting, proteins solubilized in Laemmli buffer were separated under denaturing conditions on SDS-polyacrylamide gel electrophoresis. Ten micrograms of protein was loaded on each lane of a 10% SDS polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes (Protran BA83 or BA79; Whatman, Dassel, Germany). Precision Plus Prestained Standards from Bio-Rad Laboratories (Hercules, CA) were used as markers. Equal transfer was controlled by Ponceau red staining of the membrane. After incubation in 10% Rotiblock (Roth, Nurnberg, Germany) solution for 1 h at room temperature and repeated washes with PBS with 0.05% Tween 20, the membrane was probed with COX-2 (1:1000), PPARα (1:200), PPARβ/δ (1:200), or PPARγ (1:200) antibody, washed with PBS with 0.05% Tween 20, and incubated for 1 h at room temperature again with a species-specific polyclonal antibody labeled with horseradish peroxidase. Protein bands were visualized by enhanced chemiluminescence (SuperSignal West Pico; Pierce, Bonn, Germany). For β-tubulin analysis, the blot was stripped at room temperature for 30 min with stripping buffer [2% (v/v) SDS, 62.5 mM Tris-HCl, and 100 mM β-mercaptoethanol] and washed five times. Membranes were reprobed with antibody against β-tubulin (1:10,000) from Sigma Chemicals and secondary anti-mouse IgG (Dianova, Hamburg, Germany) to control for protein loading. Band intensity was measured using a GS-800 calibrated densitometer and Quantity One software (Bio-Rad Laboratories) and normalized to the intensity of the respective bands obtained for β-tubulin.

**Transfection with Small Interfering RNA.** Small interfering RNAs (siRNAs) used in the present study were purchased from QIAGEN. Cells were transfected using magnet beads and magnet plate (see above). In brief, 2 h before the transfection of astrocytes in six-well plates, the medium was changed to fresh DMEM with 10% FCS with a final volume of 2 ml. The siRNA–beads complex was added to the cells, and plate or flask was put on the magnet plate for the 15 min at 37°C and 10% $CO_2$. Then the medium was changed to fresh DMEM with 10% FCS, and cells were incubated for 36 h. Knockdown was assessed by both Western blot and measurements of transcriptional activity. Efficiency of PPARα knockdown was more than 90% on protein and 97.5% on transcription activity level. Efficiency of PPARβ/δ knockdown was more than 90% on protein and 94% on transcription activity level. Efficiency of PPARγ knockdown was more than 90% on protein and 93% on transcription activity level.

**Measurement of $PGE_2$ Production.** For studies of $PGE_2$ production, astrocytes were grown in six-well plates. After the experiment, the supernatant was collected and stored at −80°C until $PGE_2$ concentration was detected using an enzyme-linked immunoassay (Cayman Chemical) according to the manufacturer's instructions. The results were expressed as nanograms per milliliter of cell culture medium.

**Transcription Activity of PPARs.** Transcription activity of PPARα and PPARβ/δ was assayed using an enzyme-linked immunosorbent assay-based PPARα, -δ, -γ Complete Transcription Factor Assay Kit (Cayman Chemical). Nuclear proteins were extracted from astrocytes according to the manufacturer's instructions, and protein concentration was determined by the Bradford method using BSA as standard. Transcription factor DNA binding activity was measured using the commercially available PPARα, -δ, -γ Complete Transcription Factor Assay Kit according to the manufacturer's instructions (Cayman Chemical). In brief, a double-stranded DNA sequence containing the PPAR response element was linked to the bottom of wells (96-well plate). PPARs within the nuclear fraction bind specifically to this sequence and isoforms are detected using primary antibodies directed against the individual PPARs. Clarified cell lysates were supplied for each PPAR isoform and acted as effective positive controls for PPAR DNA binding. Specificity of binding was also demonstrated using wells with no nuclear protein added. In these wells, no binding was detected (data not shown). Binding activity was measured at 450 nm (minus the blank) and calculated as an activity ratio, with the lysates from untreated astrocytes serving as the reference value.

**Experimental Data Analysis and Statistics.** All data are presented in the text and figures as means and S.E. of samples from three experiments, each characterized in groups of at least three independent replicas. Data were subjected to an analysis of variance with Dunnett's post hoc comparison. Statistical significance was established at $P < 0.05$.

## Results

**Agonists of PPARγ, but Not PPARα or PPARβ/δ, Potentiate COX-2 Expression in LPS-Stimulated Astrocytes.** Modulation of responses of astrocytes to inflammatory stimuli by agonists of PPARγ and PPARα has been shown previously (Luna-Medina et al., 2005; Xu et al., 2006a; Xu and Drew, 2007). However, the influence of all three types of PPAR agonists in parallel in astrocytes was not tested before. Thus, we compared the effects of three types of PPAR agonists on COX-2 expression levels in astrocytes that were stimulated by LPS for 4 h. We used GW7647 as PPARα agonist, L-165041 as PPARβ/δ agonist, and the thiazolidinediones rosiglitazone or ciglitazone as PPARγ agonists (Seimandi et al., 2005).

Stimulation of astrocytes by LPS for 4 h leads to a 4.5-fold increase of COX-2 expression levels, compared with basal conditions (Fig. 1; at concentration of PPAR agonist "0 μM"). The addition of GW7647 or L-165041 had no influence on LPS-induced COX-2 expression in the range of concentrations from 0.1 to 20 μM (Fig. 1). The addition of rosiglitazone (1–20 μM) and ciglitazone (1–20 μM) up to 2-fold increases the LPS-induced expression of COX-2 mRNA (Fig. 1A). Rosiglitazone at 20 μM and ciglitazone at 40 μM (data for that concentration not shown in Fig. 1A) cause the same level of COX-2 increase. Therefore, we used these concentrations for our further experiments. To confirm the biological relevance of our findings, we also measured $PGE_2$ levels. We obtained that the increase in COX-2 expression (Fig. 1A) correlated with $PGE_2$ production (Fig. 1B).

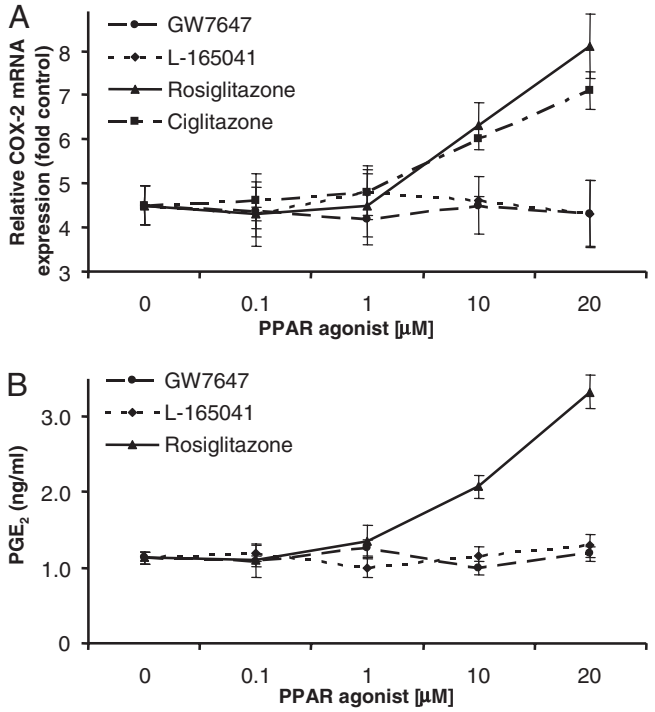


**Fig. 1.** Modulation of LPS-induced COX-2 expression (A) and $PGE_2$ production (B) in rat brain astrocytes by synthetic PPARγ agonists. Cells were pretreated for 10 min with the indicated concentrations (micromolar) of GW7647, L-165041, and rosiglitazone or ciglitazone. LPS (100 ng/ml) was then added to astrocytes for 4 h before measurement of mRNA or $PGE_2$. A, COX-2 mRNA levels were quantified by real-time RT-PCR. Results are expressed as -fold changes relative to unstimulated astrocytes. Values normalized to GAPDH mRNA level, represent the mean ± S.E.M. from three independent experiments. B, $PGE_2$ production was determined by enzyme immunoassay, as described under *Materials and Methods*. Values represent the mean ± S.E.M. from three independent experiments.

**Rosiglitazone and Ciglitazone Potentiate COX-2 Expression via a PPARγ-Dependent Process.** It is known that PPARγ agonists, besides promoting selective activation of PPARγ receptors, may act via PPARγ-independent mechanisms (Park et al., 2003; Luna-Medina et al., 2005). To investigate whether a PPARγ-dependent mechanism underlies the rosiglitazone and ciglitazone-induced potentiation of COX-2 expression, we used two independent approaches: application of the PPARγ antagonist GW9662 (1 μM), and the use of PPARγ knockdown astrocytes. The results are shown in Fig. 2. Treatment of cells with GW9662 results in the irreversible loss of ligand binding, whereas PPARγ knockdown results in the loss of receptor on protein level. The efficacy of PPARγ knockdown was confirmed by the Western blot shown in Fig. 2C. The COX-2 expression levels for both the PPARγ antagonist application and silencing of PPARγ were similar. Inhibition of PPARγ activity by GW9662 treatment or PPARγ knockdown eliminated the PPARγ agonist-induced increase of COX-2 expression in LPS-stimulated astrocytes (Fig. 2, A and B). Correlation of mRNA level with COX-2 protein expression was analyzed by Western blot, as demonstrated in Fig. 2B. Thus, similar results were obtained on mRNA (Fig. 2A) and protein levels (Fig. 2B). These data demonstrate that the effect of rosiglitazone or ciglitazone on the expression of COX-2 in LPS-stimulated astrocytes is PPARγ-dependent. COX-2 expression was not changed after inhibition of PPARγ by GW9662

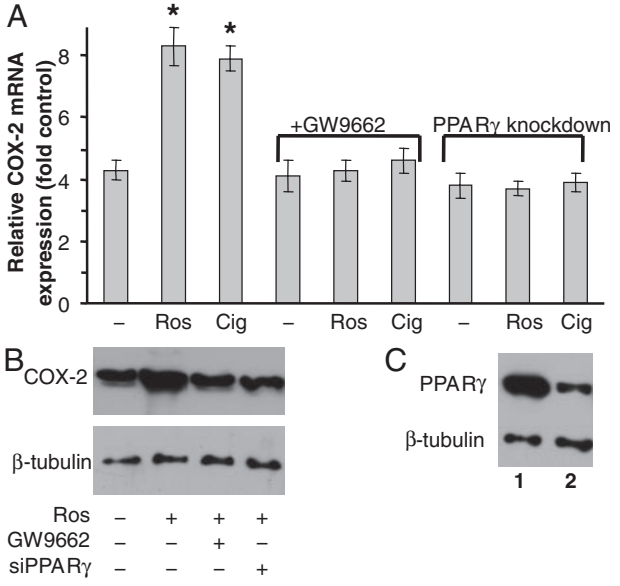


**Fig. 2.** Effect of rosiglitazone and ciglitazone on COX-2 expression in LPS-stimulated astrocytes is PPARγ-dependent. Cells were either preincubated during 10 min with GW9662 (1 μM) or transfected by PPARγ siRNA. Where indicated, cells were treated for 10 min with 20 μM rosiglitazone (Ros) or 40 μM ciglitazone (Cig). LPS (100 ng/ml) was then added to astrocytes for 4 h before measurement of mRNA expression. A, COX-2 mRNA levels were quantified by real-time RT-PCR. Results are expressed as -fold changes, relative to unstimulated astrocytes, and all values are normalized to GAPDH mRNA level. Values represent the mean ± S.E.M. from three independent experiments. ∗, $P < 0.05$, compared with the LPS-stimulated cells (−). B, COX-2 protein was evaluated by Western blotting. Example is representative for three independent experiments. C, efficacy of PPARγ knockdown verified by Western blot; lane 1, control; lane 2, PPARγ knockdown astrocytes.

or by PPARγ knockdown in LPS-stimulated astrocytes. Thus, activation of PPARγ is not required for the induction of COX-2 expression by LPS itself but is required for the overstimulation of LPS-induced COX-2 expression. Therefore, all further experiments were made with LPS-stimulated astrocytes.

**Effects of Combinations of Synthetic PPAR Agonists on COX-2 Expression.** For testing our hypothesis of the existence of a cross-talk between different isoforms of PPAR, we applied the combinations of the PPAR agonists. The combination of the PPARα and PPARβ/δ agonists had no additional effect (Fig. 3A, columns 1 and 5). Combination of PPARγ agonists rosiglitazone or ciglitazone together with the PPARα agonist GW7647 induced no changes compared with the effects of the PPARγ agonists themselves (Fig. 3A, compare columns 2 and 3). A remarkable potentiation was obtained by simultaneous addition of PPARγ and PPARβ/δ agonists. This combination leads to a 3-fold increase in COX-2 expression (Fig. 3A, column 4), compared with that in LPS-stimulated astrocytes (Fig. 3A, column 1). We were surprised to find that the PPARα agonist GW7647 strongly inhibited the potentiation of COX-2 expression exerted by the combined application of rosiglitazone and L-165041 in LPS-stimulated astrocytes (Fig. 3A, compare columns 6 and 4). For these experiments, in all cases, correlation of mRNA levels with COX-2 protein expression was confirmed by Western blot (Fig. 3B).

The addition of the PPARβ/δ agonist sensitized astrocytes for rosiglitazone treatment (Fig. 3C). Rosiglitazone being added alone, at a concentration of 1 μM, had no influence on COX-2 expression. However, in combination with the 1 μM concentration of the PPARβ/δ agonist L-165041, rosiglitazone at the same low concentration clearly enhanced COX-2 expression (Fig. 3C). Taken together, these data suggest that there is an interplay between agonists of different isoforms of PPARs in the PPARγ-dependent modulation of COX-2 gene expression.

**L-165041 Increases the PPARγ-Induced Potentiation of COX-2 Expression Only in the Presence of PPARβ/δ and PPARγ.** To check the involvement of PPARγ receptors in the effects of L-165041 on COX-2 expression in rosiglitazone-pretreated and LPS-stimulated astrocytes, we used two independent approaches: application of the PPARγ antagonist GW9662 (1 μM), and the PPARγ knockdown astrocytes. Application of GW9662 abolished the increase of COX-2 expression induced by the combined application of rosiglitazone and L-165041, as shown in Fig. 4. In agreement with this pharmacological approach, knockdown of PPARγ similarly abolished the potentiation of COX-2 expression levels (compared with the control astrocytes, which were transfected with scrambled siRNA) (Fig. 4).

To investigate the role of PPARβ/δ receptors in the COX-2 expression in LPS-stimulated astrocytes, we used PPARβ/δ knockdown astrocytes in which we tested the combined application of rosiglitazone and L-165041. The efficacy of PPARβ/δ silencing was confirmed by Western blot (Fig. 5, inset). The level of LPS-induced COX-2 expression being measured without PPAR agonists was not changed in PPARβ/δ knockdown astrocytes compared with the control astrocytes transfected with scrambled siRNA (Fig. 5, columns 2 and 1). We observed that knockdown of PPARβ/δ not only abolished the effect of the combined rosiglitazone and L-165041 application (Fig. 5; compare columns 5 and 6) but also suppressed the effect of rosiglitazone application itself (Fig. 5, compare columns 3 and 4). Thus, the combination of PPARβ/δ and PPARγ agonists modulates COX-2 expression via simultaneous involvement of PPARβ/δ and PPARγ receptors. Moreover, PPARβ/δ plays a crucial role in rosiglitazone-dependent increase of COX-2 expression.

**Role of PPARα in the GW7647-Mediated Effect of Combined Agonist Application.** Combined application of PPARβ/δ (L-165041) and PPARγ (rosiglitazone or ciglitazone) agonists increased COX-2 expression, whereas the ad-

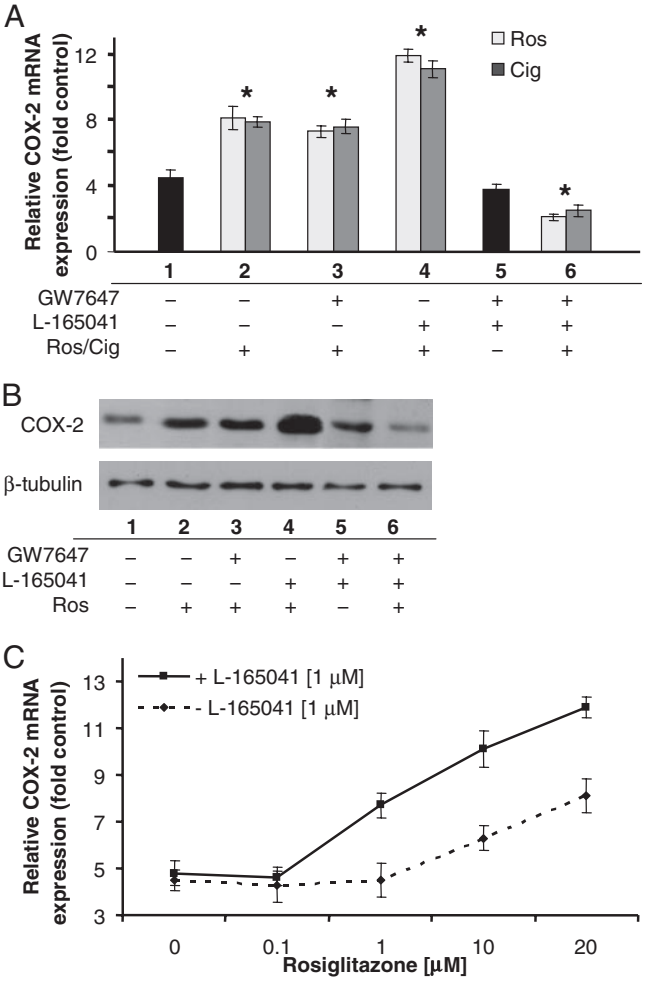


**Fig. 3.** Influence of different combinations of PPAR agonists on COX-2 expression levels in LPS-stimulated astrocytes. Cells were incubated for 10 min with indicated combinations of PPARα agonist GW7647 (1 μM), PPARβ/δ agonist L-165041 (1 μM), and PPARγ agonists rosiglitazone (Ros; 20 μM) or ciglitazone (Cig; 40 μM). LPS (100 ng/ml) was then added to astrocytes for 4 h before measurement of mRNA expression. A, COX-2 mRNA levels were quantified by real-time RT-PCR. Results are expressed as -fold changes relative to unstimulated astrocytes, and all values are normalized to GAPDH mRNA level. Values represent the mean ± S.E.M. from three independent experiments. *, $P < 0.05$, compared with the LPS-stimulated cells (column 1). B, respective protein expression for COX-2 is evaluated by Western blotting. Example is representative for three independent experiments. C, cells were pretreated for 10 min with the indicated concentration (micromolar) of rosiglitazone in the absence (−) or presence (+) of L-165041 (1 μM). LPS (100 ng/ml) was then added, and after 4 h incubation with both agents, total RNA was isolated. COX-2 mRNA levels were quantified by real-time RT-PCR. Results are expressed as -fold changes relative to unstimulated astrocytes, and all values are normalized to GAPDH mRNA level. Values represent the mean ± S.E.M. from three independent experiments.

ditional application of the PPARα agonist GW7647 abolished this effect, as shown in Fig. 3, A and B. To confirm that the action of GW7647 was PPARα-dependent, we used PPARα knockdown astrocytes to check the role of PPARα receptors in the effect of GW7647 (Fig. 6). The efficacy of the PPARα silencing was confirmed by Western blot analysis (Fig. 6, inset). The level of LPS-induced COX-2 expression measured in the absence of PPAR agonists was not changed in PPARα knockdown astrocytes (Fig. 6, columns 2 and 1). The knockdown of PPARα also did not influence the level of COX-2 expression after combined application of rosiglitazone and L-165041 (Fig. 6; columns 3 and 4). However, the knockdown of PPARα totally abolished the inhibitory effect of GW7647 (column 6), which was clearly seen in scrambled siRNA-transfected astrocytes (Fig. 6, column 5). Thus, Fig. 6 demonstrates that the negative action of GW7647 on the effect of combined application of PPARγ and PPARβ/δ agonists occurs via PPARα-dependent pathway.

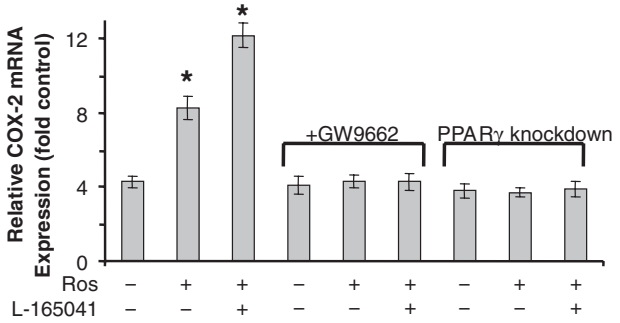


**Fig. 4.** PPARβ/δ agonist L-165041 enhances rosiglitazone-induced increase of COX-2 expression in LPS-stimulated astrocytes via a PPARγ-dependent pathway. Cells were preincubated during 10 min with 1 μM GW9662 or were transfected by PPARγ siRNA. Where indicated, cells were treated for 10 min with 20 μM rosiglitazone (Ros) in the absence or presence of 1 μM L-165041. LPS (100 ng/ml) was then added, and after 40h incubation, total RNA was isolated. COX-2 mRNA levels were quantified by real-time RT-PCR. Results are expressed as -fold changes relative to unstimulated astrocytes, and all values are normalized to the GAPDH mRNA level. Values represent the mean ± S.E.M. from three independent experiments. *, $P < 0.05$ compared with the LPS-stimulated cells.

**Overexpression of PPARβ/δ Reveals a Key Role of This Receptor in the Control of COX-2 Gene Expression.** We demonstrated that the PPARβ/δ knockdown not only abolished the effect of combined rosiglitazone and L-165041 application but also reduced the effect of rosiglitazone application itself (Fig. 5). These data suggest that PPARβ/δ has a key function in control of COX-2 gene expression. To test this possibility, we constructed astrocytes transfected by PPARβ/δ overexpression vector and investigated the influence of different combinations of PPAR agonists in this cell model (Fig. 7).

We observed that the increase in PPARβ/δ level resulted in potent, nearly 2-fold increase of COX-2 expression even in the absence of agonist stimulation (Fig. 7; compare columns 2 and 1). In cells with PPARβ/δ overexpression, L-165140 induces COX-2 expression without additional stimulation by rosiglitazone (compare column 4 cells with PPARβ/δ overexpression and column 3 wild-type cells). With application of the PPARβ/δ agonist L-165140, potentiation of COX-2 expression reached a 4-fold level (Fig. 7, column 4 versus 3). This potentiation was not sensitive to additional application of rosiglitazone (Fig. 7, compare columns 6 and 4) or PPARα agonist GW7647 (data not shown) or combined application of the three tested agonists (Fig. 7, compare columns 10 and 4). Figure 7 also demonstrates that in empty vector-transfected cells, the PPAR agonists caused the same effects on COX-2 expression as in the control cells studied above. We also observed that cells with PPARβ/δ overexpression were nonsensitive for positive/negative regulation by PPARγ agonists and PPARα agonists, because the levels of L-165140-stimulated COX-2 expression in astrocytes with PPARβ/δ overexpression were the same in cells with simultaneous PPARβ/δ overexpression and PPARγ knockdown (Fig. 7, compare col-

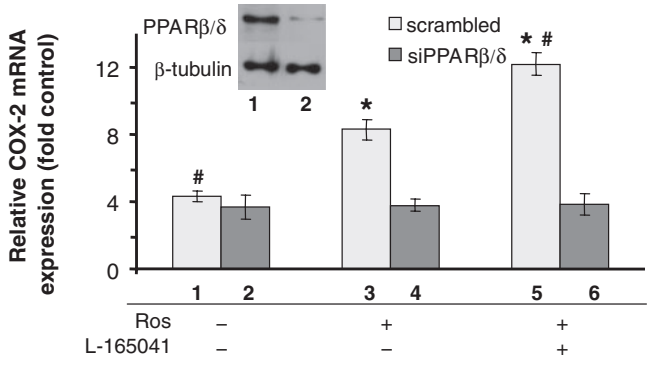


**Fig. 5.** PPARβ/δ agonist L-165041 increases rosiglitazone-induced potentiation of COX-2 expression in LPS-stimulated astrocytes via PPARβ/δ-dependent pathway. Cells were transfected with PPARβ/δ siRNA or scrambled siRNA. Where indicated, cells were treated for 10 min with 20 μM rosiglitazone (Ros) in the absence or presence of L-165041 (1 μM). LPS (100 ng/ml) was then added, and after 40h incubation, total RNA was isolated. COX-2 mRNA levels were quantified by real-time RT-PCR. Results are expressed as -fold changes relative to unstimulated astrocytes (lane 1), and all values are normalized to the GAPDH mRNA level. Values represent the mean ± S.E.M. from three independent experiments. *, $P < 0.05$, compared with the untreated and LPS-stimulated cells. #, $P < 0.05$, compared with the rosiglitazone-treated and LPS-stimulated astrocytes (lane 3 versus 5). Inset, efficacy of PPARβ/δ knockdown is verified by Western blot; lane 1, control; and lane 2, PPARβ/δ knockdown astrocytes.

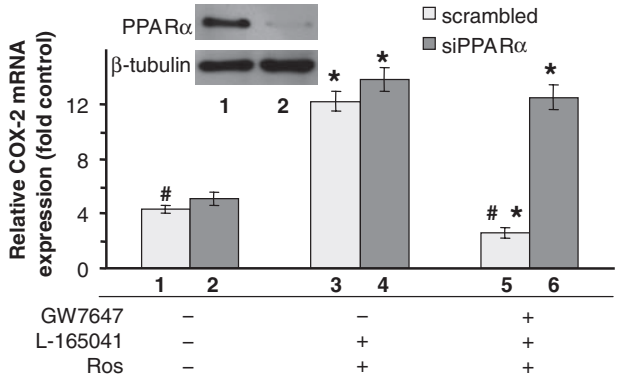


**Fig. 6.** GW7647 abolishes combined rosiglitazone/L-165041-induced potentiation of COX-2 expression in LPS-stimulated astrocytes via PPARα-dependent pathway. Cells were transfected by PPARα siRNA or scrambled siRNA as indicated. Where indicated, cells were treated for 10 min, in the absence or presence of GW7647, with L-165041 (1 μM) plus rosiglitazone (Ros; 20 μM). LPS (100 ng/ml) was then added, and after 4-h incubation, total RNA was isolated. COX-2 mRNA levels were quantified by real-time RT-PCR. Results are expressed as -fold changes, relative to unstimulated astrocytes, and all values are normalized to GAPDH mRNA level. Values represent the mean ± S.E.M. from three independent experiments. *, $P < 0.05$, compared with the LPS-stimulated cells (columns 1 and 2). #, $P < 0.05$, compared with the rosiglitazone plus L-165041-treated and LPS-stimulated astrocytes (lane 3 versus 5). Inset, efficacy of PPARα knockdown is verified by Western blot; lane 1, control; and lane 2, PPARα knockdown astrocytes.

umns 7, 8, and 4). In addition, in cells with simultaneous PPARβ/δ overexpression and PPARα knockdown, the PPARα agonist-elicited inhibitory effect was eliminated (Fig. 7, compare columns 11 and 12 with 9 and 10, and see column 4). These data prove that PPARβ/δ activation by PPARβ/δ agonist results in potentiation of COX-2 expression in LPS-induced astrocytes. To our knowledge, this is the first report showing an increase of COX-2 expression via PPARβ/δ activation in normal, noncancer cells.

**Rosiglitazone Induces the Expression of PPARβ/δ via a PPARγ-Dependent Pathway.** Because the PPARβ/δ agonist L-165041 had no effect on COX-2 expression itself (Fig. 1), whereas on the other side, overexpression of PPARβ/δ enhanced COX-2 expression even without agonist application (Fig. 7), we supposed that in basal conditions there is not enough PPARβ/δ receptor available in the tested system. From that, we hypothesized that application of rosiglitazone or ciglitazone, via PPARγ-dependent pathway, could increase the PPARβ/δ receptor expression level. This would increase the relative activity of PPARβ/δ. To check this concept, we added different concentrations of rosiglitazone to LPS-stimulated astrocytes and measured the level of PPARβ/δ receptors. In the absence of rosiglitazone, LPS did not induce a significant change of the level of the PPARβ/δ receptors (Fig. 8, concentration "0"). When we added the PPARγ agonist rosiglitazone together with LPS, rosiglitazone concentration-dependently increased the PPARβ/δ mRNA. A 3-fold increase was obtained for 20 μM rosiglitazone (Fig. 8A). The mRNA expression was correlated with the increase in the protein levels (Fig. 8B). This protein expression also corresponded to a 2.5-fold increased PPARβ/δ activity in the presence of rosiglitazone (Fig. 8C).

To distinguish between PPARγ-dependent and possible PPARγ-independent mechanisms of rosiglitazone-induced up-regulation of PPARβ/δ expression, we used GW9662, a PPARγ antagonist. Pretreatment with GW9662 (1 μM) eliminated the rosiglitazone-induced expression of PPARβ/δ. This was observed both on mRNA level (Fig. 8D) and similarly on protein level (data not shown). Comparable results were obtained in experiments using PPARγ knockdown astrocytes (Fig. 8D). Thus, rosiglitazone concentration-dependently in-

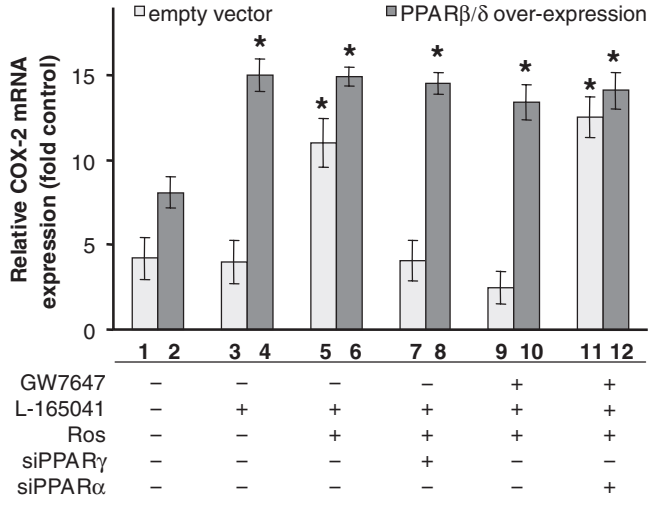


**Fig. 7.** Overexpression of PPARβ/δ reveals a key role of PPARβ/δ in control of COX-2 gene expression. Cells were transfected by PPARβ/δ overexpression plasmid or empty vector. After this, cells were transfected by PPARα or PPARγ or scrambled siRNAs, as indicated. Where additionally indicated, cells were treated for 10 min, in the absence or presence of GW7647 (1 μM), with L-165041 (1 μM) plus rosiglitazone (Ros; 20 μM). LPS (100 ng/ml) was then added, and after 4-h incubation, total RNA was isolated. COX-2 mRNA levels were quantified by real-time RT-PCR. Results are expressed as -fold changes relative to unstimulated astrocytes, and all values are normalized to the GAPDH mRNA level. Values represent the mean ± S.E.M. from three independent experiments. *, $P < 0.05$, compared with the LPS-stimulated cells.

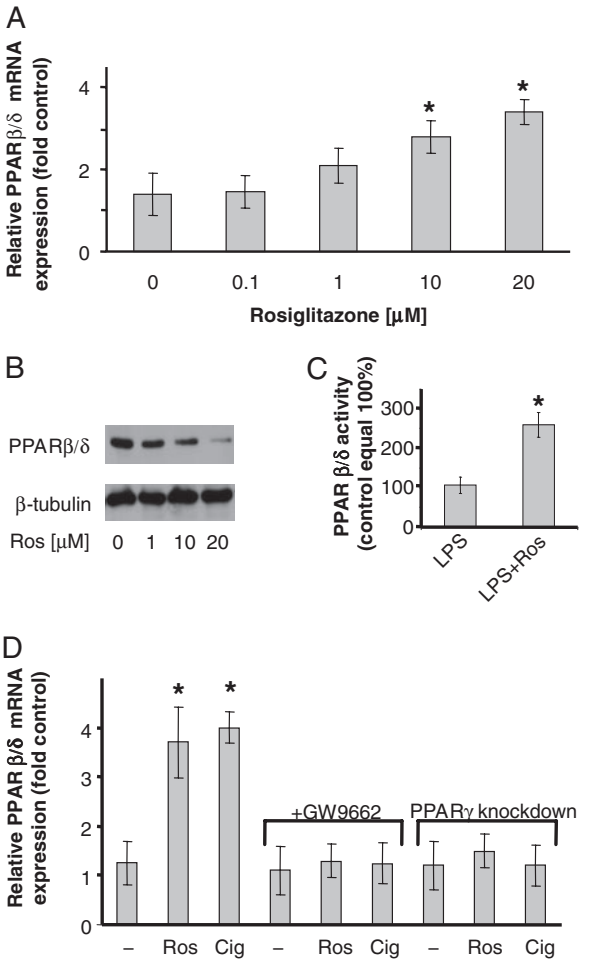


**Fig. 8.** Rosiglitazone induces PPARβ/δ expression and activity in LPS-stimulated astrocytes via PPARγ-dependent pathway. A, cells were pretreated for 10 min with the indicated concentrations (micromolar) of PPARγ agonist rosiglitazone (Ros). LPS (100 ng/ml) was then added, and after 4-h incubation with both agents, total RNA was isolated. PPARβ/δ mRNA levels were quantified by real-time RT-PCR. Results are expressed as -fold changes relative to unstimulated astrocytes, and all values are normalized to the GAPDH mRNA level. Values represent the mean ± S.E.M. from three independent experiments. *, $P < 0.05$, compared with the LPS-stimulated cells. B, protein expression for PPARβ was evaluated by Western blotting. Example is representative of three independent experiments. C, where indicated, cells were incubated for 10 min with 20 μM rosiglitazone. LPS (100 ng/ml) was then added for 4 h. PPARβ/δ transcriptional activity was determined as described under *Materials and Methods*. Values represent the mean ± S.E.M. from three independent experiments. *, $P < 0.05$, compared with the untreated LPS-stimulated cells. D, cells were preincubated for 10 min with 1 μM GW9662 or were treated with PPARγ siRNA or scrambled siRNA. Where indicated, cells were treated for 10 min with 20 μM rosiglitazone (Ros) or 40 μM ciglitazone (Cig). LPS (100 ng/ml) was then added, and after 4-h incubation, total RNA was isolated. PPARβ/δ mRNA levels were quantified by real-time RT-PCR. Results are expressed as -fold changes relative to unstimulated astrocytes, and all values are normalized to GAPDH mRNA level. Values represent the mean ± S.E.M. from three independent experiments. *, $P < 0.05$, compared with the LPS-stimulated cells (–).

creases PPARβ/δ expression and PPARβ/δ activity in LPS-stimulated astrocytes. This increase is mediated by a PPARγ-dependent mechanism.

**PPARα Agonist GW7647 Negatively Regulates the Level of PPARβ/δ Receptors.** The results demonstrated above support the hypothesis that PPARγ positively controls COX-2 expression in astrocytes via a PPARβ/δ-mediated pathway. Changes of PPARβ/δ expression levels play a key role in the regulation of COX-2 expression in LPS-stimulated astrocytes. Furthermore, we have demonstrated that GW7647 inhibits the effects of combined application of rosiglitazone and L-165041 via a PPARα-dependent pathway (Fig. 6). This raises the question of what role activation of PPARα receptors plays in the regulation of PPARβ/δ receptors. We used the PPARα knockdown model to answer this question, as shown in Fig. 9. Real-time RT-PCR analysis revealed that the knockdown of PPARα receptors leads to a 2.5-fold increase in the PPARβ/δ expression in LPS-stimulated cells (Fig. 9, compare columns 2 and 1). This shows that in resting conditions, PPARβ/δ expression is under a negative control of PPARα receptors. The negative control exerted by PPARα-dependent activation results in an increase of PPARβ/δ expression after the combined application of rosiglitazone and L-165041 in PPARα knockdown cells (Fig. 9, compare columns 3 and 4 with column 1) The changes in mRNA level were correlated with similar changes in protein level (Fig. 9B). As expected, the addition of GW7647 to the tested system had no effect in the PPARα knockdown cells (Fig. 9A, column 6) but did have an effect in the control cells treated with scrambled siRNA (Fig. 9A, column 5).

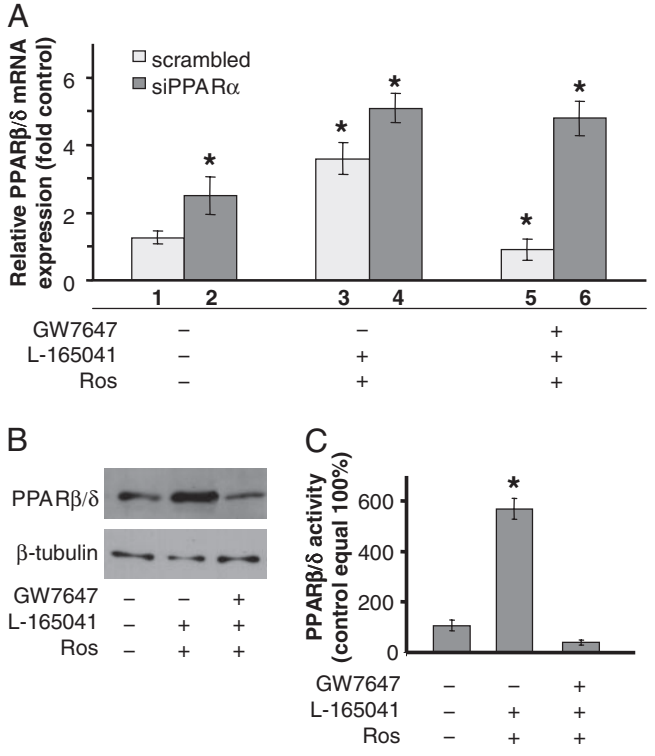


**Fig. 9.** GW7647 abolishes potentiation of PPARβ/δ expression, induced by combined rosiglitazone plus L-165041 treatment via PPARα-dependent pathway. A, cells were transfected by PPARα siRNA or scrambled siRNA, as indicated. Where indicated, cells were treated for 10 min with 1 μM L-165041 plus 20 μM rosiglitazone (Ros) in the absence or presence of 1 μM GW7647. LPS (100 ng/ml) was then added, and after 4-h incubation, total RNA was isolated. PPARβ/δ mRNA levels were quantified by real-time RT-PCR. Results are expressed as -fold changes relative to unstimulated astrocytes, and all values are normalized to GAPDH mRNA level. Values represent the mean ± S.E.M. from three independent experiments. *, $P < 0.05$, compared with the LPS-stimulated cells. B and C, cells were incubated for 10 min with 1 μM L-165041 plus 20 μM rosiglitazone in the absence or presence of 1 μM GW7647. LPS (100 ng/ml) was then added for 4 h. B, COX-2 protein was evaluated by Western blotting. Example is representative of three independent experiments. C, PPARβ/δ transcriptional activity was determined, as described under *Materials and Methods*. Values represent the mean ± S.E.M. from three independent experiments. *, $P < 0.05$, compared with the LPS-stimulated cells.

**L-165041 Increases PPARα Expression and Activity in LPS-Stimulated Astrocytes via a PPARβ/δ-Dependent Mechanism.** We have shown in Fig. 7 that PPARα negatively controls COX-2 expression in astrocytes through a PPARβ/δ-mediated pathway. However, it remains unclear why GW7647 acted only in combination with rosiglitazone and L-165041, which are PPARγ and PPARβ/δ agonists, respectively. We suggest that there is a finely tuned interplay between PPARα and PPARβ/δ receptors. PPARα negatively controls the expression of PPARβ/δ receptors, whereas PPARβ/δ might positively control PPARα receptors. Thus, rosiglitazone-induced PPARβ/δ expression level and relative PPARβ/δ activity could increase the amount and activity of PPARα. To check this hypothesis, we measured the level of PPARα receptors in LPS-treated astrocytes (Fig. 10).

It is interesting to note that LPS added alone decreased the PPARα level by approximately 60% (Fig. 10A, column 1), whereas application of the PPARβ/δ agonist L-165041 in combination with rosiglitazone increased the PPARα mRNA up to the normal level in nontreated cells (Fig. 10A, compare columns 1 and 7). That increase correlated with PPARα protein levels (Fig. 10B). PPARβ/δ knockdown abolished this increase (Fig. 10A, compare columns 8 and 7). These changes in the PPARα mRNA level also correlated with changes in the protein level of PPARα (Fig. 10B). The addition of the PPARα agonist GW7647 simultaneously with rosiglitazone did not potentiate the activity of PPARα, but the further addition of L-165041 together with these two compounds notably, that is more than 4-fold, induced the PPARα activity (Fig. 10C).

Finally, the key role of PPARβ/δ as positive regulator of PPARα expression was confirmed in cells with PPARβ/δ overexpression (Fig. 10D). Excess PPARβ/δ protein, which was activated by the specific agonist L-165140, leads to a significant increase in PPARα mRNA expression level (Fig. 10D, columns 3 and 4). This reveals that the PPARβ/δ receptor is sufficient as a positive regulator of the PPARα expression. Moreover, in astrocytes transfected by PPARβ/δ overexpression vector, the additional silencing of PPARγ had no effect on the PPARα expression (Fig. 10D, column 8). The experiments, in which it is shown that rosiglitazone is essential for the induction of PPARα expression (Fig. 10A, column 7), taken together with the data with PPARβ/δ overexpression

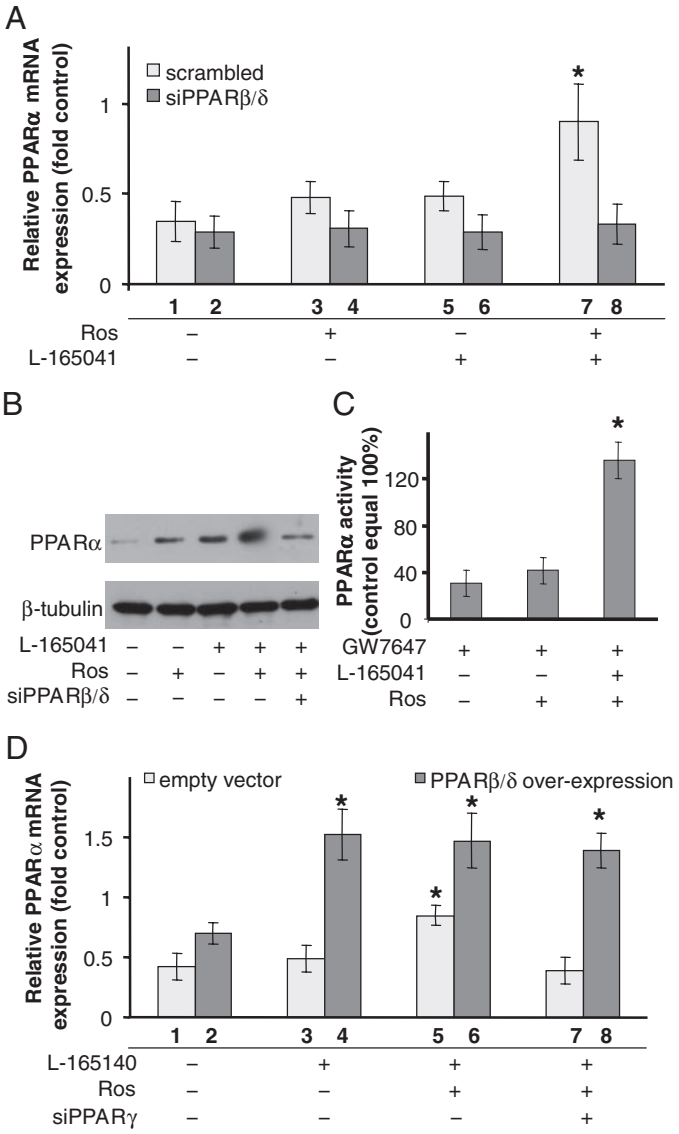


**Fig. 10.** PPARβ/δ agonist L-165041 increases the expression of PPARα in LPS-stimulated astrocytes via PPARβ/δ-dependent pathway. A and B, cells were transfected by PPARβ/δ siRNA or scrambled siRNA, as indicated. Where indicated, cells were treated for 10 min with 20 μM rosiglitazone (Ros) in the absence or presence of L-165041 (1 μM). LPS (100 ng/ml) was then added for 4 h. A, PPARα mRNA levels were quantified by real-time RT-PCR. Results are expressed as -fold changes relative to unstimulated astrocytes, and all values are normalized to the GAPDH mRNA level. Values represent the mean ± S.E.M. from three independent experiments. *, $P < 0.05$, compared with the LPS-stimulated cells. B, protein expression for PPARα was evaluated by Western blotting. Example is representative of three independent experiments. C, cells were incubated for 10 min with indicated combinations of PPARα agonist GW7647 (1 μM), PPARβ/δ agonist L-165041 (1 μM), and PPARγ agonist rosiglitazone (Ros; 20 μM). LPS (100 ng/ml) was then added for 4 h. PPARα transcriptional activity was determined as described under *Materials and Methods*. Values represent the mean ± S.E.M. of three independent experiments. *, $P < 0.05$, compared with the LPS-stimulated cells. D, cells were transfected by PPARβ/δ overexpression plasmid or empty vector. After that, cells were transfected by PPARγ siRNA or scrambled siRNA as indicated. Where indicated, cells were treated for 10 min with rosiglitazone (20 μM) or L-165041 (1 μM). LPS (100 ng/ml) was then added, and after 4-h incubation, total RNA was isolated. PPARα mRNA levels were quantified by real-time RT-PCR. Results are expressed as -fold changes relative to unstimulated astrocytes, and all values are normalized to the GAPDH mRNA level. Values represent the mean ± S.E.M. from three independent experiments. *, $P < 0.05$, compared with the LPS-stimulated cells.

(Fig. 10D), make it obvious that rosiglitazone activates PPARγ, which then induces the expression of PPARβ/δ. PPARβ/δ, in turn, after stimulation by the agonist L-165041, induced the expression of PPARα (Fig. 10A) and induced PPARα activity (Fig. 10C).

## Discussion

The present study provides evidence for a regulatory network between PPARs activated by synthetic agonists trough regulation of expression of different PPAR isoforms. This manifests itself in control of the COX-2 target gene expression. Application of single PPAR agonists reveals that only rosiglitazone, but not PPARα or PPARβ/δ agonists, can increase COX-2 expression and activity. At first sight, it is tempting to suppose from these results that COX-2 expression is under the direct positive control of PPARγ in astrocytes. The detailed analysis of the combined applications of PPARα, PPARβ/δ, and PPARγ agonists, however, revealed unexpected mechanisms of target gene regulation. In LPS-stimulated rat brain astrocytes, the COX-2 expression is under positive control by PPARγ agonists rosiglitazone or ciglitazone and under negative control by PPARα agonist GW7647 through the PPARβ/δ-dependent pathway. Our data suggest a model of PPAR-dependent control of COX-2 expression in LPS-stimulated astrocytes, where PPARβ/δ is the point of convergence of PPARγ and PPARα pathways, as schematically shown in Fig. 11. Activation of PPARγ has positive influence, whereas activation of PPARα has negative influence on the PPARβ/δ expression level. Thus, regulation of COX-2 expression by application of PPARγ or PPARα agonists is realized on the level of regulation of PPARβ/δ expression. This mechanism opens new strategies in the regulation of astrocyte functions and reveals a new role of PPARβ/δ to gate the activities of PPARα and PPARγ in the brain.

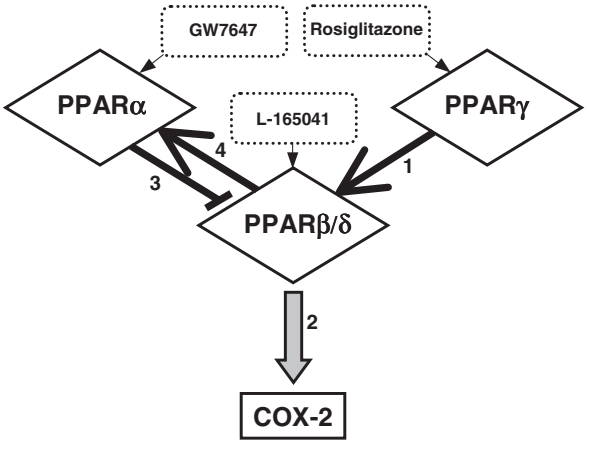


**Fig. 11.** PPARβ/δ is the point of convergence of PPARα and PPARγ pathways in control of COX-2 gene expression in LPS-stimulated astrocytes. PPARγ agonist rosiglitazone increases PPARβ/δ expression level in LPS-stimulated astrocytes via PPARγ-dependent mechanism (pathway 1). Rosiglitazone-induced increase of PPARβ/δ expression level leads to potentiation of COX-2 expression in LPS-stimulated astrocytes (pathway 2). PPARα agonist GW7647 decreases PPARβ/δ level and eliminates PPARβ/δ-mediated rosiglitazone-induced potentiation of COX-2 expression via PPARα-dependent mechanism (pathway 3). PPARβ/δ agonist L-165041 increases PPARα expression level in LPS-stimulated astrocytes via PPARβ/δ-dependent mechanism (pathway 4), generating a positive/negative feedback loop.

It is known that because of the close similarity between the three PPAR isotypes, there are no 100% selective substances for each isotype. Indeed, GW7647 and L-165041, which are considered potent PPARα and PPARβ/δ ligands, respectively, have much less effect on the two other PPAR isotypes, and the PPARγ agonist rosiglitazone has weak activity on PPARβ/δ (Seimandi et al., 2005). For the PPARγ-specific agonist rosiglitazone, PPARγ-independent effects were shown (Park et al., 2003; Luna-Medina et al., 2005). Therefore, we tested the application of agonist in naive astrocytes and in three different types of PPAR knockdown astrocytes. Our data showed that the effect of any agonist was reversed by knockdown of the respective PPAR isotype. This allowed us to conclude that the effect of the tested substances were neither PPAR-independent nor dependent on other types of PPAR.

We observed in the astrocytes a PPARβ/δ-dependent upregulation of PPARα at the level of mRNA transcription, protein, and PPARα activity. That makes astrocytes more sensitive to PPARα agonists. On the other hand, PPARα agonists are important negative control of PPARβ/δ expression, in the case of astrocytes, in which the activation of PPARβ/δ has positive influence on PPARα expression. We further found that activation of PPARγ did not influence the PPARα expression. This finding is consistent with findings by others in C6 glioma cells (Leisewitz et al., 2008). The positive-negative loop, as found in the present investigation, can control target gene expression via a complex tuning mechanism, as suggested by the full scheme in Fig. 11. In contrast with these data received in normal cells, it was shown in genetically engineered cells that an increase of PPARβ/δ expression resulted in the decrease of activity of PPARα-dependent enzymes (Shi et al., 2002).

The influence of PPARβ/δ on PPARα- and PPARγ-activated transcription via mechanisms of repression is well known (Ricote and Glass, 2007; Bensinger and Tontonoz, 2008). However, here we have shown a novel property of the PPARβ/δ receptor and its interplay with the other PPAR isoforms. The PPARβ/δ is a central switch for the regulation of target gene expression levels and serves as a connection of PPARγ- and PPARα-dependent signals to target genes. The signals from PPARγ and PPARα are positive or negative, respectively. A crossroad between PPARs was already suggested previously. PPARβ/δ activation by agonists or PPARβ/δ overexpression inhibits PPARγ (Shi et al., 2002; Zuo et al., 2006) and PPARα activity (Shi et al., 2002) in both cancer and normal cells. It was also shown that the synthetic PPARβ/δ agonists increase PPARγ-induced differentiation of adipocytes (Matsusue et al., 2004) or C6 glioma cells (Leisewitz et al., 2008). Thus, the interplay between PPAR isoforms and the type of PPAR, which might play the role of the central switch, depend on the cellular context or the target gene. In this way, not only the absolute levels of any one PPAR isotype but also the ratio of the PPAR isotype levels will control the activity of each PPAR isoform. As a consequence, the addition of a specific agonist of one PPAR isoform may indirectly change target gene responses via the increase/decrease of the expression level of other PPAR isoforms. It is important to consider this complexity for the development of new synthetic PPAR agonists.

COX-2 is a key enzyme in prostaglandin synthesis and the role of PPAR isoforms in the regulation of the expression of COX-2 in different cells is intensely investigated but still controversial (Luna-Medina et al., 2005; Xu et al., 2006b). For application of PPARγ or PPARα agonists, both activation (Ikawa et al., 2001; Seimandi et al., 2005; Zhang et al., 2006; Ulivi et al., 2008) and inhibition (Luna-Medina et al., 2005; Grau et al., 2006) of COX-2 expression were found. For PPARβ/δ, only a few data were obtained from studies using cancer cell lines. They show PPARβ/δ-induced COX-2 expression (Glinghammar et al., 2003; Xu et al., 2006b). This is consistent with our data on rat primary astrocytes. It is noteworthy that, in our test system, overexpression of PPARβ/δ leads to an increase of LPS-induced COX-2 expression without agonist addition. This reveals that increased expression of COX-2 may be accompanied by an increase of PPARβ/δ, the characteristic feature of many cancer cells (Glinghammar et al., 2003; Xu et al., 2006b; Michalik and Wahli, 2008). It is attractive to suggest that negative regulation from PPARα for the expression of PPARβ/δ, which we saw in normal astrocytes, is lost in cancer cells, and thus, this regulatory circle is disrupted. Our experiments with overexpressing PPARβ/δ cells point to this possibility.

Our data reveal a new role for PPARβ/δ in the regulation of astrocyte function in response to proinflammatory stimulation. Astrocytes are believed to contribute to the development of neurodegenerative diseases (Heales et al., 2004; Drew et al., 2006), and thus, agents that block the activation of astrocytes are considered to be effective in the treatment of these diseases (Consilvio et al., 2004; Drew et al., 2006). It was demonstrated that PPARα agonists inhibit the clinical signs of experimental autoimmune encephalomyelitis (Drew et al., 2006). This was correlated with the fact that PPARα agonists inhibit production of NO, tumor necrosis factor-α, interleukin-1β, interleukin-6, and monocyte chemotactic protein-1 in LPS-stimulated primary mouse astrocytes (Pahan et al., 2002; Xu et al., 2006a). The neuroprotective activity of PPARγ agonists both in vivo (Tzeng et al., 2005; Bordet et al., 2006; Heneka and Landreth, 2007; Kapadia et al., 2008) and in vitro through regulation of astrocyte function (Bernardo and Minghetti, 2008) is well proven (Culman et al., 2007). Rosiglitazone and other synthetic PPARγ agonists inhibit expression of inducible nitric-oxide synthase (Luna-Medina et al., 2005) and production of cytokines (Xu and Drew, 2007) in astrocytes.

There is no information concerning the effects of PPARβ/δ agonists on astrocytes, whereas neuroprotective efficacy of these substances was shown both in vitro and in vivo (Iwashita et al., 2007). In the central nervous system, PPARβ/δ has been linked to myelinogenesis, glial cell maturation, and neuroprotection (Saluja et al., 2001; Iwashita et al., 2007). PPARα and PPARγ agonists possess anti-inflammatory activity in the brain (Heneka and Landreth, 2007). Activation of all PPAR isoforms, but especially of PPARγ, was shown to prevent postischemic inflammation and neuronal damage in several in vitro and in vivo models, negatively regulating the expression of genes induced by ischemia/reperfusion, including COX-2 expression in neurons (Collino et al., 2008). In light of these evidences, it is not clear what role COX-2 activation plays in astrocyte responses for the inflammatory stimulation and for prevention of ischemia-induced brain responses. It is possible that the time of the tested effect is important, because we investigated 4-h treatments, and in vivo effects are tested at times greater than

12 h. We observed that rosiglitazone increases COX-2 expression. In another study, inhibition of COX-2 by a PPARγ agonist was shown in astrocytes (Luna-Medina et al., 2005), but this may reflect different experimental protocols. For instance, the LPS concentration used there was 100 times greater than in our study. Increase of COX-2 expression by positive interplay between PPARβ/δ and PPARγ was demonstrated in our study at the level of COX-2 protein expression in Western blot and its functional consequences by determining the $PGE_2$ synthesis. The role of COX-2 expression and increase in prostaglandin synthesis in neuroinflammation is judged controversial, because both pro- and anti-inflammatory properties have been described previously (Consilvio et al., 2004; Tzeng et al., 2005; Aid et al., 2008). Taken together, our data on rosiglitazone-induced COX-2 expression and inhibition of other important inflammatory responses (Xu and Drew, 2007) in astrocytes support the hypothesis of anti-inflammatory properties of COX-2 in brain.

The PPAR interactions described here have shown that combined application of PPAR agonists leads to effects that differ from the sum of individual effects of PPAR agonists. In the present study, we demonstrated a ligand-dependent cross-talk between different PPAR isotypes at the level of regulation of their expression, which is finalized at the level of target gene regulation. Our data clearly show that in addition to the positive control of PPARβ/δ expression by PPARγ, there is a negative feedback loop between PPARα and PPARβ/δ. This is a novel finding that may have implications not only for understanding fundamental PPAR biology but also for planning the strategies for regulation of PPARs, important drug targets.

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