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PPARγ positively and PPARα negatively control COX-2 expression in rat brain astrocytes through a convergence on PPARβ/δ via mutual control of PPAR expression levels

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Running title: PPARα, β/δ and γ interaction pathway in astrocytes

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ABBREVIATIONS: COX, cyclooxygenase; DMEM, Dulbecco's modified Eagle's medium; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PPAR, peroxisome proliferators-activated receptor; 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

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ABSTRACT

PPAR (peroxisome proliferator-activated receptor) transcription factors are pharmaceutical drug targets for treating diabetes, atherosclerosis, and inflammatory degenerative diseases. The possible mechanism of interaction between the three PPARs isotypes (α, β/δ, γ) is not yet clear. However, this is important both for understanding transcription factor regulation and development of new drugs. The present study was designed to compare effects of combinations of synthetic agonists of PPARα (GW7647), PPARβ/δ (L-165041) and PPARγ (rosiglitazone, ciglitazone) on inflammatory gene regulation in rat primary astrocytes. We measured COX-2 expression and prostaglandin E_2 synthesis in LPS-stimulated cells. PPARα, PPARβ/δ, 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, 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 PPARs expression levels. A PPARβ/δ over-expression model confirmed that PPARβ/δ expression level is the point, where PPARγ and PPARα pathways converge in control of COX-2 gene expression. Thus, we discovered that in primary astrocytes, PPARγ has positive influence and PPARα has 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 signalling by synthetic PPAR agonists that involves modulating the interaction between PPARα, β/δ, γ-isoforms on the level of their expression.

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Introduction

Transcription factors peroxisome proliferators-activated receptors (PPARs) control expression of genes related to lipid and glucose homeostasis and inflammatory responses (Bensinger and Tontonoz, 2008; Michalik et al., 2006; Michalik and Wahli, 2008). Three subtypes, PPARα, PPARβ/δ and PPARγ, have been described (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 (Bensinger and Tontonoz, 2008; Heneka and Landreth, 2007; Michalik et al., 2006). Thus, mechanisms of PPAR-induced pathways are under intensive investigation.

Some synthetic PPAR agonists are FDA-approved drugs. PPARα is the target for the 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 anti-atherosclerotic activity (Bensinger and Tontonoz, 2008; Heneka and Landreth, 2007; Michalik and Wahli, 2008). PPARβ/δ agonists are considered useful for treating dyslipidemia, obesity and for 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 (RXR) and subsequently bind to PPAR response elements (PPREs) 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 from 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

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suggested for repression of the PPARγ- and PPARα-mediated activation of target gene expression following PPARβ/δ activation (Shi et al., 2002), and also 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 analyse 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 brain, and disturbance in their activity is related to neurodegenerative diseases (Bernardo and Minghetti, 2008; Heales et al., 2004; Heneka and Landreth, 2007). Second, PPARγ and PPARα agonists modulate inflammatory responses in astrocytes (Luna-Medina et al., 2005; Pahan et al., 2002; J. Xu et al., 2006). All three PPAR isoforms are present in primary astrocytes (Cristiano et al., 2005). Nevertheless, an investigation of PPARβ 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, PPARγ knockdown models and application of specific PPAR agonists to characterize the contribution of each PPAR isotype in regulation of COX-2 expression. Additionally, using PPARβ/δ over-expression 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 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. Further, the PPARβ/δ-activation resulted in an increase of PPARα expression level, thus forming positive/negative feedback loop. These findings suggest a novel mechanism of signalling by PPAR synthetic agonists.

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Materials and methods.

Materials

The cell culture medium was from GIBCO/BRL (Eggenstein, Germany) except for fetal calf serum (FCS), penicillin and streptomycin, which were from Biochrom (Berlin, Germany). Lipopolysaccharide (LPS) was purchased from Sigma Chemicals (Germany, Taufkirchen), LPS was dissolved in deionized water at a final concentration of 1 mg/ml and stored at -20º C. Rosiglitazone, Ciglitazone, GW7647 and GW9662 were purchased from Cayman Chemical (Tallinn, Estonia). L-165041 was purchased from Sigma Chemicals (Germany, Taufkirchen). PPARα, PPARβ/δ and β-tubulin antibodies were purchased from Sigma Chemicals (Germany, Taufkirchen). COX-2 antibodies were purchased from Cayman Chemical (Tallinn, Estonia). PPAR agonists and GW9662 were dissolved in DMSO 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 1-2 µg total RNA, 20 pmol oligod$T_{(12\text{-}18)}$ and 1 U Omniscript (Qiagen), for 1 h at 37°C. In a RT-PCR with 1 U AccuPrime (Invitrogen, Karlsruhe, Germany) and the two oligonucleotides RnPPARβStart, GATCAAGCTTGGATCCCGATGGAACAGCCACAGGAGGAGACC, and RnPPARβRev, GATCGATATCTTAGTACATGTCCTTGTAGATCTC, the full length RnPPARβ was generated. The *Hind*III/*Eco*RV fragment of this PCR product was subcloned to pBlueScriptKSII (Stratagene, La Jolla, CA, USA). Positive clones were verified via nucleotide sequence analysis (SeqLab, Göttingen, Germany) and a *Bam*HI/*Eco*RV 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

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(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 by 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, 5.5 mM glucose, pH 7.4). The brains were gently passed through nylon meshes of 250 μm and 136 μm pore width, in consecutive order. Cells were plated onto 75-cm^2 culture flasks at a starting density of 6×10^5 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 Hank's solution (50 mM NaCl, 5 mM KCl, 0.2 mM KH_2PO_4, 0.17 mM Na_2HPO_4, 5.0 mM glucose, 58.4 mM sucrose, pH 7.4). Cells were trypsinized and replated onto 6 well plates. 10^6 cells 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 instructions. Briefly, 2 hours before the transfection of astrocytes in 6-well plates the medium was changed to fresh DMEM with 10% FCS with a final volume of 2 mL. The plasmid−beads (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, 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 both Western blot.

RNA and protein preparation

Before RNA and protein preparation, cells were washed 3 times with 5 ml of cold phosphate-buffered saline (PBS) per well. Total RNA was isolated and DNAase-treated from cultured astrocytes with the total RNA isolation kit RNeasy (Qiagen, Hilden, Germany). The total RNA isolated by this method is nondegraded and free of protein and DNA contamination. For protein isolation, astrocytes were lysed in modified RIPA buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.25% Na-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 000g (4°C) for 15 min, and the pellet was discarded. The protein concentration was determined by the Bradford method using BSA as standard**.**

Measurement of the relative RNA expression level

cDNA was generated from 250 ng of total RNA with iScript™ cDNA synthesis kit (BioRad) 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

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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', 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 were solubilized in Laemmli buffer were separated under denaturing conditions on sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). Ten μg 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 Pre-stained Standards from Bio-Rad Laboratories (Hercules, CA, USA) 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 PBST (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 PBST 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 (Super-Signal 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; 100 mM β-mercaptoethanol), and washed five times. Membranes were reprobed with antibody against β-tubulin (1:10000) from Sigma Chemicals (Taufkirchen, Germany) and secondary anti-mouse IgG (Dianova, Hamburg, Germany) to control for protein loading. Band intensity was measured using a GS-800 calibrated

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densitometer and Quantity One software (Bio-Rad, Munich, Germany) and normalized to the intensity of the respective bands obtained for β-tubulin.

Transfection with small interfering RNA (siRNA)

siRNAs used in the present study were purchased from Qiagen (Hilden, Germany). PPARα: cat.# SI01963451 ;PPARβ/δ: cat.# SI01963479 ;PPARγ: cat.# SI01963507. Cells were transfected using magnet beads and magnet plate (see above). Briefly, 2 hours before the transfection of astrocytes in 6-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, 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 6-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; Ann Arbor, MI) according to the manufacturer's instructions. The results were expressed as ng per ml 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; Tallinn, Estonia). 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 (#10008878) following the manufacturers' instructions (Cayman Chemical; Tallinn, Estonia). Briefly, a dsDNA sequence containing the PPRE is linked onto the bottom of wells (96-well plate). PPARs within the nuclear fraction bind specifically to this sequence and isoforms are detected

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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 lisates from untreated astrocytes serving as the reference value.

Experimental data analysis and statistics

All data presented in text and figures as means and standard error of samples from three experiments, each characterized in groups of at least three independent replicas. Data were subjected to an ANOVA with Dunnett's posthoc comparison. Statistical significance was established at $P<0.05$.

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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 (Luna-Medina et al., 2005; J. Xu et al., 2006; 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, which 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 4.5 fold increase of COX-2 expression levels, compared to basal conditions (Fig.1; at concentration of PPAR agonist "0 μM"). The addition of GW7647 or L-165041 has no influence on LPS –induced COX-2 expression in the range of concentrations from 0.1 to 20 μM (Fig. 1). 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).

Rosiglitazone and ciglitazone potentiate COX-2 expression via PPARγ–dependent process

It is known that PPARγ agonists besides selective activation of PPARγ receptors may act via PPARγ-independent mechanisms (Luna-Medina et al., 2005; Park et al., 2003). 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 usage of PPARγ knockdown astrocytes. The results are shown in Fig. 2. Treatment of cells with GW9662 results in the irreversible loss of ligand binding, while PPARγ knockdown results in 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

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silencing of PPARγ were similar. Inhibition of PPARγ activity by GW9662 treatment or PPARγ knockdown eliminated the PPARγ agonists-induced increase of COX-2 expression in LPS-stimulated astrocytes (Fig. 2A, 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 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 in comparison 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), as compared to that in LPS-stimulated astrocytes (Fig. 3A, column 1). Surprisingly, 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 of the PPARβ/δ agonist L-165041, rosiglitazone at the same low concentration clearly enhanced COX-2 expression (Fig. 3C). Taken together, these data allow suggesting there is an interplay between agonists of different isoforms of PPARs in the PPARγ-dependent modulation of COX-2 gene expression.

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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 (as compared to 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, where 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, as compared to 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, while the additional application of the PPARα agonist GW7647 abolished this effect, as shown above in Fig. 3A, 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

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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, Figure 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.

Over-expression 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 allow suggesting 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 over-expression, L-165140 induces COX-2 expression without additional stimulation by rosiglitazone (compare column 4 cells with PPARβ/δ over-expression 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 three tested agonists (Fig. 7, compare columns 10 and 4). Figute 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 over-expression were non-sensitive for positive/negative regulation by PPARγ agonists and PPARα agonists, since the levels of L-165140-stimulated COX-2 expression in astrocytes with PPARβ/δ over-expression were the same in cells with simultaneous PPARβ/δ over-expression and PPARγ knockdown (compare columns 7, 8 and 4, Fig. 7). Also in cells with simultaneous PPARβ/δ over-expression and PPARα knockdown the PPARα agonist-elicited inhibitory effect was eleiminated (Fig. 7, compare columns 11, 12 with 9, 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

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first report showing increase of COX-2 expression via PPARβ/δ activation in normal, non-cancer cells.

Rosiglitazone induces the expression of PPARβ/δ via PPARγ-dependent pathway

As the PPARβ/δ agonist L-165041 had no effect on COX-2 expression itself (Fig. 1), while 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. In order 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 upregulation 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 increases 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 regulation of COX-2 expression in LPS-stimulated astrocytes. Further, we have demonstrated that GW7647 inhibits the effects of combined application of rosiglitazone and L-165041 via a PPARα-dependent pathway (Fig. 6). This

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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 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, 4 with column 1) The changes in mRNA level were correlated with similar changes in protein level (Fig. 9B). As expected, addition of GW7647 to the tested system had no effect in the PPARα knockdown cells (Fig. 9A, column 6), but in the control cells treated with scrambled siRNA (Fig. 9A, column 5).

Application of rosiglitazone induced a 2.5-fold increase of PPARβ/δ activity (Fig. 8C), whereas simultaneous addition of rosiglitazone and the PPARβ/δ agonist L-165041 resulted in 5.5-fold increase of PPARβ/δ activity (Fig. 9C). The latter effect was totally abolished in the presence of PPARα agonist; moreover, under PPARα agonist stimulation we obtained a 2-fold decrease of PPARβ/δ activity relative to control (Fig. 9C). Thus, we found that activation of PPARα receptors leads to reduction of PPARβ/δ receptor levels and a subsequent decrease in PPARβ/δ activity.

L-165041 increases PPARα expression and activity in LPS-stimulated astrocytes via PPARβ/δ– dependent mechanism

We have shown above in Figure 7 that PPARα negatively controls COX-2 expression in astrocytes through a PPARβ/δ-mediated pathway. However, it remained 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. While PPARα negatively controls the expression of PPARβ/δ receptors, 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 being added alone decreased the PPARα level (Fig. 10A; column 1), while application of the PPARβ/δ agonist L-165041 in combination with

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rosiglitazone increased the PPARα mRNA up to the normal level in non-treated 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 PPARα mRNA level also correlated with changes in protein level of PPARα (Fig. 10B). 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 over-expression (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 over-expression vector, the additional silencing of PPARγ had no effect on the PPARα expression (Fig. 10D, column 8). The experiments, where it is shown that rosiglitazone is essential for induction of PPARα expression (Fig. 10A, column 7), taken together with the data with PPARβ/δ over-expression (Fig. 10D) make it obvious that rosiglitazone activates PPARγ, which then induces the expression of PPARβ/δ. That PPARβ/δ in turn, after stimulation by the agonist L-165041, induced the expression of PPARα (Fig. 10A), and induced PPARα activity (Fig. 10C).

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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 temping 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 GW4756 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 Figure 11. Activation of PPARγ has positive influence, while 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 regulation of astrocyte functions and reveals a new role of PPARβ/δ to gate the activities of PPARα and PPARγ in brain.

It is known that due to 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 as 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 also PPARγ-independent effects were shown (Luna-Medina et al., 2005; Park et al., 2003). Therefore, we tested application of agonist in naïve astrocytes and in and 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 upon other types of PPAR.

We observed in the astrocytes a PPARβ/δ-dependent up-regulation of PPARα at the level of mRNA transcription, protein and PPARα activity. That makes astrocytes more sensitive to PPARα agonists. On the other side, PPARα agonists are important negative

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control of PPARβ/δ expression, in the case of astrocytes, where 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 complex tuning mechanism, as suggested by the full scheme in Figure 11. In contrast to these data received on normal cells, it was shown in genetically engineering cells that an increase of PPARβ/δ expression resulted in the decrease of activity of PPARα-dependent enzymes (Shi et al., 2002).

Influence of PPARβ/δ on PPARα- and PPARγ-activated transcription via mechanisms of repression is well known (Bensinger and Tontonoz, 2008; Ricote and Glass, 2007). 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 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 cross-road between PPARs was already suggested previously. PPARβ/δ activation by agonists or PPARβ/δ over/expression inhibits PPARγ activity (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 isotypes levels will control the activity of each PPAR isoform. As a consequence, addition of a specific agonist of one PPAR isoform may indirectly change target gene responses via 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; L. Xu et al., 2006). For application of PPARγ or PPARα agonists, both activation (Ikawa et al., 2001; Seimandi et al., 2005; Ulivi et al., 2008; Zhang et al., 2006) and inhibition (Grau et al., 2006; Luna-Medina et al., 2005) of COX-2 expression were found. For PPARβ/δ only few data were obtained from studies using cancer cell lines. They show PPARβ/δ-induced COX-2 expression (Glinghammar et al., 2003; L. Xu

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et al., 2006). This is consistent with our data on rat primary astrocytes. Interestingly, in our test system over-expression of PPARβ/δ leads to increase of LPS-induced COX-2 expression without agonist addition. This reveals that increased expression of COX-2 may be accompanied by increase of PPARβ/δ, the characteristic feature of many cancer cells (Glinghammar et al., 2003; Michalik and Wahli, 2008; L. Xu et al., 2006). It is attractive to suggest that negative regulation from PPARα for 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 over-expressing 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 (Drew et al., 2006; Heales et al., 2004) 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, TNF-α, IL-1β, IL-6, and MCP-1 in LPS-stimulated primary mouse astrocytes (Pahan et al., 2002; J. Xu et al., 2006). The neuroprotective activity of PPARγ agonists both *in vivo* (Bordet et al., 2006; Heneka and Landreth, 2007; Kapadia et al., 2008; Tzeng et al., 2005) and *in vitro* through regulation of astrocyte function (Bernardo and Minghetti, 2008) is well proven (Culman et al., 2007). Rosiglitazone and other synthethic PPARγ agonists inhibit expression of iNOS (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, while 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 (Iwashita et al., 2007; Saluja et al., 2001). PPARα and PPARγ agonists possess anti-inflammatory activity in brain (Heneka and Landreth, 2007). Activation of all PPAR isoforms, but especially of PPARγ, was shown to prevent post-ischemic 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 the 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

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of the tested effect is important, as we investigated 4 h treatments and *in vivo* effects are tested at times more 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 higher 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 are judged controversial, because both pro- and anti-inflammatory properties have been described (Aid et al., 2008; Consilvio et al., 2004; Tzeng et al., 2005). 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, which differ from the sum of individual effects of PPAR agonists. In the present study, we demonstrated a ligand-dependent crosstalk 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 which may have not only implications for understanding of fundamental PPAR biology, but also for planning the strategies for regulation of PPARs, important drug targets.

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This article has not been copyedited and formatted. The final version may differ from this version.

References

Aid S, Langenbach R and Bosetti F (2008) Neuroinflammatory response to lipopolysaccharide is exacerbated in mice genetically deficient in cyclooxygenase-2. *J Neuroinflammation* **5**:17.

Balakumar P, Rose M, Ganti SS, Krishan P and Singh M (2007) PPAR dual agonists: are they opening Pandora's Box? *Pharmacol Res* **56**:91-98.

Bensinger SJ and Tontonoz P (2008) Integration of metabolism and inflammation by lipid-activated nuclear receptors. *Nature* **454**:470-477.

Bernardo A and Minghetti L (2008) Regulation of Glial Cell Functions by PPAR-gamma Natural and Synthetic Agonists. *PPAR Res* **2008**:864140.

Bordet R, Ouk T, Petrault O, Gele P, Gautier S, Laprais M, Deplanque D, Duriez P, Staels B, Fruchart JC and Bastide M (2006) PPAR: a new pharmacological target for neuroprotection in stroke and neurodegenerative diseases. *Biochem Soc Trans* **34**:1341-1346.

Collino M, Patel NS and Thiemermann C (2008) PPARs as new therapeutic targets for the treatment of cerebral ischemia/reperfusion injury. *Ther Adv Cardiovasc Dis* **2**:179-197.

Consilvio C, Vincent AM and Feldman EL (2004) Neuroinflammation, COX-2, and ALS--a dual role? *Exp Neurol* **187**:1-10.

Cristiano L, Cimini A, Moreno S, Ragnelli AM and Paola Ceru M (2005) Peroxisome proliferator-activated receptors (PPARs) and related transcription factors in differentiating astrocyte cultures. *Neuroscience* **131**:577-587.

Culman J, Zhao Y, Gohlke P and Herdegen T (2007) PPAR-gamma: therapeutic target for ischemic stroke. *Trends Pharmacol Sci* **28**:244-249.

Drew PD, Xu J, Storer PD, Chavis JA and Racke MK (2006) Peroxisome proliferator-activated receptor agonist regulation of glial activation: relevance to CNS inflammatory disorders. *Neurochem Int* **49**:183-189.

Glinghammar B, Skogsberg J, Hamsten A and Ehrenborg E (2003) PPARdelta activation induces COX-2 gene expression and cell proliferation in human hepatocellular carcinoma cells. *Biochem Biophys Res Commun* **308**:361-368.

Grau R, Punzon C, Fresno M and Iniguez MA (2006) Peroxisome-proliferator-activated receptor alpha agonists inhibit cyclo-oxygenase 2 and vascular endothelial growth factor transcriptional activation in human colorectal carcinoma cells via inhibition of activator protein-1. *Biochem J* **395**:81-88.

Heales SJ, Lam AA, Duncan AJ and Land JM (2004) Neurodegeneration or neuroprotection: the pivotal role of astrocytes. *Neurochem Res* **29**:513-519.

Heneka MT and Landreth GE (2007) PPARs in the brain. *Biochim Biophys Acta* **1771**:1031-1045.

Ikawa H, Kameda H, Kamitani H, Baek SJ, Nixon JB, Hsi LC and Eling TE (2001) Effect of PPAR activators on cytokine-stimulated cyclooxygenase-2 expression in human colorectal carcinoma cells. *Exp Cell Res* **267**:73-80.

Iwashita A, Muramatsu Y, Yamazaki T, Muramoto M, Kita Y, Yamazaki S, Mihara K, Moriguchi A and Matsuoka N (2007) Neuroprotective efficacy of the peroxisome proliferator-activated receptor delta-selective agonists in vitro and in vivo. *J Pharmacol Exp Ther* **320**:1087-1096.

Kang YJ, Mbonye UR, DeLong CJ, Wada M and Smith WL (2007) Regulation of intracellular cyclooxygenase levels by gene transcription and protein degradation. *Prog Lipid Res* **46**:108-125.

Kapadia R, Yi JH and Vemuganti R (2008) Mechanisms of anti-inflammatory and neuroprotective actions of PPAR-gamma agonists. *Front Biosci* **13**:1813-1826.

Leisewitz AV, Urrutia CR, Martinez GR, Loyola G and Bronfman M (2008) A PPARs cross-talk concertedly commits C6 glioma cells to oligodendrocytes and induces enzymes involved in myelin synthesis. *J Cell Physiol* **217**:367-376.

Lemay DG and Hwang DH (2006) Genome-wide identification of peroxisome proliferator response elements using integrated computational genomics. *J Lipid Res* **47**:1583-1587.

Luna-Medina R, Cortes-Canteli M, Alonso M, Santos A, Martinez A and Perez-Castillo A (2005) Regulation of inflammatory response in neural cells in vitro by thiadiazolidinones derivatives through peroxisome proliferator-activated receptor gamma activation. *J Biol Chem* **280**:21453-21462.

Matsusue K, Peters JM and Gonzalez FJ (2004) PPARbeta/delta potentiates PPARgamma-stimulated adipocyte differentiation. *FASEB J.* **18**:1477-1479.

Michalik L, Auwerx J, Berger JP, Chatterjee VK, Glass CK, Gonzalez FJ, Grimaldi PA, Kadowaki T, Lazar MA, O'Rahilly S, Palmer CN, Plutzky J, Reddy JK, Spiegelman BM, Staels B and Wahli W (2006) International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors. *Pharmacol Rev* **58**:726-741.

Michalik L and Wahli W (2008) PPARs Mediate Lipid Signaling in Inflammation and Cancer. *PPAR Res* **2008**:134059.

Pahan K, Jana M, Liu X, Taylor BS, Wood C and Fischer SM (2002) Gemfibrozil, a lipid-lowering drug, inhibits the induction of nitric-oxide synthase in human astrocytes. *J Biol Chem* **277**:45984-45991.

Park EJ, Park SY, Joe EH and Jou I (2003) 15d-PGJ2 and rosiglitazone suppress Janus kinase-STAT inflammatory signaling through induction of suppressor of cytokine signaling 1 (SOCS1) and SOCS3 in glia. *J Biol Chem* **278**:14747-14752.

Peters JM, Hollingshead HE and Gonzalez FJ (2008) Role of peroxisome-proliferator-activated receptor beta/delta (PPARbeta/delta) in gastrointestinal tract function and disease. *Clin Sci (Lond)* **115**:107-127.

Ricote M and Glass CK (2007) PPARs and molecular mechanisms of transrepression. *Biochim Biophys Acta* **1771**:926-935.

Saluja I, Granneman JG and Skoff RP (2001) PPAR delta agonists stimulate oligodendrocyte differentiation in tissue culture. *Glia* **33**:191-204.

Seimandi M, Lemaire G, Pillon A, Perrin A, Carlavan I, Voegel JJ, Vignon F, Nicolas JC and Balaguer P (2005) Differential responses of PPARalpha, PPARdelta, and PPARgamma reporter cell lines to selective PPAR synthetic ligands. *Anal Biochem* **344**:8-15.

Shi Y, Hon M and Evans RM (2002) The peroxisome proliferator-activated receptor delta, an integrator of transcriptional repression and nuclear receptor signaling. *Proc Natl Acad Sci U S A* **99**:2613-2618.

Strokin M, Sergeeva M and Reiser G (2004) Role of Ca^{2+}-independent phospholipase A_2 and n-3 polyunsaturated fatty acid docosahexaenoic acid in prostanoid production in brain: perspectives for protection in neuroinflammation. *Int J Dev Neurosci* **22**:551-557.

Strokin M, Sergeeva M and Reiser G (2007) Prostaglandin synthesis in rat brain astrocytes is under the control of the n-3 docosahexaenoic acid, released by group VIB calcium-independent phospholipase A2. *J Neurochem* **102**:1771-1782.

Tzeng SF, Hsiao HY and Mak OT (2005) Prostaglandins and cyclooxygenases in glial cells during brain inflammation. *Curr Drug Targets Inflamm Allergy* **4**:335-340.

Ulivi V, Cancedda R and Cancedda FD (2008) 15-deoxy-delta 12,14-prostaglandin J(2) inhibits the synthesis of the acute phase protein SIP24 in cartilage: Involvement of COX-2 in resolution of inflammation. *J Cell Physiol* **217**:433-441.

Xu J, Chavis JA, Racke MK and Drew PD (2006) Peroxisome proliferator-activated receptor-alpha and retinoid X receptor agonists inhibit inflammatory responses of astrocytes. *J Neuroimmunol* **176**:95-105.

Xu J and Drew PD (2007) Peroxisome proliferator-activated receptor-gamma agonists suppress the production of IL-12 family cytokines by activated glia. *J Immunol* **178**:1904-1913.

Xu L, Han C, Lim K and Wu T (2006) Cross-talk between peroxisome proliferator-activated receptor delta and cytosolic phospholipase A(2)alpha/cyclooxygenase-2/prostaglandin E(2) signaling pathways in human hepatocellular carcinoma cells. *Cancer Res* **66**:11859-11868.

Zhang Q, Seltmann H, Zouboulis CC and Konger RL (2006) Involvement of PPARgamma in oxidative stress-mediated prostaglandin E(2) production in SZ95 human sebaceous gland cells. *J Invest Dermatol* **126**:42-48.

Zuo X, Wu Y, Morris JS, Stimmel JB, Leesnitzer LM, Fischer SM, Lippman SM and Shureiqi I (2006) Oxidative metabolism of linoleic acid modulates PPAR-beta/delta suppression of PPAR-gamma activity. *Oncogene* **25**:1225-1241.

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FOOTNOTES

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Figure legends

Figure 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 (μM) 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 ± SEM from three independent experiments. **(B)** PGE_2 production was determined by IEA, as described in Materials and methods. Values represent the mean ± SEM from three independent experiments.

Figure 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 were transfected by PPARγ siRNA. Where indicated, cells were treated for 10 min with 20 μM of rosiglitazone (Ros) or 40 μM of 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 ± SEM 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, and lane 2- PPARγ knockdown astrocytes.

Figure 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 ± SEM from three independent experiments. *$P < 0.05$, compared with the LPS-stimulated cells (column 1). **(B)** Respective protein expression for COX-2 is

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evaluated by Western blotting. Example is representative for three independent experiments**. (C)** Cells were pretreated for 10 min with the indicated concentration ns (μM) 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 ± SEM from three independent experiments.

Figure 4. PPARβ/δ agonist L-165041 enhances rosiglitazone-induced increase of COX-2 expression in LPS-stimulated astrocytes via PPARγ–dependent pathway. Cells were preincubated during 10 min with 1 μM of GW9662 or were transfected by PPARγ siRNA. Where indicated, cells were treated for 10 min with 20 μM of rosiglitazone (Ros) in the absence or presence of 1 μM of L-165041. 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 ± SEM from three independent experiments. *P < 0.05, compared with the LPS-stimulated cells.

Figure 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 by PPARβ/δ siRNA or scrambled siRNA. Where indicated, cells were treated for 10 min with 20 μM of rosiglitazone (Ros) in the absence or presence of L-165041 (1 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 (lane 1), and all values are normalized to GAPDH mRNA level. Values represent the mean ± SEM 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 vs 5). **(Inset)** Efficacy of PPARβ/δ knockdown is verified by Western blot**,** lane 1-control and lane 2- PPARβ/δ knockdown astrocytes.

Figure 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

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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 ± SEM 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 vs 5). **(Inset)** Efficacy of PPARα knockdown is verified by Western blot**,** lane 1- control and lane 2- PPARα knockdown astrocytes.

Figure 7. Over-expression of PPARβ/δ reveals a key role of PPARβ/δ in control of COX-2 gene expression. Cells were transfected by PPARβ/δ over-expression 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 GAPDH mRNA level. Values represent the mean ± SEM from three independent experiments. *P < 0.05, compared with the LPS-stimulated cells.

Figure 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 (μM) 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 GAPDH mRNA level. Values represent the mean ± SEM from three independent experiments. *P < 0.05, compared with the LPS-stimulated cells. **(B)** Protein expression for PPARβ is evaluated by Western blotting. Example is representative for three independent experiments**. (C)** Where indicated, cells were incubated for 10 min with 20 μM of rosiglitazone. LPS (100 ng/ml) was then added for 4 h. PPARβ/δ transcriptional activity was determined as described in Materials and Methods. Values represent the mean ± SEM from three independent experiments. *P < 0.05,

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compared with the untreated LPS-stimulated cells. **(D)** Cells were preincubated during 10 min with 1 μM of GW9662 or were treated with PPARγ siRNA or scrambled siRNA. Where indicated, cells were treated for 10 min with 20 μM of rosiglitazone (Ros) or 40 μM of 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 ± SEM from three independent experiments. *P < 0.05, compared with the LPS-stimulated cells (-).

Figure 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 it is indicated cells were treated for 10 min with 1 μM of L-165041 plus 20 μM of rosiglitazone (Ros) in the absence or presence of 1 μM of 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 ± SEM from three independent experiments. *P < 0.05, compared with the LPS-stimulated cells. **(B, C)** Cells were incubated for 10 min with 1 μM of L-165041 plus 20 μM of rosiglitazone in the absence or presence of 1 μM of GW7647. LPS (100 ng/ml) was then added for 4 h. **(B)** COX-2 protein was evaluated by Western blotting. Example is representative for three independent experiments. **(C)** PPARβ/δ transcriptional activity was determined, as described in Materials and Methods. Values represent the mean ± SEM from three independent experiments. *P < 0.05, compared with the LPS-stimulated cells.

Figure 10. PPARβ/δ agonist L-165041 increases expression of PPARα in LPS-stimulated astrocytes via PPARβ/δ–dependent pathway. (A, B) Cells were transfected by PPARβ/δ siRNA or scrambled siRNA, as indicated. Where it is indicated cells were treated for 10 min with 20 μM of 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 GAPDH mRNA level. Values represent the mean ± SEM from three independent experiments. *P < 0.05, compared with the LPS-stimulated cells. **(B)** Protein

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expression for PPARα was evaluated by Western blotting. Example is representative for 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 in Materials and Methods. Values represent the mean ± SEM from 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 it is 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 GAPDH mRNA level. Values represent the mean ± SEM from three independent experiments. *P < 0.05, compared with the LPS-stimulated cells.

Figure 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.

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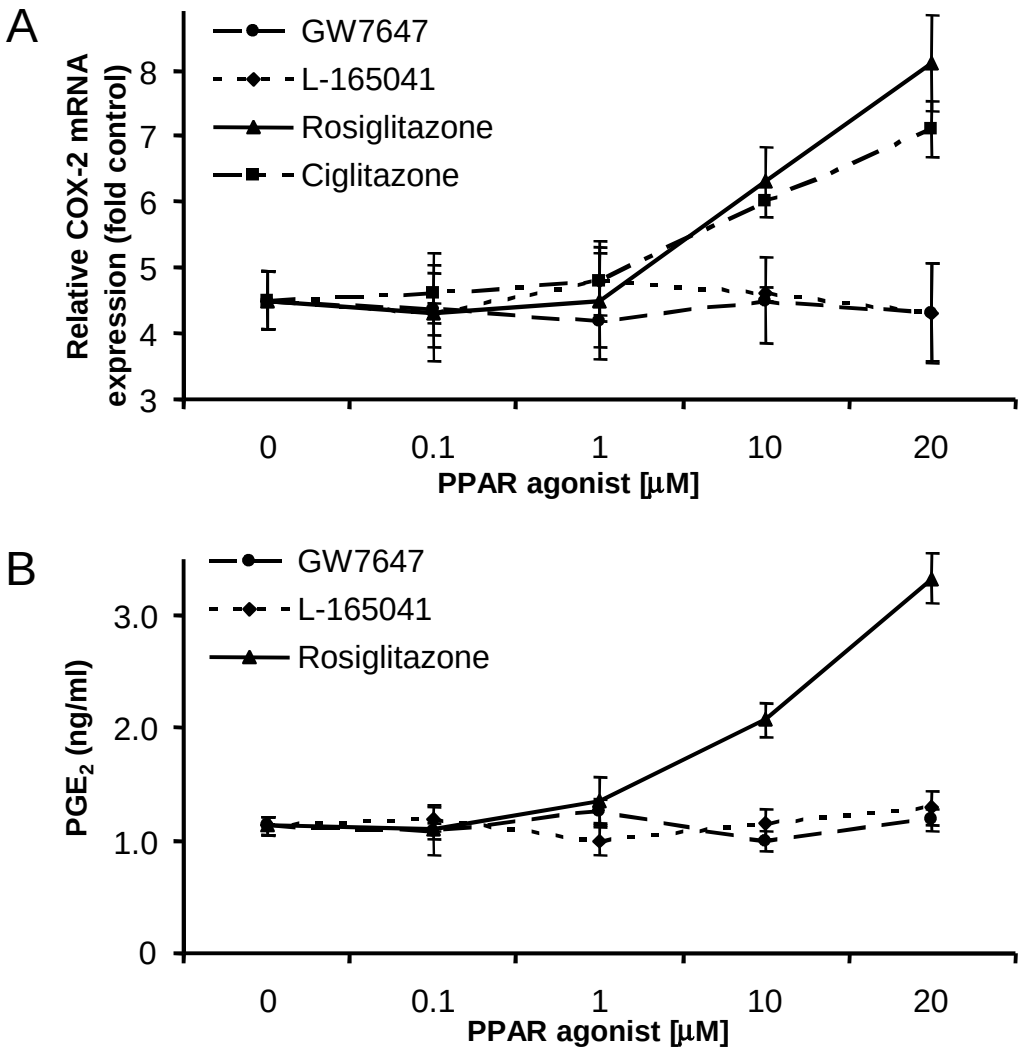

Figure 1

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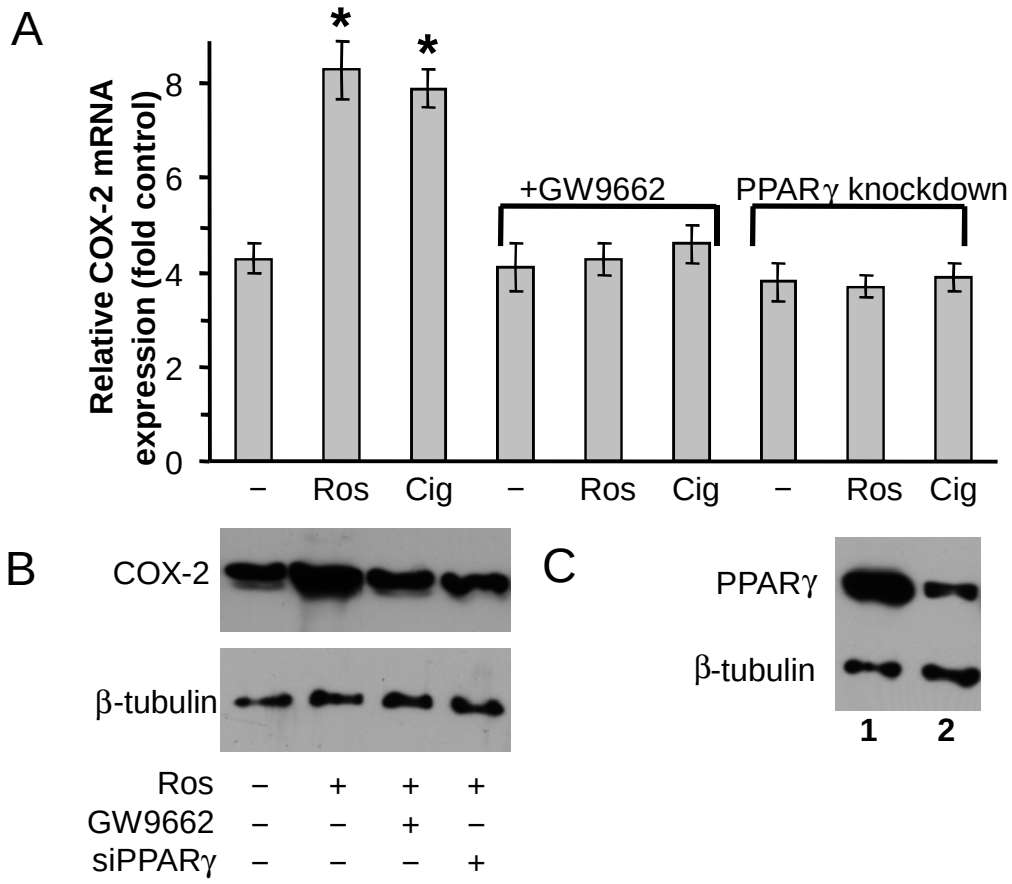

Figure 2

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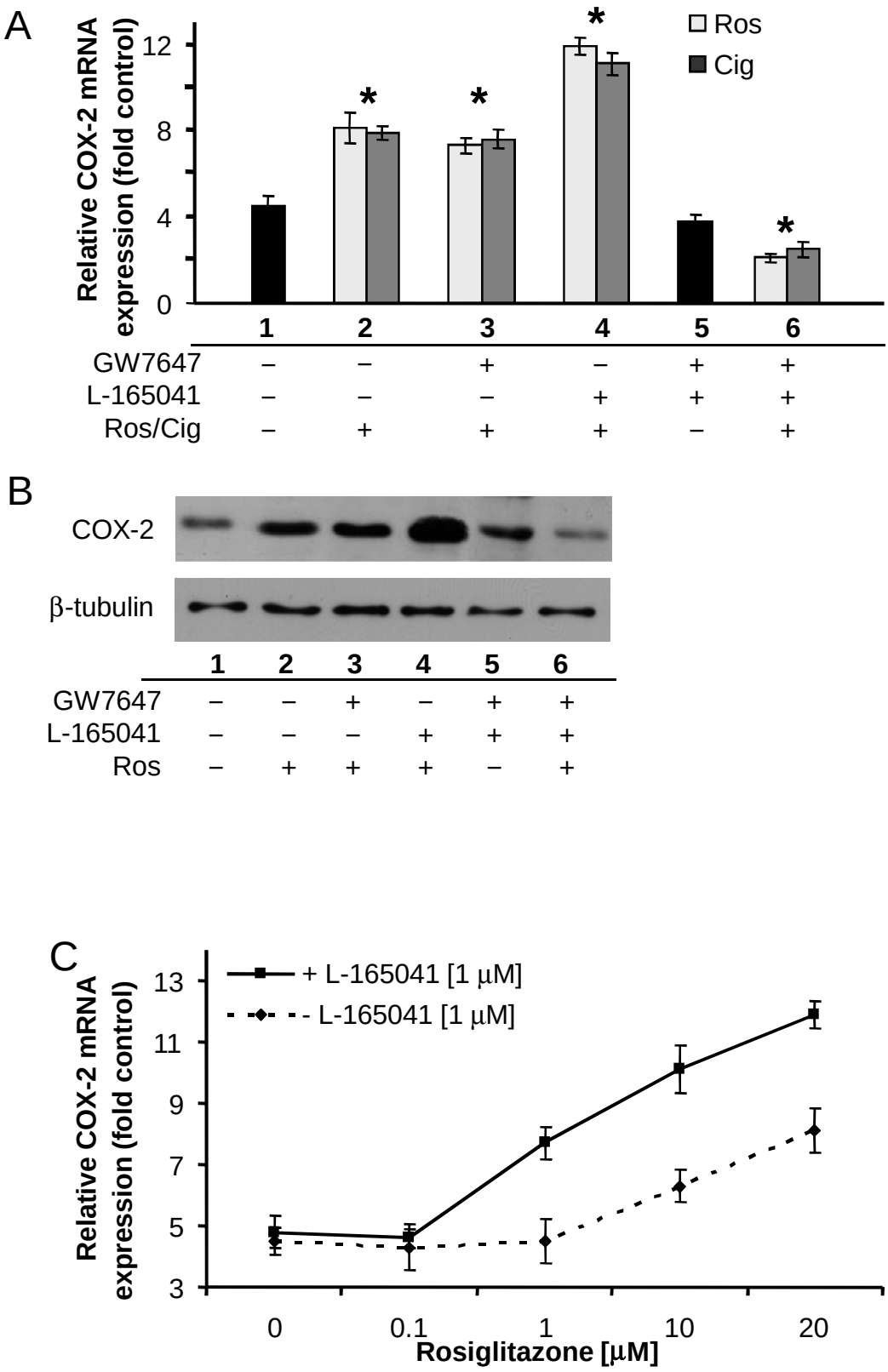

Figure 3

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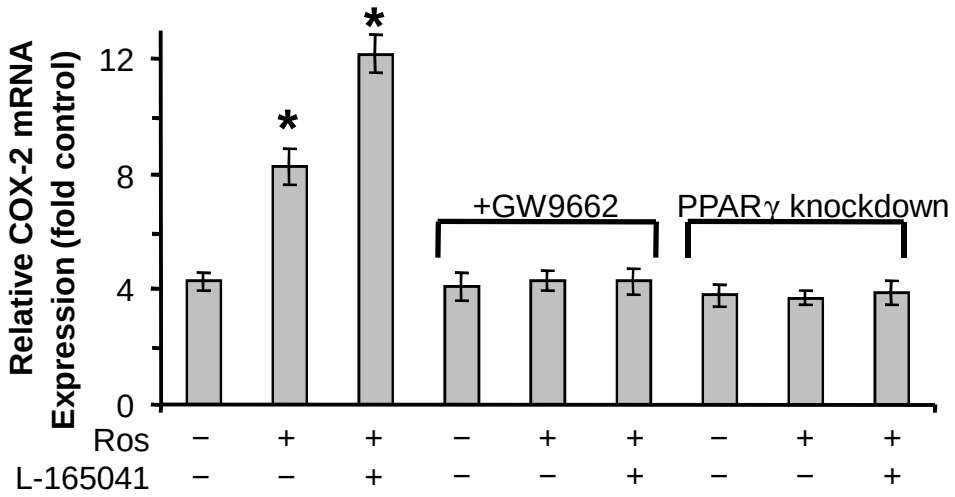

Figure 4

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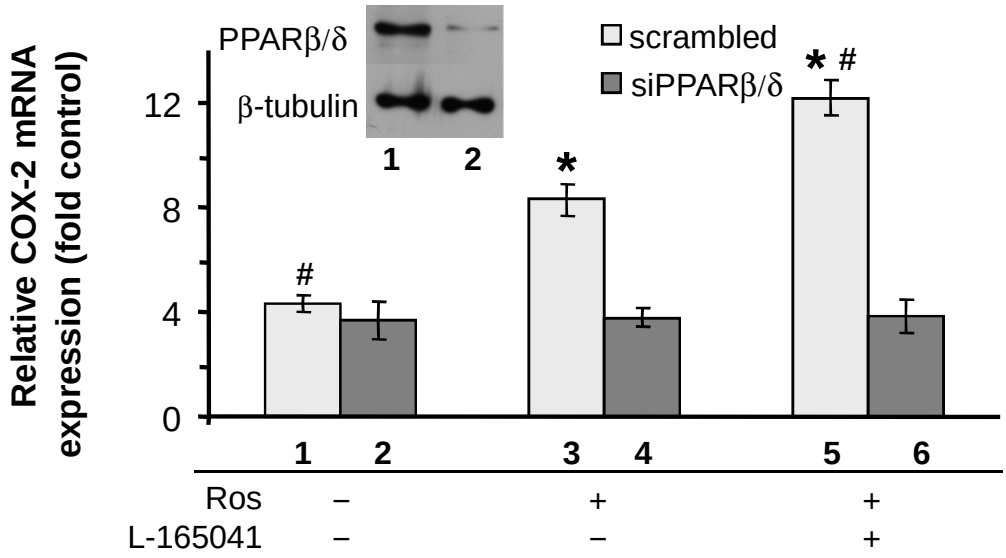

Figure 5

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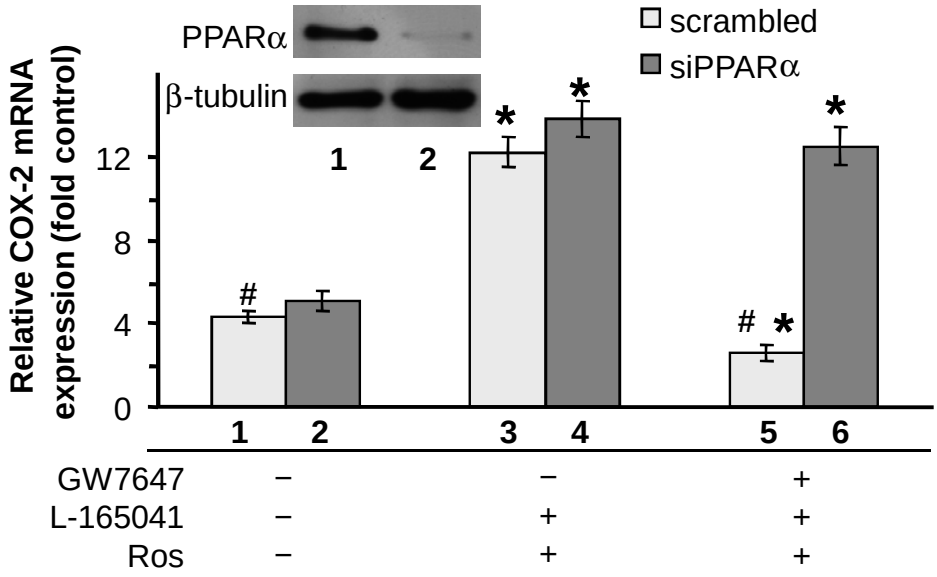

Figure 6

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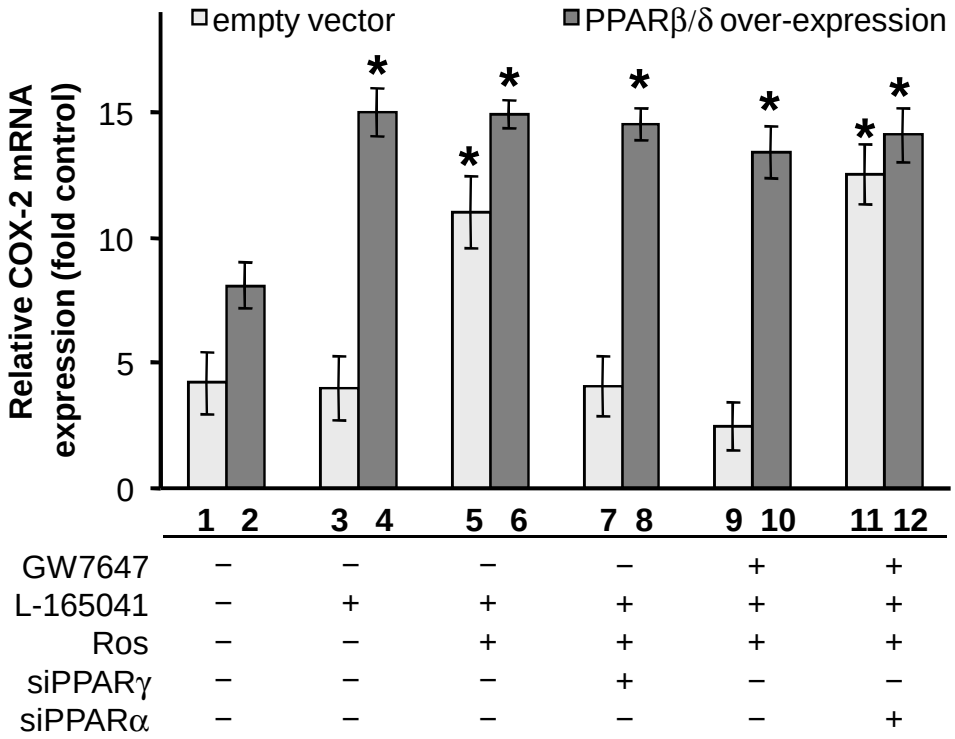

Figure 7

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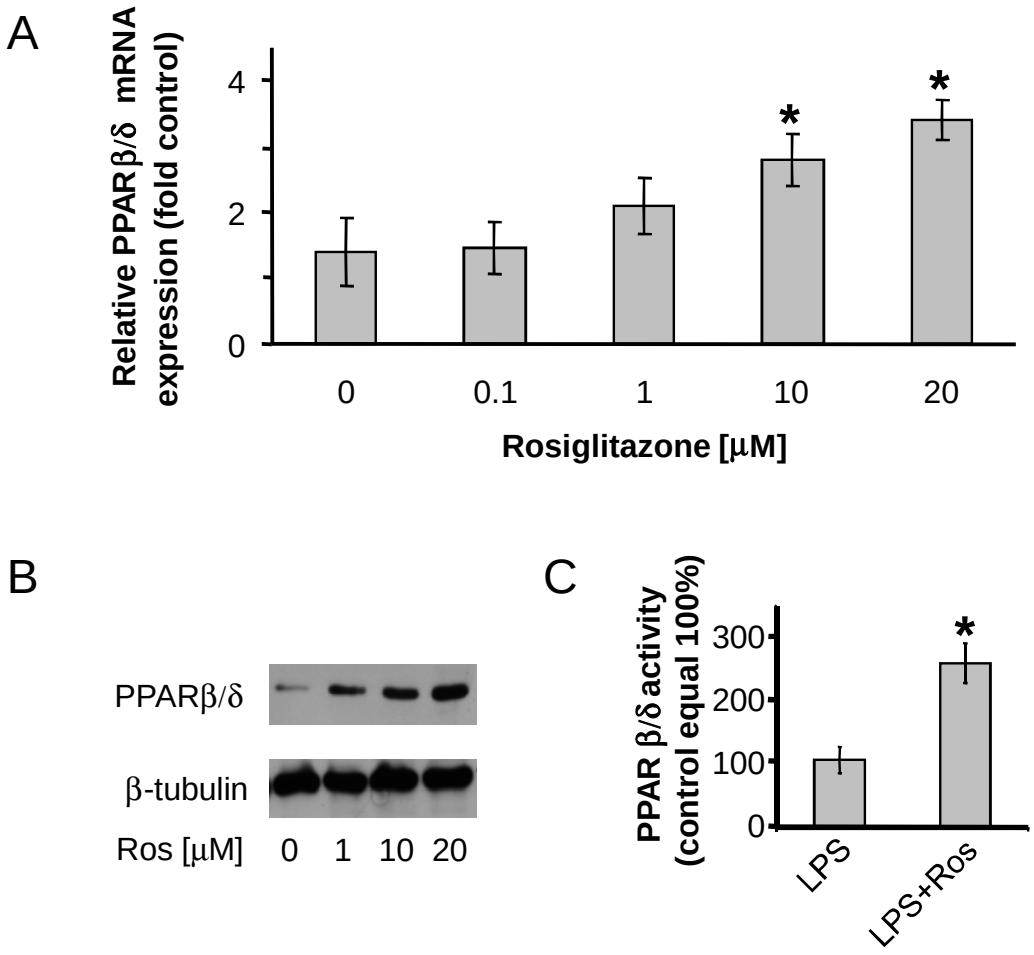

Figure 8

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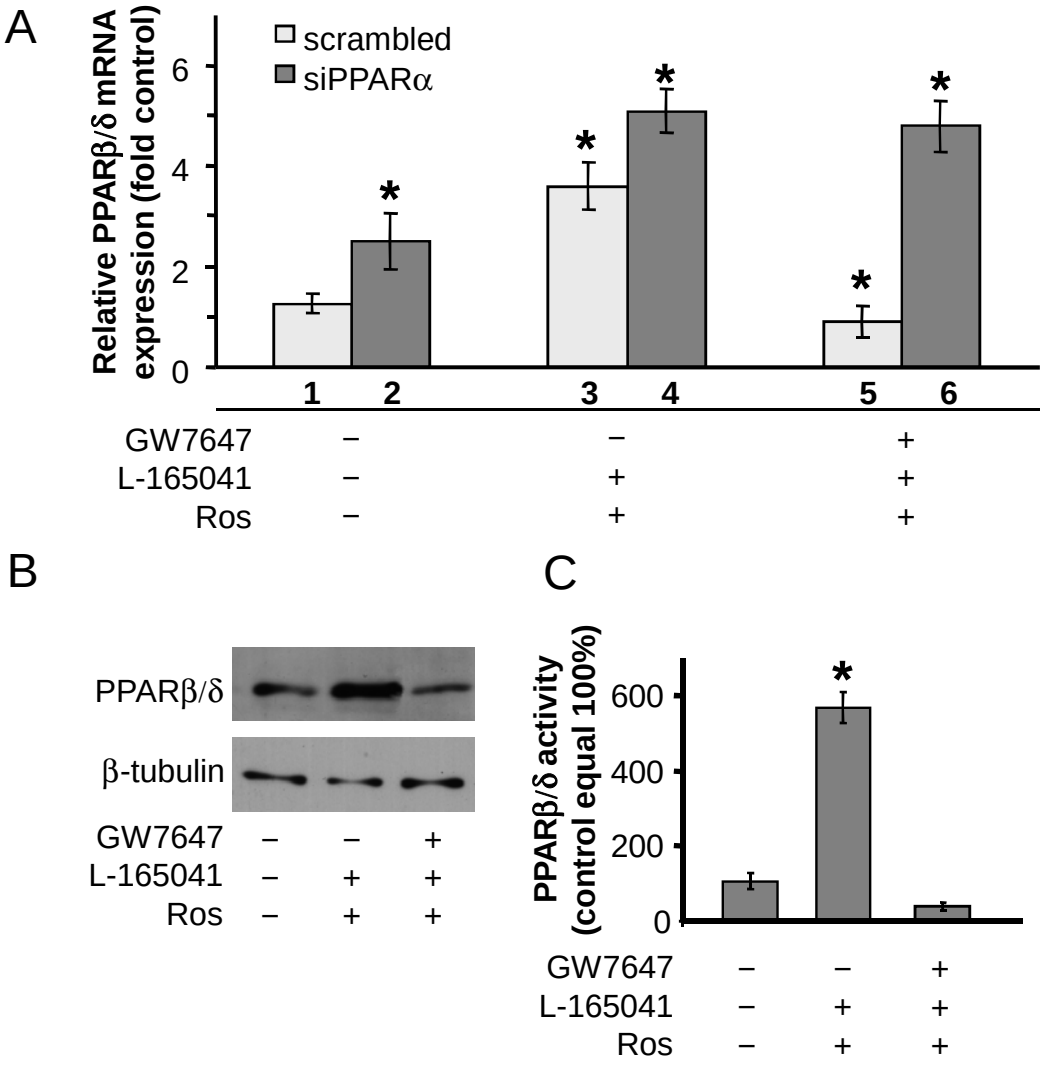

Figure 9

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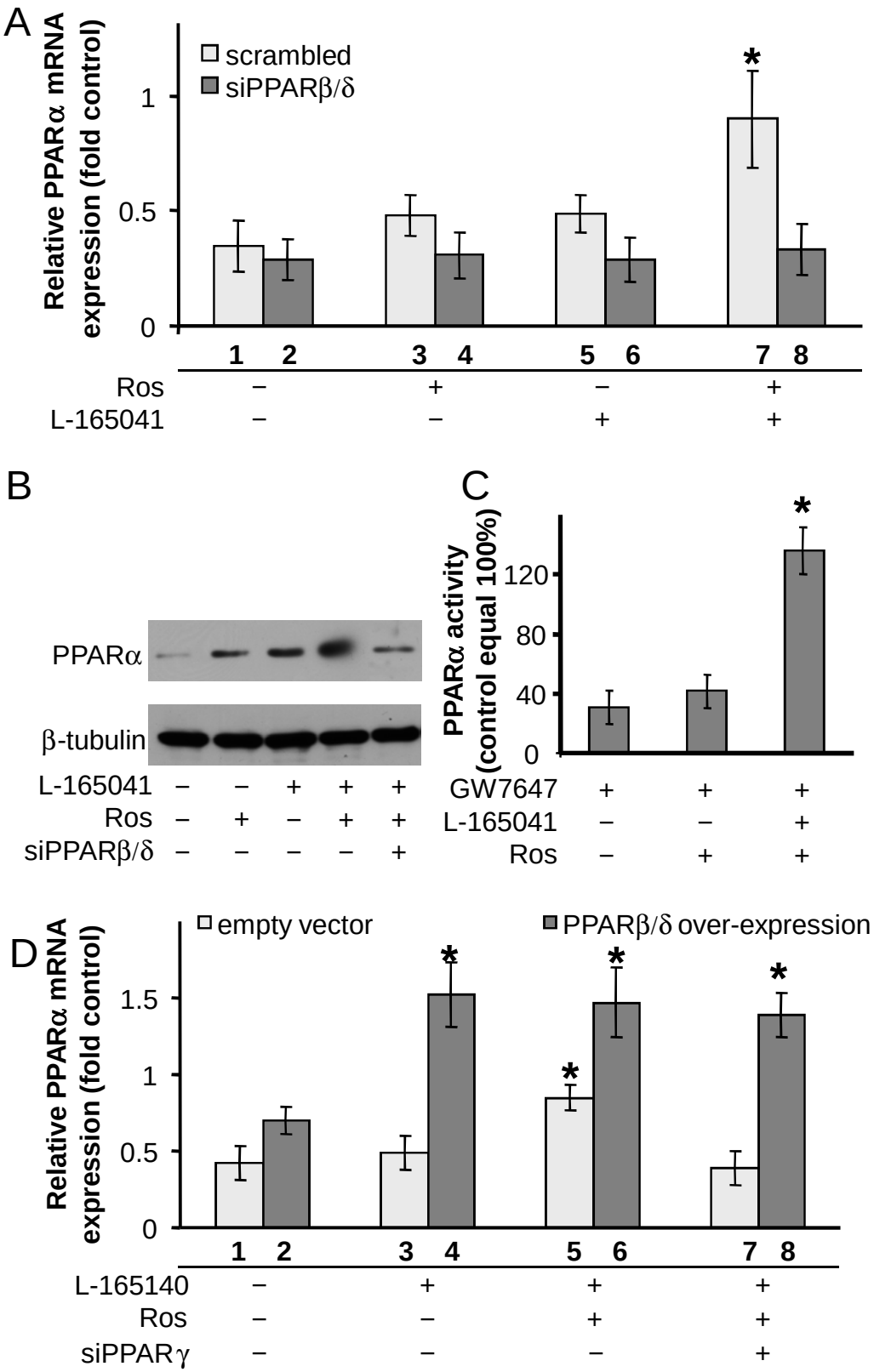

Figure 10

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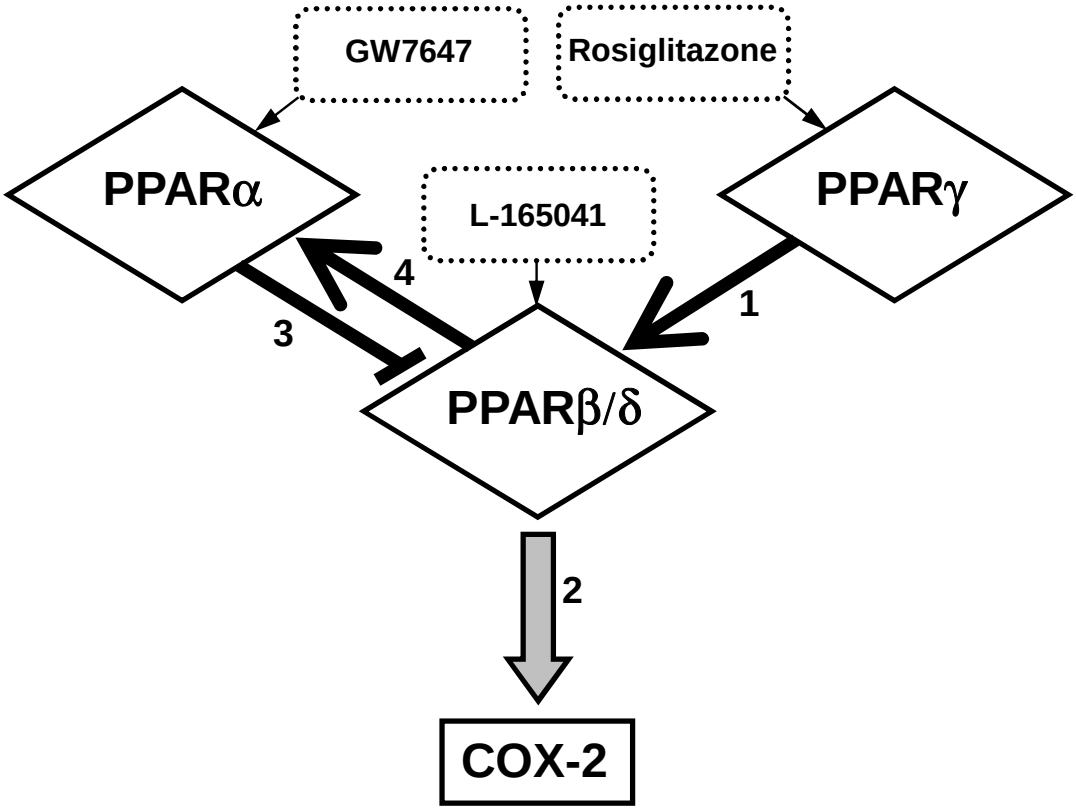

Figure 11