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**PPAR $\gamma$  positively and PPAR $\alpha$  negatively control COX-2 expression in rat brain astrocytes through a convergence on PPAR $\beta/\delta$  via mutual control of PPAR expression levels**

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**Running title:** PPAR $\alpha$ ,  $\beta/\delta$  and  $\gamma$  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 ( $\alpha$ ,  $\beta/\delta$ ,  $\gamma$ ) 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 PPAR $\alpha$  (GW7647), PPAR $\beta/\delta$  (L-165041) and PPAR $\gamma$  (rosiglitazone, ciglitazone) on inflammatory gene regulation in rat primary astrocytes. We measured COX-2 expression and prostaglandin E<sub>2</sub> synthesis in LPS-stimulated cells. PPAR $\alpha$ , PPAR $\beta/\delta$ , PPAR $\gamma$  knockdown models served to delineate the contribution of each PPAR isotype. Thiazolidinediones enhanced the LPS-induced COX-2 expression via PPAR $\gamma$ -dependent pathway, whereas L-165041 and GW7647 had no influence. However, addition of L-165041 potentiated the effect of PPAR $\gamma$  activation, through PPAR $\beta/\delta$ -dependent mechanism. On the contrary, PPAR $\alpha$  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 PPAR $\beta/\delta$  over-expression model confirmed that PPAR $\beta/\delta$  expression level is the point, where PPAR $\gamma$  and PPAR $\alpha$  pathways converge in control of COX-2 gene expression. Thus, we discovered that in primary astrocytes, PPAR $\gamma$  has positive influence and PPAR $\alpha$  has negative influence on PPAR $\beta/\delta$  expression and activity. A positive/negative feedback-loop is formed by PPAR $\beta/\delta$ -dependent increase in PPAR $\alpha$  expression level. These findings elucidate a novel principle of regulation in the signalling by synthetic PPAR agonists that involves modulating the interaction between PPAR $\alpha$ ,  $\beta/\delta$ ,  $\gamma$ -isoforms on the level of their expression.

## 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, PPAR $\alpha$ , PPAR $\beta/\delta$  and PPAR $\gamma$ , 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. PPAR $\alpha$  is the target for the fibrate class of hypolipidemic drugs, and PPAR $\gamma$  for the thiazolidinediones class of insulin-sensitizing drugs (Bensinger and Tontonoz, 2008). Synthetic PPAR $\alpha$  and PPAR $\gamma$  agonists have anti-inflammatory and anti-atherosclerotic activity (Bensinger and Tontonoz, 2008; Heneka and Landreth, 2007; Michalik and Wahli, 2008). PPAR $\beta/\delta$  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 PPAR $\alpha$  and PPAR $\gamma$  activities are in clinical trials (Balakumar et al., 2007). However, such dual agonists with PPAR $\alpha$ /PPAR $\gamma$  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 PPAR $\gamma$ - and PPAR $\alpha$ -mediated activation of target gene expression following PPAR $\beta/\delta$  activation (Shi et al., 2002), and also for PPAR $\beta/\delta$ -dependent PPAR $\gamma$ -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 PPAR $\alpha$  (GW7647), PPAR $\beta/\delta$  (L-165041) and PPAR $\gamma$  (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, PPAR $\gamma$  and PPAR $\alpha$  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 PPAR $\beta$  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 PPAR $\alpha$ , PPAR $\beta/\delta$ , and PPAR $\gamma$ . Here, we used PPAR $\alpha$ , PPAR $\beta/\delta$ , PPAR $\gamma$  knockdown models and application of specific PPAR agonists to characterize the contribution of each PPAR isotype in regulation of COX-2 expression. Additionally, using PPAR $\beta/\delta$  over-expression model, we demonstrated that PPAR $\beta/\delta$  is the point of convergence in the control of COX-2 gene expression. In summary, thiazolidinediones increase COX-2 expression via PPAR $\gamma$ -dependent increase of PPAR $\beta/\delta$  receptors. We found that positive influence of PPAR $\gamma$  and negative influence of PPAR $\alpha$  on PPAR $\beta/\delta$  transcriptional activity occurs via regulation of the expression level of PPAR $\beta/\delta$ . Further, the PPAR $\beta/\delta$ -activation resulted in an increase of PPAR $\alpha$  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 -20° C. Rosiglitazone, Ciglitazone, GW7647 and GW9662 were purchased from Cayman Chemical (Tallinn, Estonia). L-165041 was purchased from Sigma Chemicals (Germany, Taufkirchen). PPAR $\alpha$ , PPAR $\beta/\delta$  and  $\beta$ -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 PPAR $\beta$  from rat (RnPPAR $\beta$ ) 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  $\mu$ g total RNA, 20 pmol oligodT<sub>(12-18)</sub> 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 RnPPAR $\beta$ Start, GATCAAGCTTGGATCCCGATGGAACAGCCACAGGAGGAGACC, and RnPPAR $\beta$ Rev, GATCGATATCTTAGTACATGTCCTTG TAGATCTC, the full length RnPPAR $\beta$  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 RnPPAR $\beta$  was cloned into pcDNA3.1-A-myhis vector (Invitrogen). From the empty vector pcDNA3.1-A-myhis and pcDNA3.1-A-myhisRnPPAR $\beta$  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<sub>2</sub>PO<sub>4</sub>, 0.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 5.5 mM glucose, pH 7.4). The brains were gently passed through nylon meshes of 250  $\mu$ m and 136  $\mu$ m pore width, in consecutive order. Cells were plated onto 75-cm<sup>2</sup> 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 Hank's solution (50 mM NaCl, 5 mM KCl, 0.2 mM KH<sub>2</sub>PO<sub>4</sub>, 0.17 mM Na<sub>2</sub>HPO<sub>4</sub>, 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 PPAR $\beta$  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  $\mu$ g 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<sub>2</sub>. 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<sub>3</sub>VO<sub>4</sub>, 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  $\mu$ l according to the manufacturer's protocol. Real-time PCR was performed on the iCycler (Bio-Rad) in 20  $\mu$ l 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'-CAAATCAAGTGGGGCGATGCT-3', Antisense 5'-  
ACCACCTGGTGCTCAGTGTAGC-3', COX-2 Sense 5'-  
GCAGCTTCCTGATTCAAATGAG-3', Antisense 5'-ATCATCTCTGCCTGAGTATCT-3',  
PPAR $\alpha$  Sense 5'-TGCGGACTACCAGTACTTAG-3', Antisense 5'-  
CGACACTCGATGTTTCAGTGC-3', PPAR $\beta/\delta$  Sense 5'-CTCCTGCTGACTGACAGATG-  
3', Antisense 5'-TCTCCTCCTGTGGCTGTTC-3'.

The sets of primers for the target genes (COX-2, PPAR $\alpha$  and PPAR $\beta/\delta$ ) 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, PPAR $\alpha$ , PPAR $\beta/\delta$ , and PPAR $\gamma$ 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  $\mu$ g 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), PPAR $\alpha$  (1:200), PPAR $\beta/\delta$  (1:200) or PPAR $\gamma$  (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  $\beta$ -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  $\beta$ -mercaptoethanol), and washed five times. Membranes were reprobated with antibody against  $\beta$ -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  $\beta$ -tubulin.

### ***Transfection with small interfering RNA (siRNA)***

siRNAs used in the present study were purchased from Qiagen (Hilden, Germany). PPAR $\alpha$ : cat.# SI01963451 ;PPAR $\beta/\delta$ : cat.# SI01963479 ;PPAR $\gamma$ : 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<sub>2</sub>. 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 PPAR $\alpha$  knockdown was more than 90% on protein and 97.5% on transcription activity level. Efficiency of PPAR $\beta/\delta$  knockdown was more than 90% on protein and 94% on transcription activity level. Efficiency of PPAR $\gamma$  knockdown was more than 90% on protein and 93% on transcription activity level.

### ***Measurement of PGE<sub>2</sub> production.***

For studies of PGE<sub>2</sub> production, astrocytes were grown in 6-well plates. After the experiment the supernatant was collected and stored at –80°C until PGE<sub>2</sub> 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 PPAR $\alpha$  and PPAR $\beta/\delta$  was assayed using an enzyme-linked immunosorbent assay-based PPAR $\alpha$ ,  $\delta$ ,  $\gamma$  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 PPAR $\alpha$ ,  $\delta$ ,  $\gamma$  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 lysates 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 PPAR $\gamma$ , but not PPAR $\alpha$ or PPAR $\beta/\delta$ , potentiate COX-2 expression in LPS-stimulated astrocytes**

Modulation of responses of astrocytes to inflammatory stimuli by agonists of PPAR $\gamma$  and PPAR $\alpha$  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 PPAR $\alpha$  agonist, L-165041 as PPAR $\beta/\delta$  agonist, and the thiazolidinediones rosiglitazone or ciglitazone as PPAR $\gamma$  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  $\mu$ M”). 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  $\mu$ M (Fig. 1). Addition of rosiglitazone (1-20  $\mu$ M) and ciglitazone (1-20  $\mu$ M) up to 2- fold increases the LPS-induced expression of COX-2 mRNA (Fig. 1A). Rosiglitazone at 20  $\mu$ M and Ciglitazone at 40  $\mu$ M (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<sub>2</sub> levels. We obtained that the increase in COX-2 expression (Fig. 1A) correlated with PGE<sub>2</sub> production (Fig. 1B).

### **Rosiglitazone and ciglitazone potentiate COX-2 expression via PPAR $\gamma$ –dependent process**

It is known that PPAR $\gamma$  agonists besides selective activation of PPAR $\gamma$  receptors may act via PPAR $\gamma$ -independent mechanisms (Luna-Medina et al., 2005; Park et al., 2003). To investigate whether a PPAR $\gamma$ -dependent mechanism underlies the rosiglitazone and ciglitazone-induced potentiation of COX-2 expression we used two independent approaches: application of the PPAR $\gamma$  antagonist GW9662 (1  $\mu$ M), and the usage of PPAR $\gamma$  knockdown astrocytes. The results are shown in Fig. 2. Treatment of cells with GW9662 results in the irreversible loss of ligand binding, while PPAR $\gamma$  knockdown results in loss of receptor on protein level. The efficacy of PPAR $\gamma$  knockdown was confirmed by the Western-blot shown in Fig. 2C. The COX-2 expression levels for both the PPAR $\gamma$  antagonist application and

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silencing of PPAR $\gamma$  were similar. Inhibition of PPAR $\gamma$  activity by GW9662 treatment or PPAR $\gamma$  knockdown eliminated the PPAR $\gamma$  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 PPAR $\gamma$ -dependent. COX-2 expression was not changed after inhibition of PPAR $\gamma$  by GW9662 or by PPAR $\gamma$  knockdown in LPS-stimulated astrocytes. Thus, activation of PPAR $\gamma$  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 PPAR $\alpha$  and PPAR $\beta/\delta$  agonists had no additional effect (Fig. 3A, columns 1 and 5). Combination of PPAR $\gamma$  agonists rosiglitazone or ciglitazone together with the PPAR $\alpha$  agonist GW7647 induced no changes in comparison with the effects of the PPAR $\gamma$  agonists themselves (Fig. 3A, compare columns 2 and 3). A remarkable potentiation was obtained by simultaneous addition of PPAR $\gamma$  and PPAR $\beta/\delta$  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 PPAR $\alpha$  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 PPAR $\beta/\delta$  agonist sensitized astrocytes for rosiglitazone treatment (Fig. 3C). Rosiglitazone being added alone, at a concentration of 1  $\mu$ M, had no influence on COX-2 expression. However, in combination with the 1  $\mu$ M of the PPAR $\beta/\delta$  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 PPAR $\gamma$ -dependent modulation of COX-2 gene expression.

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### **L-165041 increases the PPAR $\gamma$ -induced potentiation of COX-2 expression only in the presence of PPAR $\beta/\delta$ and PPAR $\gamma$**

To check the involvement of PPAR $\gamma$  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 PPAR $\gamma$  antagonist GW9662 (1  $\mu$ M) and the PPAR $\gamma$  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 PPAR $\gamma$  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 PPAR $\beta/\delta$  receptors in the COX-2 expression in LPS-stimulated astrocytes we used PPAR $\beta/\delta$  knockdown astrocytes, where we tested the combined application of rosiglitazone and L-165041. The efficacy of PPAR $\beta/\delta$  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 PPAR $\beta/\delta$  knockdown astrocytes, as compared to the control astrocytes transfected with scrambled siRNA (Fig. 5, columns 2 and 1). We observed that knockdown of PPAR $\beta/\delta$  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 PPAR $\beta/\delta$  and PPAR $\gamma$  agonists modulates COX-2 expression via simultaneous involvement of PPAR $\beta/\delta$  and PPAR $\gamma$  receptors. Moreover, PPAR $\beta/\delta$  plays a crucial role in rosiglitazone-dependent increase of COX-2 expression.

### **Role of PPAR $\alpha$ in the GW7647-mediated effect of combined agonist application**

Combined application of PPAR $\beta/\delta$  (L-165041) and PPAR $\gamma$  (rosiglitazone or ciglitazone) agonists increased COX-2 expression, while the additional application of the PPAR $\alpha$  agonist GW7647 abolished this effect, as shown above in Fig. 3A, B. To confirm that the action of GW7647 was PPAR $\alpha$ -dependent, we used PPAR $\alpha$  knockdown astrocytes to check the role of PPAR $\alpha$  receptors in the effect of GW7647 (Fig. 6). The efficacy of the PPAR $\alpha$  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 PPAR $\alpha$  knockdown astrocytes (Fig. 6, columns 2 and 1). The knockdown of PPAR $\alpha$  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 PPAR $\alpha$  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 PPAR $\gamma$  and PPAR $\beta/\delta$  agonists occurs via PPAR $\alpha$ -dependent pathway.

### **Over-expression of PPAR $\beta/\delta$ reveals a key role of this receptor in the control of COX-2 gene expression**

We demonstrated that the PPAR $\beta/\delta$  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 PPAR $\beta/\delta$  has a key function in control of COX-2 gene expression. To test this possibility we constructed astrocytes transfected by PPAR $\beta/\delta$  overexpression vector and investigated the influence of different combinations of PPAR agonists in this cell model (Fig. 7).

We observed that the increase in PPAR $\beta/\delta$  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 PPAR $\beta/\delta$  over-expression, L-165140 induces COX-2 expression without additional stimulation by rosiglitazone (compare column 4 cells with PPAR $\beta/\delta$  over-expression and column 3 wild-type cells). With application of the PPAR $\beta/\delta$  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 PPAR $\alpha$  agonist GW7647 (data not shown) or combined application of 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 PPAR $\beta/\delta$  over-expression were non-sensitive for positive/negative regulation by PPAR $\gamma$  agonists and PPAR $\alpha$  agonists, since the levels of L-165140-stimulated COX-2 expression in astrocytes with PPAR $\beta/\delta$  over-expression were the same in cells with simultaneous PPAR $\beta/\delta$  over-expression and PPAR $\gamma$  knockdown (compare columns 7, 8 and 4, Fig. 7). Also in cells with simultaneous PPAR $\beta/\delta$  over-expression and PPAR $\alpha$  knockdown the PPAR $\alpha$  agonist-elicited inhibitory effect was eliminated (Fig. 7, compare columns 11, 12 with 9, 10 and see column 4). These data prove that PPAR $\beta/\delta$  activation by PPAR $\beta/\delta$  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 PPAR $\beta/\delta$  activation in normal, non-cancer cells.

### **Rosiglitazone induces the expression of PPAR $\beta/\delta$ via PPAR $\gamma$ -dependent pathway**

As the PPAR $\beta/\delta$  agonist L-165041 had no effect on COX-2 expression itself (Fig. 1), while on the other side, overexpression of PPAR $\beta/\delta$  enhanced COX-2 expression even without agonist application (Fig. 7) we supposed that in basal conditions there is not enough PPAR $\beta/\delta$  receptor available in the tested system. From that, we hypothesized that application of rosiglitazone or ciglitazone, via PPAR $\gamma$ -dependent pathway, could increase the PPAR $\beta/\delta$  receptor expression level. This would increase the relative activity of PPAR $\beta/\delta$ . In order to check this concept, we added different concentrations of rosiglitazone to LPS-stimulated astrocytes and measured the level of PPAR $\beta/\delta$  receptors. In the absence of rosiglitazone, LPS did not induce a significant change of the level of the PPAR $\beta/\delta$  receptors (Fig. 8; concentration "0"). When we added the PPAR $\gamma$  agonist rosiglitazone together with LPS, rosiglitazone concentration-dependently increased the PPAR $\beta/\delta$  mRNA. A 3-fold increase was obtained for 20  $\mu$ M 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 PPAR $\beta/\delta$  activity in the presence of rosiglitazone (Fig. 8C).

To distinguish between PPAR $\gamma$ -dependent and possible PPAR $\gamma$ -independent mechanisms of rosiglitazone-induced upregulation of PPAR $\beta/\delta$  expression, we used GW9662, a PPAR $\gamma$  antagonist. Pretreatment with GW9662 (1  $\mu$ M) eliminated the rosiglitazone-induced expression of PPAR $\beta/\delta$ . This was observed both on mRNA level (Fig. 8D) and similarly on protein level (data not shown). Comparable results were obtained in experiments using PPAR $\gamma$  knockdown astrocytes (Fig. 8D). Thus, rosiglitazone concentration-dependently increases PPAR $\beta/\delta$  expression and PPAR $\beta/\delta$  activity in LPS-stimulated astrocytes. This increase is mediated by a PPAR $\gamma$ -dependent mechanism.

### **PPAR $\alpha$ agonist GW7647 negatively regulates the level of PPAR $\beta/\delta$ receptors**

The results demonstrated above support the hypothesis that PPAR $\gamma$  positively controls COX-2 expression in astrocytes via a PPAR $\beta/\delta$ -mediated pathway. Changes of PPAR $\beta/\delta$  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 PPAR $\alpha$ -dependent pathway (Fig. 6). This

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raises the question of what role activation of PPAR $\alpha$  receptors plays in the regulation of PPAR $\beta/\delta$  receptors. We used the PPAR $\alpha$  knockdown model to answer this question, as shown in Fig. 9. Real time RT-PCR analysis revealed that the knockdown of PPAR $\alpha$  receptors leads to 2.5-fold increase in the PPAR $\beta/\delta$  expression in LPS-stimulated cells (Fig. 9; compare columns 2 and 1). This shows that in resting conditions PPAR $\beta/\delta$  expression is under a negative control of PPAR $\alpha$  receptors. The negative control exerted by PPAR $\alpha$ -dependent activation results in an increase of PPAR $\beta/\delta$  expression after the combined application of rosiglitazone and L-165041 in PPAR $\alpha$  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 PPAR $\alpha$  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 PPAR $\beta/\delta$  activity (Fig. 8C), whereas simultaneous addition of rosiglitazone and the PPAR $\beta/\delta$  agonist L-165041 resulted in 5.5-fold increase of PPAR $\beta/\delta$  activity (Fig. 9C). The latter effect was totally abolished in the presence of PPAR $\alpha$  agonist; moreover, under PPAR $\alpha$  agonist stimulation we obtained a 2-fold decrease of PPAR $\beta/\delta$  activity relative to control (Fig. 9C). Thus, we found that activation of PPAR $\alpha$  receptors leads to reduction of PPAR $\beta/\delta$  receptor levels and a subsequent decrease in PPAR $\beta/\delta$  activity.

### **L-165041 increases PPAR $\alpha$ expression and activity in LPS-stimulated astrocytes via PPAR $\beta/\delta$ - dependent mechanism**

We have shown above in Figure 7 that PPAR $\alpha$  negatively controls COX-2 expression in astrocytes through a PPAR $\beta/\delta$ -mediated pathway. However, it remained unclear why GW7647 acted only in combination with rosiglitazone and L-165041, which are PPAR $\gamma$  and PPAR $\beta/\delta$  agonists, respectively. We suggest that there is a finely tuned interplay between PPAR $\alpha$  and PPAR $\beta/\delta$  receptors. While PPAR $\alpha$  negatively controls the expression of PPAR $\beta/\delta$  receptors, PPAR $\beta/\delta$  might positively control PPAR $\alpha$  receptors. Thus, rosiglitazone-induced PPAR $\beta/\delta$  expression level and relative PPAR $\beta/\delta$  activity could increase the amount and activity of PPAR $\alpha$ . To check this hypothesis we measured the level of PPAR $\alpha$  receptors in LPS-treated astrocytes (Fig. 10).

It is interesting to note that LPS being added alone decreased the PPAR $\alpha$  level (Fig. 10A; column 1), while application of the PPAR $\beta/\delta$  agonist L-165041 in combination with



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rosiglitazone increased the PPAR $\alpha$  mRNA up to the normal level in non-treated cells (Fig. 10A, compare columns 1 and 7). That increase correlated with PPAR $\alpha$  protein levels (Fig. 10B). PPAR $\beta/\delta$  knockdown abolished this increase (Fig. 10A; compare columns 8 and 7). These changes in PPAR $\alpha$  mRNA level also correlated with changes in protein level of PPAR $\alpha$  (Fig. 10B). Addition of the PPAR $\alpha$  agonist GW7647 simultaneously with rosiglitazone did not potentiate the activity of PPAR $\alpha$ , but the further addition of L-165041 together with these two compounds notably, that is more than 4-fold, induced the PPAR $\alpha$  activity (Fig. 10C).

Finally, the key role of PPAR $\beta/\delta$  as positive regulator of PPAR $\alpha$  expression was confirmed in cells with PPAR $\beta/\delta$  over-expression (Fig. 10D). Excess PPAR $\beta/\delta$  protein, which was activated by the specific agonist L-165140, leads to a significant increase in PPAR $\alpha$  mRNA expression level (Fig. 10D, columns 3 and 4). This reveals that the PPAR $\beta/\delta$  receptor is sufficient as a positive regulator of the PPAR $\alpha$  expression. Moreover, in astrocytes, transfected by PPAR $\beta/\delta$  over-expression vector, the additional silencing of PPAR $\gamma$  had no effect on the PPAR $\alpha$  expression (Fig. 10D, column 8). The experiments, where it is shown that rosiglitazone is essential for induction of PPAR $\alpha$  expression (Fig. 10A, column 7), taken together with the data with PPAR $\beta/\delta$  over-expression (Fig. 10D) make it obvious that rosiglitazone activates PPAR $\gamma$ , which then induces the expression of PPAR $\beta/\delta$ . That PPAR $\beta/\delta$  in turn, after stimulation by the agonist L-165041, induced the expression of PPAR $\alpha$  (Fig. 10A), and induced PPAR $\alpha$  activity (Fig. 10C).

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## Discussion

The present study provides evidence for a regulatory network between PPARs activated by synthetic agonists through 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 PPAR $\alpha$  or PPAR $\beta/\delta$  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 PPAR $\gamma$  in astrocytes. The detailed analysis of the combined applications of PPAR $\alpha$ , PPAR $\beta/\delta$ , and PPAR $\gamma$  agonists, however, revealed unexpected mechanisms of target gene regulation. In LPS-stimulated rat brain astrocytes, the COX-2 expression is under positive control by PPAR $\gamma$  agonists rosiglitazone or ciglitazone and under negative control by PPAR $\alpha$  agonist GW4756 through the PPAR $\beta/\delta$ -dependent pathway. Our data suggest a model of PPAR-dependent control of COX-2 expression in LPS-stimulated astrocytes, where PPAR $\beta/\delta$  is the point of convergence of PPAR $\gamma$  and PPAR $\alpha$  pathways, as schematically shown in Figure 11. Activation of PPAR $\gamma$  has positive influence, while activation of PPAR $\alpha$  has negative influence on the PPAR $\beta/\delta$  expression level. Thus, regulation of COX-2 expression by application of PPAR $\gamma$  or PPAR $\alpha$  agonists is realized on the level of regulation of PPAR $\beta/\delta$  expression. This mechanism opens new strategies in regulation of astrocyte functions and reveals a new role of PPAR $\beta/\delta$  to gate the activities of PPAR $\alpha$  and PPAR $\gamma$  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 PPAR $\alpha$  and PPAR $\beta/\delta$  ligands, respectively, have much less effect on the two other PPAR isotypes and the PPAR $\gamma$  agonist rosiglitazone has weak activity on PPAR $\beta/\delta$  (Seimandi et al., 2005). For the PPAR $\gamma$ -specific agonist rosiglitazone also PPAR $\gamma$ -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 PPAR $\beta/\delta$ -dependent up-regulation of PPAR $\alpha$  at the level of mRNA transcription, protein and PPAR $\alpha$  activity. That makes astrocytes more sensitive to PPAR $\alpha$  agonists. On the other side, PPAR $\alpha$  agonists are important negative

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control of PPAR $\beta/\delta$  expression, in the case of astrocytes, where the activation of PPAR $\beta/\delta$  has positive influence on PPAR $\alpha$  expression. We further found that activation of PPAR $\gamma$  did not influence the PPAR $\alpha$  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 PPAR $\beta/\delta$  expression resulted in the decrease of activity of PPAR $\alpha$ -dependent enzymes (Shi et al., 2002).

Influence of PPAR $\beta/\delta$  on PPAR $\alpha$ - and PPAR $\gamma$ -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 PPAR $\beta/\delta$  receptor and its interplay with the other PPAR isoforms. The PPAR $\beta/\delta$  is a central switch for regulation of target gene expression levels and serves as a connection of PPAR $\gamma$ - and PPAR $\alpha$ -dependent signals to target genes. The signals from PPAR $\gamma$  and PPAR $\alpha$  are positive or negative, respectively. A cross-road between PPARs was already suggested previously. PPAR $\beta/\delta$  activation by agonists or PPAR $\beta/\delta$  over/expression inhibits PPAR $\gamma$  activity (Shi et al., 2002; Zuo et al., 2006) and PPAR $\alpha$  activity (Shi et al., 2002) in both cancer and normal cells. It was also shown that the synthetic PPAR $\beta/\delta$  agonists increase PPAR $\gamma$ -induced differentiation of adipocytes (Matusue 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 PPAR $\gamma$  or PPAR $\alpha$  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 PPAR $\beta/\delta$  only few data were obtained from studies using cancer cell lines. They show PPAR $\beta/\delta$ -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 PPAR $\beta/\delta$  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 PPAR $\beta/\delta$ , 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 PPAR $\alpha$  for expression of PPAR $\beta/\delta$ , which we saw in normal astrocytes, is lost in cancer cells and, thus, this regulatory circle is disrupted. Our experiments with over-expressing PPAR $\beta/\delta$  cells point to this possibility.

Our data reveal a new role for PPAR $\beta/\delta$  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 PPAR $\alpha$  agonists inhibit the clinical signs of experimental autoimmune encephalomyelitis (Drew et al., 2006). This was correlated with the fact that PPAR $\alpha$  agonists inhibit production of NO, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and MCP-1 in LPS-stimulated primary mouse astrocytes (Pahan et al., 2002; J. Xu et al., 2006). The neuroprotective activity of PPAR $\gamma$  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 synthetic PPAR $\gamma$  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 PPAR $\beta/\delta$  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, PPAR $\beta/\delta$  has been linked to myelinogenesis, glial cell maturation, and neuroprotection (Iwashita et al., 2007; Saluja et al., 2001). PPAR $\alpha$  and PPAR $\gamma$  agonists possess anti-inflammatory activity in brain (Heneka and Landreth, 2007). Activation of all PPAR isoforms, but especially of PPAR $\gamma$ , 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 PPAR $\gamma$  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 PPAR $\beta/\delta$  and PPAR $\gamma$  was demonstrated in our study at the level of COX-2 protein expression in Western blot and its functional consequences by determining the PGE<sub>2</sub> 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 PPAR $\beta/\delta$  expression by PPAR $\gamma$  there is a negative feedback loop between PPAR $\alpha$  and PPAR $\beta/\delta$ . 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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## FOOTNOTES

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## Figure legends

**Figure 1. Modulation of LPS-induced COX-2 expression (A) and PGE<sub>2</sub> production (B) in rat brain astrocytes by synthetic PPAR $\gamma$  agonists.** Cells were pretreated for 10 min with the indicated concentrations ( $\mu$ M) 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<sub>2</sub>. **(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  $\pm$  SEM from three independent experiments. **(B)** PGE<sub>2</sub> production was determined by IEA, as described in Materials and methods. Values represent the mean  $\pm$  SEM from three independent experiments.

**Figure 2. Effect of rosiglitazone and ciglitazone on COX-2 expression in LPS-stimulated astrocytes is PPAR $\gamma$ -dependent.** Cells were either preincubated during 10 min with GW9662 (1  $\mu$ M) or were transfected by PPAR $\gamma$  siRNA. Where indicated, cells were treated for 10 min with 20  $\mu$ M of rosiglitazone (Ros) or 40  $\mu$ M 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  $\pm$  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 PPAR $\gamma$  knockdown verified by Western blot, lane 1-control, and lane 2- PPAR $\gamma$  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 PPAR $\alpha$  agonist GW7647 (1  $\mu$ M), PPAR $\beta/\delta$  agonist L-165041 (1  $\mu$ M), and PPAR $\gamma$  agonists rosiglitazone (Ros; 20  $\mu$ M), or ciglitazone (Cig; 40  $\mu$ M). 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  $\pm$  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 ( $\mu\text{M}$ ) of rosiglitazone in the absence (-) or presence (+) of L-165041 (1  $\mu\text{M}$ ). 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  $\pm$  SEM from three independent experiments.

**Figure 4. PPAR $\beta/\delta$  agonist L-165041 enhances rosiglitazone-induced increase of COX-2 expression in LPS-stimulated astrocytes via PPAR $\gamma$ -dependent pathway.** Cells were preincubated during 10 min with 1  $\mu\text{M}$  of GW9662 or were transfected by PPAR $\gamma$  siRNA. Where indicated, cells were treated for 10 min with 20  $\mu\text{M}$  of rosiglitazone (Ros) in the absence or presence of 1  $\mu\text{M}$  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  $\pm$  SEM from three independent experiments. \* $P < 0.05$ , compared with the LPS-stimulated cells.

**Figure 5. PPAR $\beta/\delta$  agonist L-165041 increases rosiglitazone-induced potentiation of COX-2 expression in LPS-stimulated astrocytes via PPAR $\beta/\delta$ -dependent pathway.** Cells were transfected by PPAR $\beta/\delta$  siRNA or scrambled siRNA. Where indicated, cells were treated for 10 min with 20  $\mu\text{M}$  of rosiglitazone (Ros) in the absence or presence of L-165041 (1  $\mu\text{M}$ ). 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  $\pm$  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 PPAR $\beta/\delta$  knockdown is verified by Western blot, lane 1-control and lane 2- PPAR $\beta/\delta$  knockdown astrocytes.

**Figure 6. GW7647 abolishes combined rosiglitazone / L-165041-induced potentiation of COX-2 expression in LPS-stimulated astrocytes via PPAR $\alpha$ -dependent pathway.** Cells were transfected by PPAR $\alpha$  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  $\mu$ M) plus rosiglitazone (Ros; 20  $\mu$ M). 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  $\pm$  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 PPAR $\alpha$  knockdown is verified by Western blot, lane 1- control and lane 2- PPAR $\alpha$  knockdown astrocytes.

**Figure 7. Over-expression of PPAR $\beta/\delta$  reveals a key role of PPAR $\beta/\delta$  in control of COX-2 gene expression.** Cells were transfected by PPAR $\beta/\delta$  over-expression plasmid or empty vector. After this, cells were transfected by PPAR $\alpha$  or PPAR $\gamma$  or scrambled siRNAs, as indicated. Where additionally indicated, cells were treated for 10 min, in the absence or presence of GW7647 (1  $\mu$ M), with L-165041 (1  $\mu$ M) plus rosiglitazone (Ros; 20  $\mu$ M). 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  $\pm$  SEM from three independent experiments. \*P < 0.05, compared with the LPS-stimulated cells.

**Figure 8. Rosiglitazone induces PPAR $\beta/\delta$  expression and activity in LPS-stimulated astrocytes via PPAR $\gamma$ -dependent pathway.** (A) Cells were pretreated for 10 min with the indicated concentrations ( $\mu$ M) of PPAR $\gamma$  agonist rosiglitazone (Ros). LPS (100 ng/ml) was then added, and after 4 h incubation with both agents, total RNA was isolated. PPAR $\beta/\delta$  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  $\pm$  SEM from three independent experiments. \*P < 0.05, compared with the LPS-stimulated cells. (B) Protein expression for PPAR $\beta$  is evaluated by Western blotting. Example is representative for three independent experiments. (C) Where indicated, cells were incubated for 10 min with 20  $\mu$ M of rosiglitazone. LPS (100 ng/ml) was then added for 4 h. PPAR $\beta/\delta$  transcriptional activity was determined as described in Materials and Methods. Values represent the mean  $\pm$  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  $\mu$ M of GW9662 or were treated with PPAR $\gamma$  siRNA or scrambled siRNA. Where indicated, cells were treated for 10 min with 20  $\mu$ M of rosiglitazone (Ros) or 40  $\mu$ M of ciglitazone (Cig). LPS (100 ng/ml) was then added, and after 4 h incubation, total RNA was isolated. PPAR $\beta/\delta$  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  $\pm$  SEM from three independent experiments. \*P < 0.05, compared with the LPS-stimulated cells (-).

**Figure 9. GW7647 abolishes potentiation of PPAR $\beta/\delta$  expression, induced by combined rosiglitazone plus L-165041 treatment via PPAR $\alpha$ -dependent pathway.** **(A)** Cells were transfected by PPAR $\alpha$  siRNA or scrambled siRNA, as indicated. Where it is indicated cells were treated for 10 min with 1  $\mu$ M of L-165041 plus 20  $\mu$ M of rosiglitazone (Ros) in the absence or presence of 1  $\mu$ M of GW7647. LPS (100 ng/ml) was then added, and after 4 h incubation, total RNA was isolated. PPAR $\beta/\delta$  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  $\pm$  SEM from three independent experiments. \*P < 0.05, compared with the LPS-stimulated cells. **(B, C)** Cells were incubated for 10 min with 1  $\mu$ M of L-165041 plus 20  $\mu$ M of rosiglitazone in the absence or presence of 1  $\mu$ M 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)** PPAR $\beta/\delta$  transcriptional activity was determined, as described in Materials and Methods. Values represent the mean  $\pm$  SEM from three independent experiments. \*P < 0.05, compared with the LPS-stimulated cells.

**Figure 10. PPAR $\beta/\delta$  agonist L-165041 increases expression of PPAR $\alpha$  in LPS-stimulated astrocytes via PPAR $\beta/\delta$ -dependent pathway.** **(A, B)** Cells were transfected by PPAR $\beta/\delta$  siRNA or scrambled siRNA, as indicated. Where it is indicated cells were treated for 10 min with 20  $\mu$ M of rosiglitazone (Ros) in the absence or presence of L-165041 (1  $\mu$ M). LPS (100 ng/ml) was then added for 4 h. **(A)** PPAR $\alpha$  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  $\pm$  SEM from three independent experiments. \*P < 0.05, compared with the LPS-stimulated cells. **(B)** Protein

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expression for PPAR $\alpha$  was evaluated by Western blotting. Example is representative for three independent experiments. (C) Cells were incubated for 10 min with indicated combinations of PPAR $\alpha$  agonist GW7647 (1  $\mu$ M), PPAR $\beta/\delta$  agonist L-165041 (1  $\mu$ M) and PPAR $\gamma$  agonist rosiglitazone (Ros; 20  $\mu$ M). LPS (100 ng/ml) was then added for 4 h. PPAR $\alpha$  transcriptional activity was determined as described in Materials and Methods. Values represent the mean  $\pm$  SEM from three independent experiments. \*P < 0.05, compared with the LPS-stimulated cells. (D) Cells were transfected by PPAR $\beta/\delta$  overexpression plasmid or empty vector. After that, cells were transfected by PPAR $\gamma$  siRNA or scrambled siRNA, as indicated. Where it is indicated cells were treated for 10 min with rosiglitazone (20  $\mu$ M) or L-165041 (1  $\mu$ M). LPS (100 ng/ml) was then added, and after 4 h incubation total RNA was isolated. PPAR $\alpha$  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  $\pm$  SEM from three independent experiments. \*P < 0.05, compared with the LPS-stimulated cells.

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

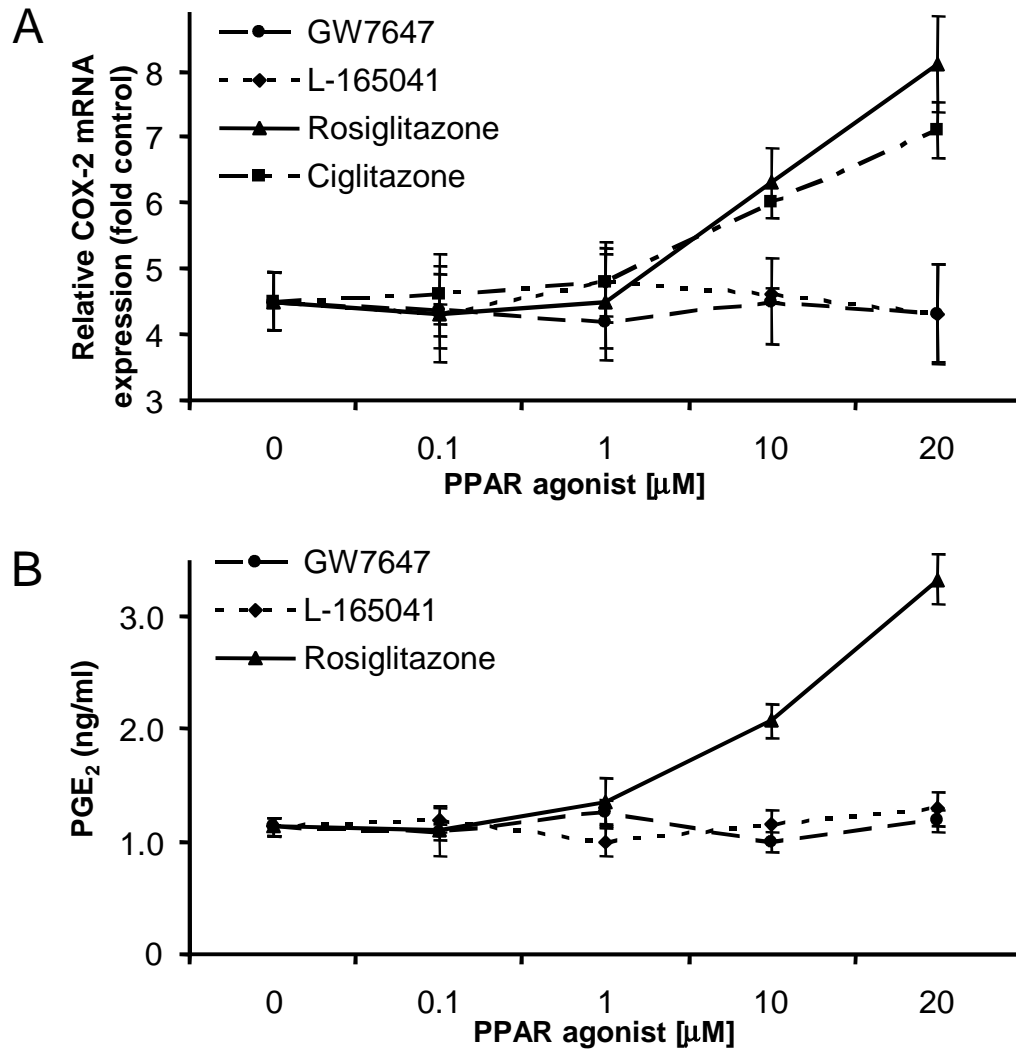


Figure 1



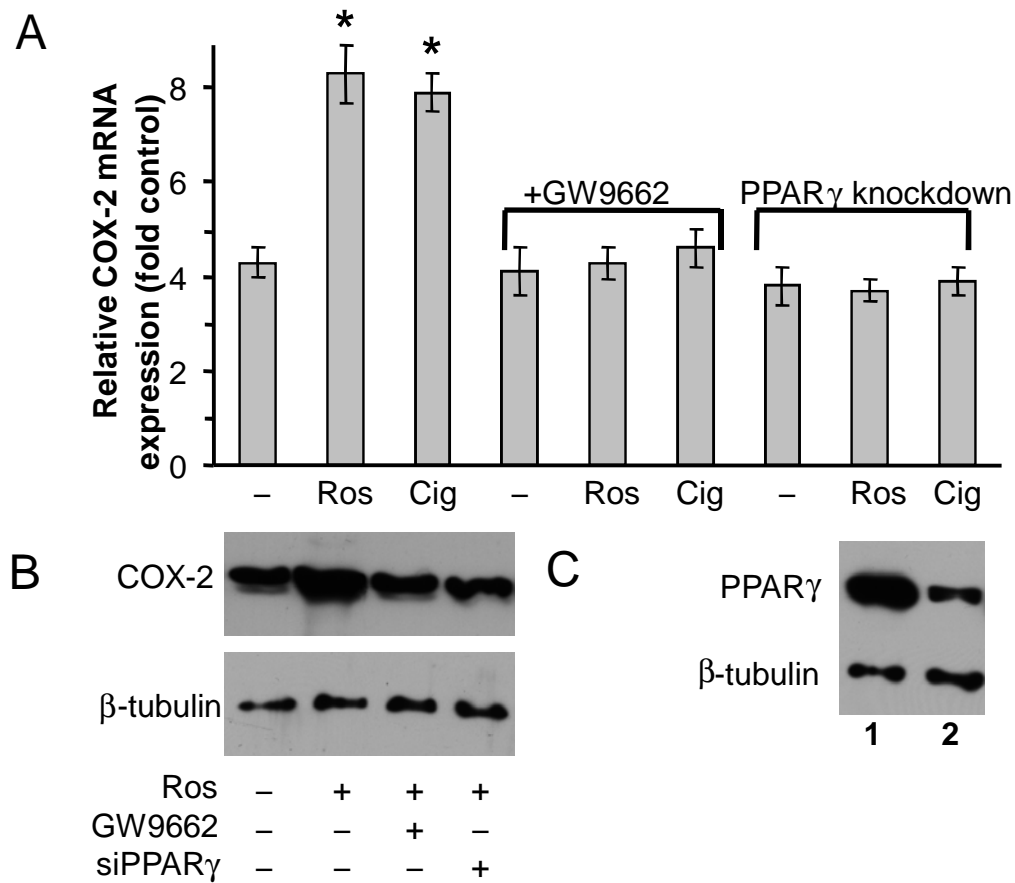


Figure 2

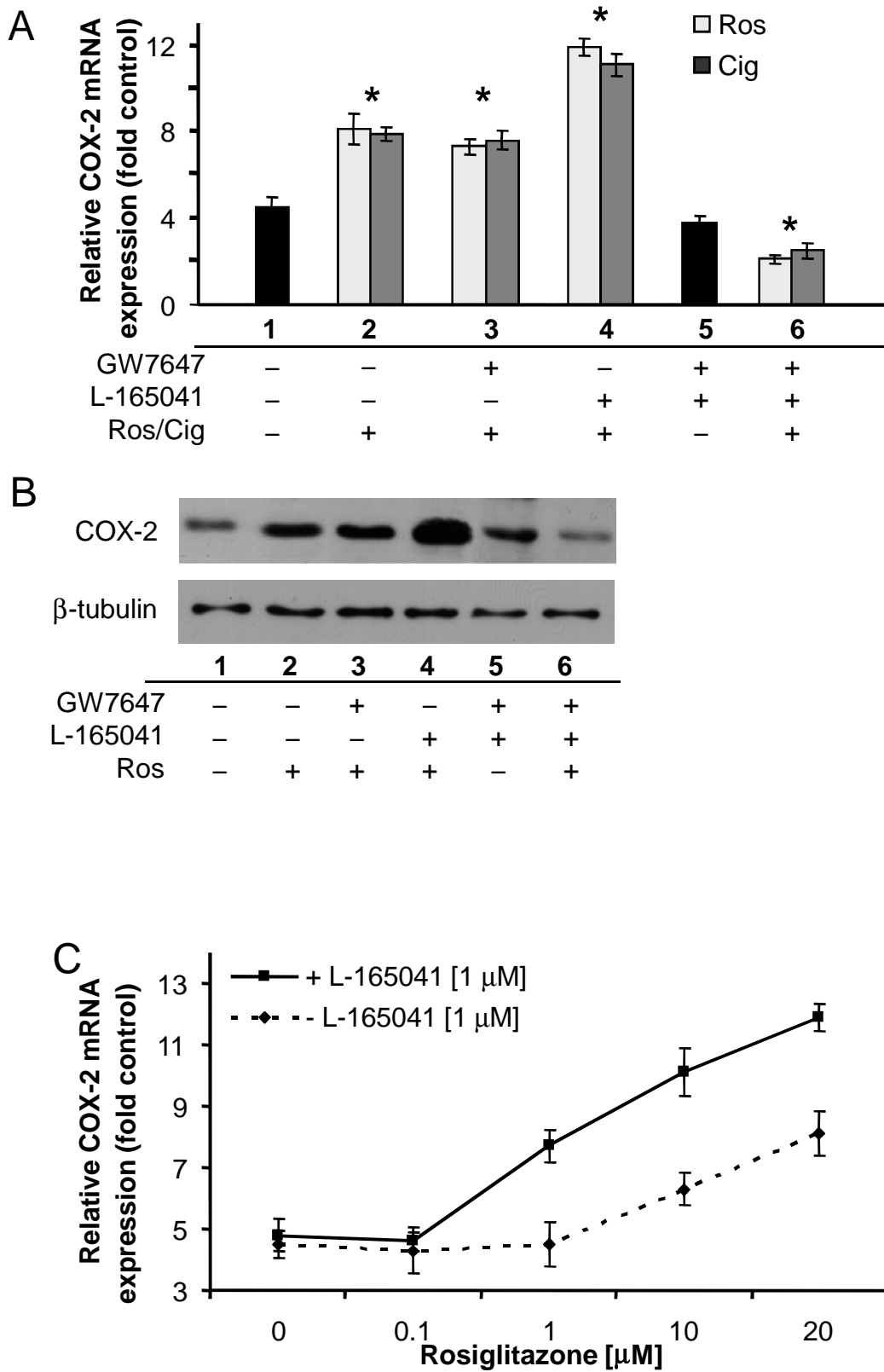


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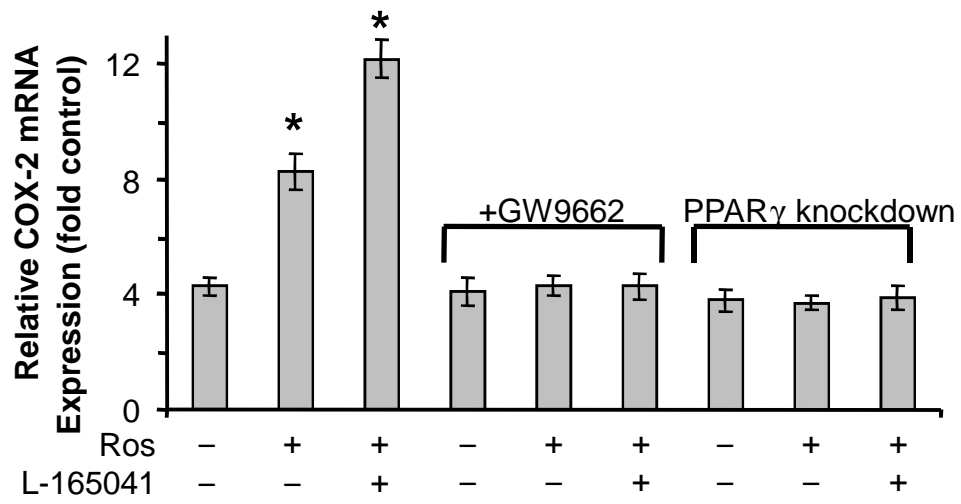


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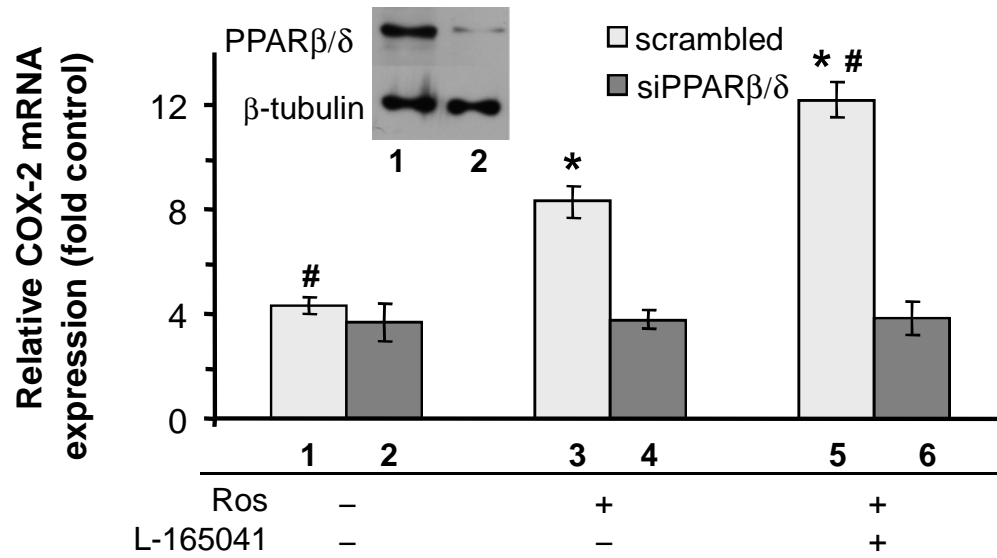


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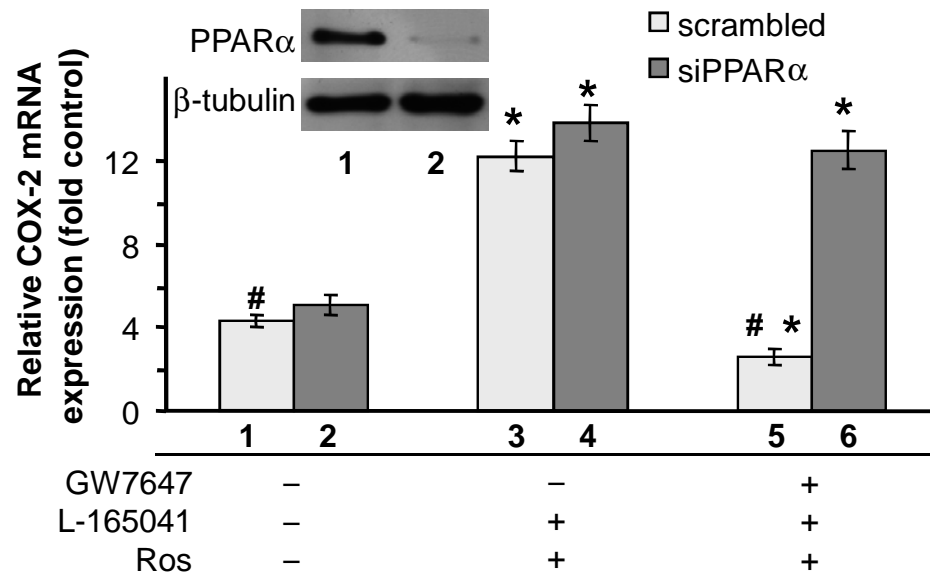


Figure 6

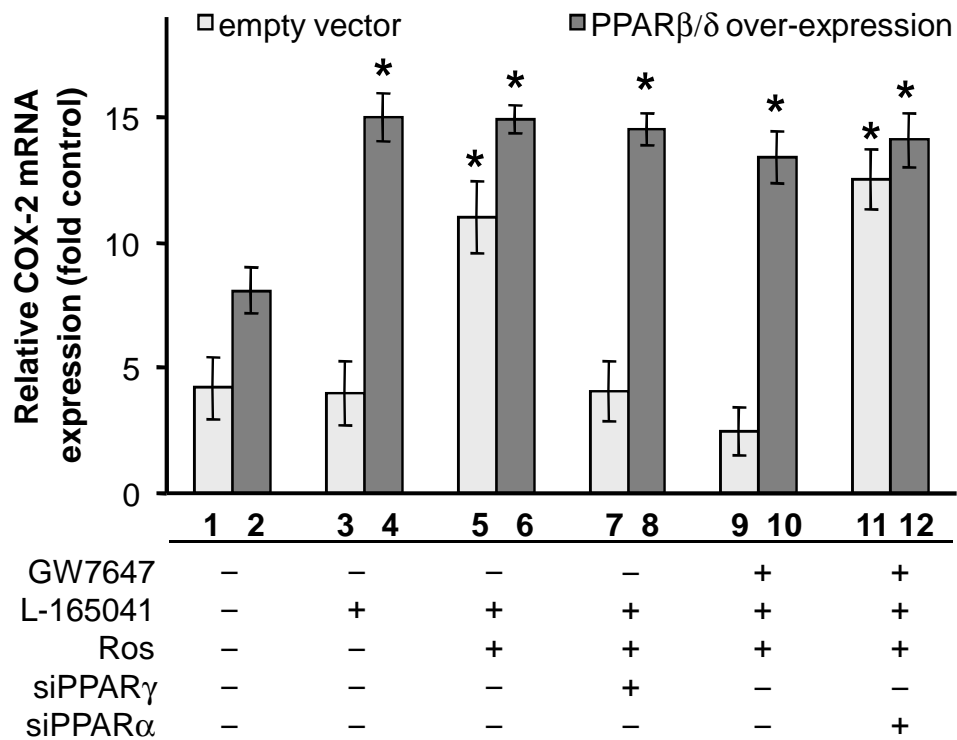


Figure 7

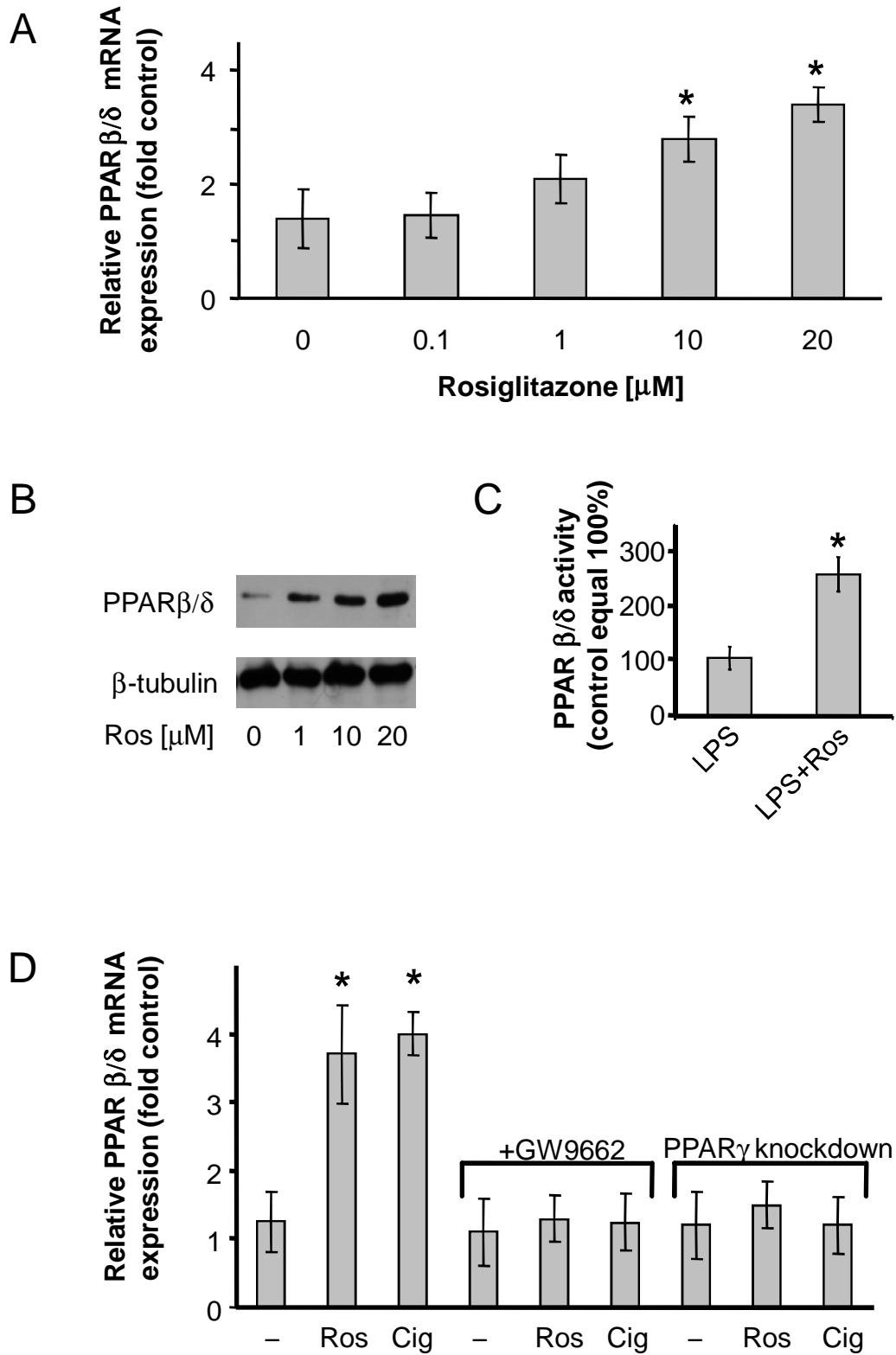


Figure 8

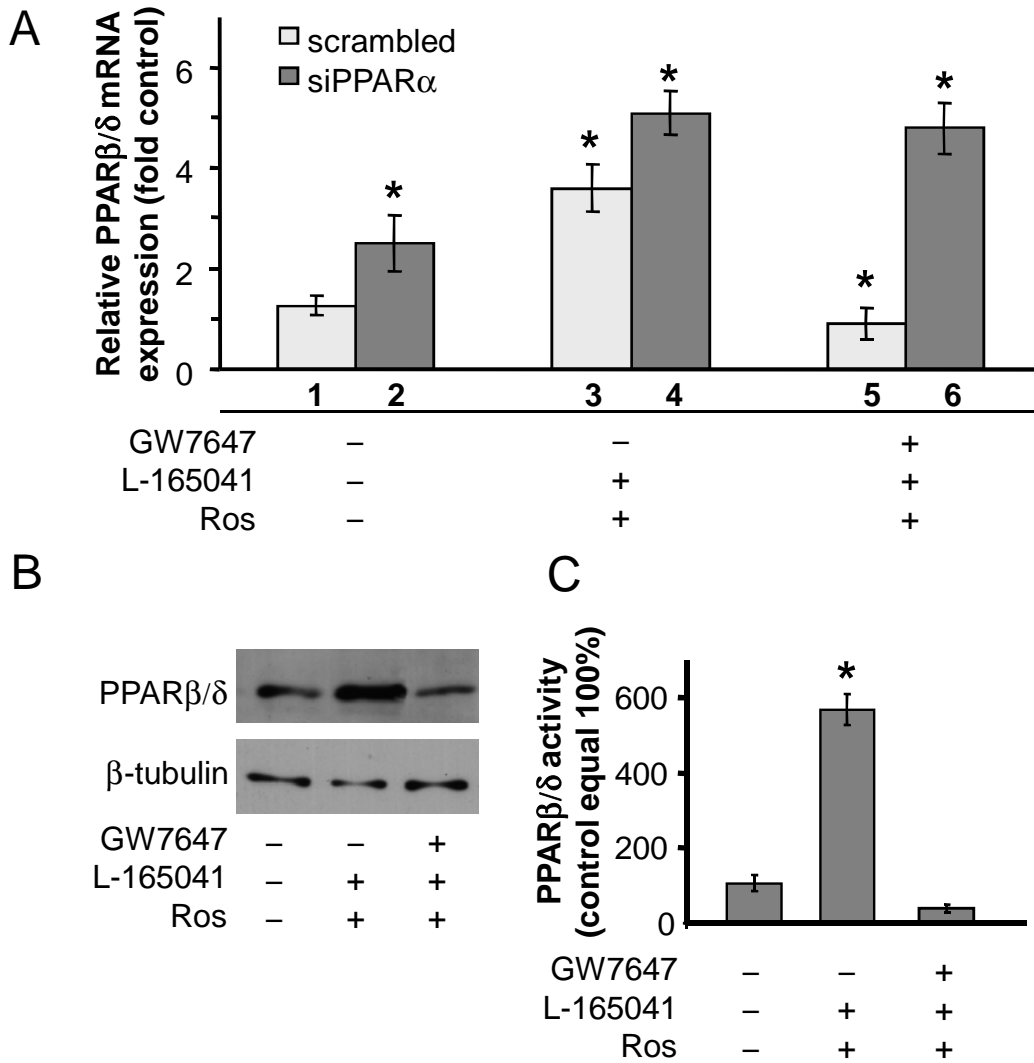


Figure 9



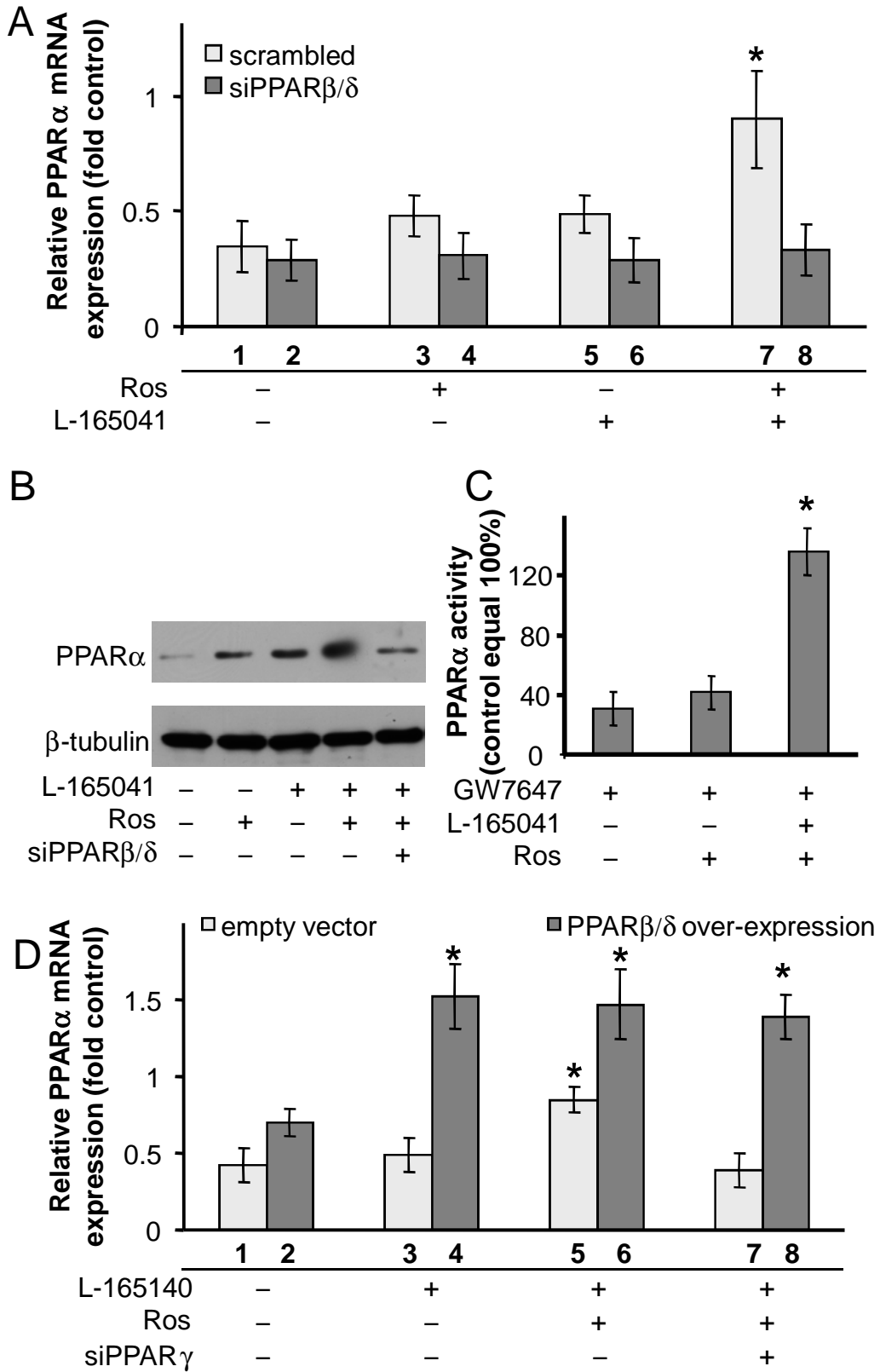


Figure 10

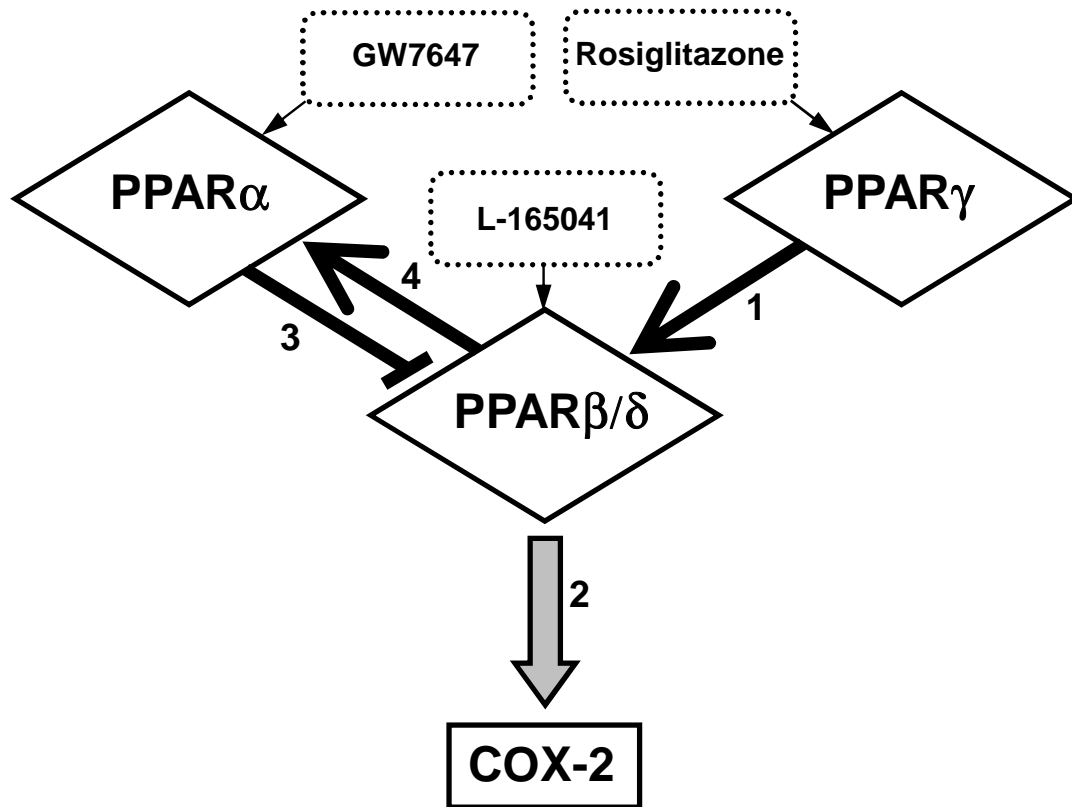


Figure 11