

## **The Interleukin-1 receptor antagonist is a direct target gene of PPARalpha in liver.**

Rinke Stienstra, Stéphane Mandard, Nguan Soon Tan, Walter Wahli, Christian Trautwein, Terrilyn Richardson, Elgin Lichtenauer-Kaligis, Sander Kersten, Michael Müller

### ► **To cite this version:**

Rinke Stienstra, Stéphane Mandard, Nguan Soon Tan, Walter Wahli, Christian Trautwein, et al.. The Interleukin-1 receptor antagonist is a direct target gene of PPARalpha in liver.: PPARalpha controls the expression of the sIL-1ra in liver. *Journal of Hepatology*, Elsevier, 2007, 46 (5), pp.869-77. <10.1016/j.jhep.2006.11.019>. <inserm-00576110>

**HAL Id: inserm-00576110**

**<http://www.hal.inserm.fr/inserm-00576110>**

Submitted on 12 Mar 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

*The Interleukin 1 receptor antagonist is a direct target gene of  
PPAR $\alpha$  in liver*

Rinke Stienstra <sup>1</sup>, Stéphane Mandard <sup>1</sup>, Nguan Soon Tan <sup>2</sup>, Walter Wahli <sup>2</sup>, Christian Trautwein <sup>3</sup>, Terrilyn A. Richardson <sup>4</sup>, Elgin Lichtenauer-Kaligis <sup>1</sup>, Sander Kersten <sup>1</sup>, Michael Müller <sup>1\*</sup>

1. Nutrition, Metabolism and Genomics group, Division of Human Nutrition, Wageningen University, The Netherlands

2. Center for Integrative Genomics, University of Lausanne, Switzerland

3. Department of Medicine III, University Hospital Aachen, Aachen University, Aachen, Germany

4. Department of Pharmacology, Emory University, School of Medicine, Atlanta, GA, USA

Short title: PPAR $\alpha$  controls the expression of the sIL-1ra in liver

\*Corresponding Author: Michael Müller, PhD, Division of Human Nutrition, Wageningen University, PO Box 8129, 6700 EV Wageningen, The Netherlands, Phone: 31-317-482590, Fax: 31-317-483342, E-mail: [michael.muller@wur.nl](mailto:michael.muller@wur.nl)

## **Abstract**

*Background/Aims:* The Peroxisome Proliferator-Activated Receptor (PPAR)  $\alpha$  belongs to the superfamily of Nuclear Receptors and plays an important role in numerous cellular processes, including lipid metabolism. It is known that PPAR $\alpha$  also has an anti-inflammatory effect, which is mainly achieved by down-regulating pro-inflammatory genes. The objective of this study was to further characterize the role of PPAR $\alpha$  in inflammatory gene regulation in liver.

*Results:* According to Affymetrix micro-array analysis, the expression of various inflammatory genes in liver was decreased by treatment of mice with the synthetic PPAR $\alpha$  agonist Wy14643 in a PPAR $\alpha$ -dependent manner. In contrast, expression of Interleukin-1 receptor antagonist (IL-1ra), which was acutely stimulated by LPS treatment, was induced by PPAR $\alpha$ . Up-regulation of IL-1ra by LPS was lower in PPAR $\alpha$   $-/-$  mice compared to Wt mice. Transactivation and chromatin immunoprecipitation studies identified IL-1ra as a direct positive target gene of PPAR $\alpha$  with a functional PPRE present in the promoter. Up-regulation of IL-1ra by PPAR $\alpha$  was conserved in human HepG2 hepatoma cells and the human monocyte/macrophage THP-1 cell line.

*Conclusions:* In addition to down-regulating expression of pro-inflammatory genes, PPAR $\alpha$  suppresses the inflammatory response by direct up-regulation of genes with anti-inflammatory properties.

**Keywords:** PPAR, Liver, Inflammation, Microarray, Interleukin-1 receptor antagonist

## 1. Introduction

Inflammation describes the comprehensive reaction of the host to various types of injury, which is generally protective and aimed at promoting tissue repair. The inflammatory response is mediated by a diverse group of cytokines and other signaling molecules that are able to profoundly influence cellular function. At the cellular level, numerous signaling pathways and transcription factors conspire in a complex network to produce the appropriate response. In recent years it has become clear that the Peroxisome Proliferator Activated Receptors (PPARs) modulate this response in a variety of organs. PPARs are members of the superfamily of Nuclear Receptors that play a pivotal role in mediating the effect of small lipophilic ligands on gene transcription (1). The three isotypes of the PPAR-family, PPAR $\alpha$ , PPAR $\beta/\delta$  and PPAR $\gamma$ , have been implicated in numerous processes, including lipid and glucose metabolism, and inflammation. Activation of the receptor occurs by binding of various ligands, ranging from natural compounds such as fatty acids to highly specific synthetic agonists. Upon ligand-activation, binding to so called PPAR Response Elements (PPRE) located in the promoter of target genes results in increased gene transcription. To accomplish activation of gene transcription it is essential that PPAR forms a heterodimer with the Retinoid X Receptor (RXR) (2).

Besides their ability to enhance gene transcription (3), PPARs are also able to suppress gene expression. For example, it has been shown that activated PPAR $\alpha$  lowers the expression of several enzymes connected with amino acid metabolism (4), although the mechanism behind the observed down-regulation remains unknown. In addition, the effects of PPAR $\alpha$  on inflammation are mainly achieved by suppressing gene expression. Since the initial observation that PPAR $\alpha$ -/- mice have a prolonged inflammatory response (5), an important role for PPARs in regulating inflammatory responses has clearly emerged. Although numerous studies have demonstrated the protective and anti-inflammatory effects of PPAR $\alpha$ -

activation in liver (6) (7), information about the precise molecular mechanisms involved is somewhat limited. One of the mechanisms by which this nuclear receptor exerts its anti-inflammatory action is through modulation of the NF- $\kappa$ B pathway. Physical interaction of PPAR $\alpha$  with NF- $\kappa$ B prevents its activation and downstream pro-inflammatory effects (8). Moreover, PPAR $\alpha$  has been shown to up-regulate the expression of I $\kappa$ B, the natural NF- $\kappa$ B inhibitor that prevents the nuclear translocation and activation of the pro-inflammatory transcription factor (9).

Anti-inflammatory properties have also been assigned to the other two PPAR isotypes. Activation of PPAR $\gamma$  controls the inflammatory status of the intestinal tract (10) and is responsible for the down-regulation of a specific subset of pro-inflammatory genes in macrophages (11). The recent generation of macrophages lacking PPAR $\beta/\delta$  has also revealed a specific role for PPAR $\beta/\delta$  in regulating inflammatory processes (12).

To better understand the regulatory role of PPAR $\alpha$  in liver and to identify possible new target genes under the control of PPAR $\alpha$ , we studied PPAR $\alpha$ -dependent gene expression levels in mouse liver by means of Affymetrix microarray analysis. Activation of PPAR $\alpha$  was achieved by treating Wildtype (Wt) and PPAR $\alpha$   $-/-$  mice with the synthetic PPAR $\alpha$  agonist Wy-14643. While numerous inflammatory genes were found to be down regulated by PPAR $\alpha$  activation, the IL-1 receptor antagonist, however, was highly up-regulated by PPAR $\alpha$ . Additional experiments indicated that the IL-1 receptor antagonist is a direct target gene of PPAR $\alpha$ . Our data suggest that PPAR $\alpha$  may modulate inflammation by direct up-regulation of target genes.

## 2. Material and Methods

### 2.1. Chemicals

Wy-14643 was obtained from ChemSyn Laboratories. Cell culture medium, fetal calf serum and penicillin/streptomycin/fungizone were from BioWhittaker Europe (Cambrex Bioscience). SYBR green was from Eurogentec (Seraing, Belgium).

The human and mouse antibody against IL-1ra and the recombinant hIL-1 $\beta$  were from R&D systems (R&D Systems Europe Ltd, Abingdon, UK). Otherwise, chemicals were from Sigma-Aldrich (Zwijndrecht, The Netherlands).

### 2.2. Animal experiments

Sv129 PPAR $\alpha$ <sup>-/-</sup> mice and corresponding Wt mice were purchased at the Jackson Laboratory (Bar Harbor, Maine, USA). For the fasting experiment, male mice were fasted for 24 hours starting at the onset of the light cycle. For the feeding experiments with Wy-14643 (0.1%), L165041 (0.025%) and Rosiglitazone (0.01%), ligands were mixed in the food and given to female mice for 5 days. Liver was dissected and directly frozen into liquid nitrogen. Lipopolysaccharide (LPS) (*E. coli*; Sigma-Aldrich, St-Louis, MO) was administered at a dose of 4 mg/kg IP. After 3 hours of treatment, liver was dissected and frozen into liquid nitrogen. The animal experiments were approved by the animal experimentation committee of the Wageningen University, the Netherlands and the district government of Lower Saxony, Germany.

### 2.3. Oligonucleotide microarray

Total RNA was isolated from mouse liver using Trizol reagent (Invitrogen, Breda, The Netherlands) following the supplier's protocol. For the microarray experiment, 10  $\mu$ g of total

liver RNA pooled from 5 or 3 mice was used for cRNA synthesis. To confirm integrity of the RNA, bioanalyzer (Agilent) analysis was done before the hybridization process was started. The Affymetrix Mouse Expression array 430A was used and results were analyzed using Microarray Suite and Data Mining Tool (DMT) software following instructions of the manufacturer. Heat Map analysis was done using Spotfire DecisionSite software (Spotfire Inc, Somerville, MA).

#### *2.4. RNA Isolation and RT-PCR*

RNA from animal tissue or cells was extracted with Trizol reagent using the supplier's instructions. After treatment with Dnase I amplification grade (Invitrogen), 4 or 5  $\mu$ g of RNA was used for reverse transcription with Superscript II RT Rnase H (Invitrogen) using oligo (dT) primers following manufacturer's instructions.

#### *2.5. Real Time Quantitative PCR*

PCR was performed with platinum Taq polymerase (Invitrogen) and SYBR green using an iCycler PCR machine (Bio-Rad Laboratories BV, Veenendaal, The Netherlands). The primers were designed using Primer3 software ([http://cbr-rbc.nrc-cnrc.gc.ca/cgi-bin/primer3\\_www.cgi](http://cbr-rbc.nrc-cnrc.gc.ca/cgi-bin/primer3_www.cgi)) and are listed in table I. Only primer pairs yielding unique amplification products were used for real-time PCR analysis. Generated PCR-product sizes were between 90-260 bp. As an internal control, the expression of the housekeeping gene  $\beta$ -actin was measured which remained constant during all of the experimental conditions studied.

## 2.6. Plasmids and DNA constructs

Mouse genomic DNA (mouse strain C57/B6) was used to PCR-amplify 1900 bp of the soluble IL-1ra promoter. The forward primer sIL-1ra-Fprom 5'CCGCTCGAGCGGTGAGCAAATAGAATAGTC 3' and the reverse primer sIL-1ra-Rprom 5' CCCAAGCTTGGGACAGAAGGATGAGAAGGA 3' including restrictions sites for both XhoI and HindIII were used to PCR-amplify 1900 bp of the sIL-1ra promoter. The generated fragment was subcloned into the XhoI and HindIII sites of the pGL-3 basic vector (Promega Corp., Leiden, The Netherlands). Mutations of the PPRE were obtained using two separate partially overlapping PCR fragments generated using the wildtype sIL-1ra promoter as a template. The forward primer sIL-1ra-mutF 5' TTTCTCTAGGGCTGAGGACAGCAAACCTTCT 3' combined with primer sIL-1ra-Rprom and the reverse primer sIL-1ra-mutR 5' AGAAGTTTGCTGTCCTCAGCCCTAGAGAAA 3' combined with primer sIL-1ra-Fprom were used to generate the two partially overlapping PCR fragments. In a final PCR, the two fragments with overlapping ends were used to amplify the mutated sIL-1ra promoter with the forward primer sIL-1ra-Fprom and the reverse primer sIL-1ra-Rprom. The final product was cloned into the XhoI and HindIII sites of the pGL-3 basic vector. cDNA corresponding to mPPAR $\alpha$  was cloned into pSG5 (Stratagene). After cloning, fragments were sequenced to confirm the integrity of the constructs. The RXR $\alpha$  expression plasmid was a generous gift of Dr. S.A. Kliewer.

## 2.7. Primary mouse hepatocyte isolation

Primary mouse and rat hepatocytes were isolated as described previously [13]. Briefly, after cannulation of the portal vein, the liver was perfused with calcium free HBSS which was pre-gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. Next, the liver was perfused with a collagenase solution until



swelling and degradation of the internal liver structure was observed. The hepatocytes were released, filtered and washed several times using Krebs buffer. The viability was assessed by Trypan blue staining and was at least 80%. Cells were cultured in William's Medium E supplemented with 10% FCS, penicillin/ streptomycin/fungizone, insulin and dexamethasone. Cells were plated in collagen (Serva Feinbiochemica, Heidelberg, Germany) coated wells with a density of  $0.5 \times 10^6$  cells/ml. After 4 h of incubation, the medium was removed and replaced with fresh medium. The next day, hepatocytes were used for experiments and treated with IL-1 $\beta$  (5 ng/ml) for 24 h.

### *2.8. Cell culture and transfection*

Human hepatoma HepG2 cells were obtained from the ATCC (Manassas, VA, USA) and grown in DMEM containing 10% FCS and PSF. THP-1 cells were from ATCC and grown in RPMI-1640 containing 10% FCS and PSF. HepG2 cells were transfected using calcium phosphate precipitation. A  $\beta$ -galactosidase reporter was co-transfected to normalize for differences in transfection efficiency. After transfection, cells were treated with the PPAR $\alpha$  ligand Wy14643 at 50  $\mu$ M or vehicle (DMSO) for 24 h prior to lysis. Promega luciferase assay (Promega) and standard  $\beta$ -galactosidase assay with 2-nitrophenyl-BD galactopyranoside were used to measure the relative activity of the promoter. For expression experiments in HepG2 cells, FCS was removed from the medium when ligand or cytokines were added. THP-1 cells were differentiated towards macrophages using phorbol myristic acid (Sigma–Aldrich) at a concentration of 100  $\mu$ M.

### *2.9. Chromatin Immunoprecipitation (ChIP)*

Wt or PPAR $\alpha$   $-/-$  mice were used and fed by gavage with either Wy-14643 (50 mg/kg/day) or vehicle (0.5 % carboxymethyl cellulose) for 5 days or fasted for 24h. After treatment, mice

were sacrificed by cervical dislocation and the liver was perfused with prewarmed (37 °C) phosphate-buffered saline for 5 minutes followed 0.2% collagenase for 10 min. The liver was diced and forced through a stainless steel sieve and the hepatocytes were collected into DMEM containing 1% formaldehyde. After incubation at 37 °C for 15 min, the hepatocytes were pelleted and ChIP was performed using a mouse PPAR $\alpha$ -specific antibody as previously described (14). The sequences of the primers used for PCR were 5'-CAGATGCAGAATTGGGAAAAGATG-3' for the forward primer and 5'-GCAAGCAATAGGGCCTGGTGAAC-3' for the reverse primer. Control primers used were 5'-CTCCCTTCCCCTTCTGTCCCTCTCATT-3' for the forward primer and 5'-TTCCCAAACCTCCCCACCCCATCC-3' for the reverse primer.

#### 2.10. Western Blot

Western blotting was carried out using an ECL system (Amersham Biosciences) according to the manufacturer's instructions. Acetone precipitated protein from equal amounts of medium from HepG2 cells or equal amounts of mouse total liver cell lysates as determined by Bio-Rad Protein Assay reagent (Bio-Rad Laboratories BV) were used and resolved by SDS/PAGE on a 12% polyacrylamide gel. A protein marker (Invitrogen) was used to determine the sizes of the separated proteins. Separated proteins were transferred to Immobilon-P transfer membranes (Millipore). The primary antibody was used at a dilution 1:1000 and the membranes were incubated overnight at 4 °C. The secondary antibody was used at a dilution of 1:5000. All incubations were performed in 1X Tris-buffered saline, pH 7.5, with 0.1 % Tween 20 and 5% dry milk. In the final washings, dry milk was removed from the solution.

#### 2.11. Statistical analysis

The Student's T-test was used to calculate statistical significant differences.

### 3. Results

#### *3.1 PPAR $\alpha$ , but not PPAR $\beta/\delta$ and PPAR $\gamma$ , is involved in the negative regulation of inflammatory related-genes in liver*

To screen for potential novel target genes of PPAR $\alpha$  involved in inflammation in liver we performed micro-array analysis. A comparison was made between liver RNA from Wt and PPAR $\alpha$ <sup>-/-</sup> mice treated or not with the synthetic PPAR $\alpha$  ligand Wy14643. The expression of a large number of genes involved in inflammation was decreased by Wy14643 in Wt but not PPAR $\alpha$ <sup>-/-</sup> mice (Fig. 1A). As shown in the Fig. 1B, the expression of all of these genes was up-regulated during Lipopolysaccharide (LPS)-induced acute inflammation. These included serum amyloid A, orosomucoid, metallothionein, Signal Transducer and Activator of Transcription 3, IL-18, and IL-1RAcP. An exception to this pattern of regulation was IL-1Ra, which in contrast to the numerous other genes induced by LPS, was up-regulated by PPAR $\alpha$  activation. Real-time quantitative PCR (qPCR) confirmed the marked suppression of the selected genes involved in inflammation by Wy14643 in Wt but not PPAR $\alpha$ <sup>-/-</sup> mice (Fig. 2). To evaluate whether the down-regulation of inflammatory genes in liver is specific to PPAR $\alpha$  activation, we studied the effect of synthetic PPAR $\beta/\delta$  and PPAR $\gamma$  agonists. Whereas Wy14643 markedly reduced expression of the selected set of genes, no changes or an opposite response was observed after administration of L165041 and rosiglitazone (Fig. 3).

### *3.2. PPAR $\alpha$ activation enhances gene expression of the anti-inflammatory IL-1 receptor antagonist*

While many genes involved in inflammation were down-regulated by Wy14643, hepatic expression of Interleukin 1 receptor antagonist (IL-1ra) was highly increased by PPAR $\alpha$  activation (Fig. 1). IL-1ra is believed to have mainly anti-inflammatory properties (15). The IL-1ra gene is expressed in multiple forms using two different promoters, resulting in three intracellular forms transcribed via the 5' promoter and one soluble form transcribed via a different promoter (16). To more closely examine the regulation of IL-1ra by PPAR $\alpha$  in liver, specific primers were developed to distinguish between the intracellular (icIL-1ra) and soluble (sIL-1ra) form of IL-1ra. QPCR showed that the soluble form but not the intracellular form is regulated by Wy14643 and fasting in mouse liver in a PPAR $\alpha$ -dependent manner (Fig. 4A,B). It should be mentioned that icIL-1ra mRNA was difficult to detect compared to sIL-1ra and to our knowledge no function has been assigned to icIL-1ra in liver. Further analysis showed that only the PPAR $\alpha$  isotype is involved in the upregulation of sIL-1ra expression in liver (Fig. 4C). Protein levels of IL-1ra analyzed in liver cell lysates matched the gene expression pattern (Fig. 5).

### *3.3 PPAR $\alpha$ is essential to induce expression of sIL-1ra during inflammatory conditions in liver*

To investigate whether PPAR $\alpha$  is involved in the regulation of sIL-1ra during inflammatory conditions, Wt and PPAR $\alpha$   $-/-$  mice were exposed to LPS. As expected, LPS treatment increased sIL-1ra gene expression, however the increase was significantly higher in Wt mice compared to PPAR $\alpha$   $-/-$  mice (Fig. 6A). Induction of IL-1 $\beta$  expression was similar between Wt and PPAR $\alpha$   $-/-$  mice after exposure to LPS (data not shown). Subsequently, the ratio of IL-1ra to IL-1 $\beta$ , which is often used to determine the activation of IL-1 signaling pathways,

was drastically improved in Wt mice compared to PPAR $\alpha$  mice after exposure to LPS, suggesting a shift towards a more anti inflammatory phenotype (Fig. 6A). Treatment of Wt and PPAR $\alpha$   $-/-$  primary mouse hepatocytes with IL-1 $\beta$  to induce sIL-1ra gene expression gave comparable results, as shown by a more pronounced increase in sIL-1ra gene expression in hepatocytes expressing PPAR $\alpha$  compared to PPAR $\alpha$   $-/-$  hepatocytes (Fig. 6B).

#### 3.4. sIL-1ra is a true target gene of PPAR $\alpha$ in mouse liver

To determine whether the sIL-1ra promoter is directly regulated by PPAR $\alpha$ , 1.9 kb of promoter region of the soluble isoform was cloned into a luciferase reporter vector and used in transactivation assays. Co-transfection with PPAR $\alpha$  and RXR $\alpha$  expression plasmids revealed that while RXR $\alpha$  alone did not have any effect on the promoter activity (data not shown), transfection of both PPAR $\alpha$  and RXR $\alpha$  expression plasmids increased the activity of the sIL-1ra promoter (Fig. 6A). This effect was further enhanced by treatment of cells with Wy14643. *In silico* analysis of the sIL-1ra promoter using Nubiscan V2.0 software identified a possible PPRE located at around 700 bp upstream of the transcription start site. To investigate whether this PPRE may be responsible for PPAR $\alpha$ -mediated transactivation of the sIL-1ra gene, the response element was disabled by site-directed mutagenesis (Fig. 6B). After mutating the PPRE, the response to PPAR $\alpha$  and Wy14643 was completely abolished, suggesting that the effects of PPAR $\alpha$  are mediated by the PPRE identified (Fig. 6C).

Finally, to investigate if PPAR $\alpha$  is bound to the PPRE identified in mouse liver, *in vivo* chromatin immunoprecipitation (ChIP) was performed using an anti-PPAR $\alpha$  antibody. In mice, treatment with Wy-14643 and fasting increased the binding of PPAR $\alpha$  to this PPRE in Wt but not PPAR $\alpha$   $-/-$  mice. No binding was observed to a control sequence located 1800

base pairs upstream from the PPRE (Fig. 6D). Taken together, these data indicate that the PPRE identified mediates the effects of PPAR $\alpha$  on sIL-1ra gene expression.

### *3.5. sIL-1ra is also regulated by PPAR $\alpha$ in human cells*

To evaluate whether the effects seen in mouse liver can also be reproduced in human liver cells, HepG2 cells were used. HepG2 cells only express the soluble form of the IL-1ra gene (17) and treatment of the cells with IL-1 $\beta$ , an inducer of sIL-1ra expression, resulted in the expected increase in gene expression levels (Fig. 7A). Incubation with RXR $\alpha$  and PPAR $\alpha$  agonists alone did not result in major changes in gene transcription. However, combined treatment with both IL-1 $\beta$  and PPAR $\alpha$ /RXR agonists did synergistically increase sIL-1ra expression. Since soluble IL-1ra is secreted, medium from treated HepG2 cells was analyzed for IL-1ra protein concentration. The protein levels followed the sIL-1ra mRNA induction in these cells (Fig 7B). The upper band represents the glycosylated form of the sIL-1ra protein and the lower band the unglycosylated protein (18). Next, the expression of the sIL-1ra was examined in the human derived monocyte/macrophage THP-1 cell line (Fig 7C). Since it is known that PPAR $\alpha$  localization shifts from hepatocytes towards Kupffer cells during hepatic inflammation (19), it was of interest to test the ability of PPAR $\alpha$  to regulate the expression of sIL-1ra in this cell type. The expression of sIL-1ra was increased by differentiation of the cells towards macrophages using phorbol myristic acid (PMA). In contrast to the expression levels in monocytes, the expression level of sIL-1ra in macrophages was increased by Wy14643. These data indicate that sIL-1ra is a target of PPAR $\alpha$  in human hepatocytes and macrophages.

#### 4. Discussion

Since the initial discovery of a possible role for PPAR $\alpha$  in controlling the inflammatory response (5), considerable progress has been made towards resolving the function of PPAR $\alpha$  during inflammation (8, 9, 20). It is now clear that PPAR $\alpha$  mainly governs inflammation by down-regulating expression of genes, including several acute phase genes.

Here, we show that soluble Interleukin 1 Receptor antagonist is a direct target gene of PPAR $\alpha$  with a functional PPRE in its promoter, suggesting that PPAR $\alpha$  may also govern inflammation by direct up-regulation of target genes. IL-1ra is an inhibitor of cytokine signaling and is produced by many cells including hepatocytes. During the acute phase response, the expression of IL-1ra is strongly induced and IL-1ra is therefore referred to as a positive acute phase protein. In our hands, hepatic sIL-1ra expression was highly up-regulated after LPS treatment. By binding to the IL-1 receptor with almost equal affinity as IL-1, it prevents activation of the pro-inflammatory IL-1 pathway and downstream NF- $\kappa$ B activation (21). Under non-inflammatory conditions, PPAR $\alpha$  activation by Wy14643 or fasting markedly increased sIL-1ra gene expression in liver, which was not observed in PPAR $\alpha$  *-/-* mice. Thus, PPAR $\alpha$  seems to be able to increase the expression of sIL-1ra in liver independently of any inflammatory stimulus. Transactivation and chromatin immunoprecipitation experiments indicated that PPAR $\alpha$  regulates sIL-1ra expression via a PPRE located around 700 bp upstream of the transcription start site.

In human HepG2 cells PPAR $\alpha$  activation was able to increase sIL-1ra gene expression when cells were co-treated with IL-1 $\beta$  which is known to induce expression of sIL-1ra by NF- $\kappa$ B and C/EBP binding to the sIL-1ra promoter (22). In THP-1 cells differentiated towards macrophages, PPAR $\alpha$  was also capable of inducing sIL-1ra expression. Since macrophage/monocyte type cells including Kupffer cells have an important role in controlling

hepatic inflammation (23), the PPAR $\alpha$ -dependent regulation of the IL-1ra gene in these cells might contribute to the immune-modulating functions of PPAR $\alpha$  in liver. Together, these data indicate that sIL-1ra is a direct target gene in mouse and human hepatocytes, as well as in human macrophages. By inhibiting binding of IL-1 $\beta$  to its receptor via increased expression of its natural antagonist, PPAR $\alpha$  might prevent or counteract the activation of the IL-1 $\beta$ -signalling cascade including the pro-inflammatory NF- $\kappa$ B pathway.

The availability of microarray techniques offers the opportunity to screen for new PPAR $\alpha$  controlled genes and pathways with the aim of elucidating the effects of PPAR $\alpha$  on inflammation. Using this approach, our initially analysis focused on inflammation-related genes that are regulated by PPAR $\alpha$  activation in liver under normal conditions. Many of the genes that were decreased in liver of mice treated with Wy14643 were increased during acute inflammation mimicked by LPS administration. Several acute phase response genes, cytokine receptor components, and intracellular signaling transducers were affected. The results for several of these genes corresponds well with earlier studies (24, 25), whereas for other genes the linkage to PPAR $\alpha$  is entirely novel.

IL-18, a pro-inflammatory cytokine with an established role in liver injury (26), is one of the genes that was decreased after PPAR $\alpha$  activation. Furthermore, the LIF-receptor, which belongs to the IL-6 receptor family, was strongly decreased after PPAR $\alpha$ -agonist treatment in mouse liver. Downstream of this pathway, PPAR $\alpha$  is also able to suppress the expression of STAT3, a transcription factor implicated in the regulation of inflammatory signaling in liver (27). Finally, PPAR $\alpha$  seems to have a role in regulating IFN $\gamma$ -activated genes which is illustrated by the suppression of IFN $\gamma$ -inducible genes by PPAR $\alpha$ .



Our analysis clearly demonstrates an important immunosuppressive effect by PPAR $\alpha$  in liver. One limitation of the present study is that we did not address the effect of PPAR $\alpha$  activation during LPS-induced inflammation. However, a previous study showed that when the expression of specific components of the inflammatory signaling cascade was suppressed by PPAR $\alpha$  agonists prior to IL-6 treatment, the subsequent acute phase responses in liver were diminished (24). Although future experiments should aim at combining both PPAR $\alpha$  activation and inflammation in liver to appraise the importance of PPAR $\alpha$  in regulating inflammation, our data reveal several candidate genes by which PPAR $\alpha$  might exert its potent anti-inflammatory effects in liver.

In conclusion, by negatively regulating multiple components of inflammatory signalling pathways ranging from cytokines to receptor complexes and downstream target genes, PPAR $\alpha$  activation may render the liver less susceptible to the effects of inflammation. Up-regulation of soluble IL-1ra by PPAR $\alpha$  may contribute to its immune-modulating effects.

## **Acknowledgements**

We thank Jolanda van der Meijde for performing the microarray experiment and René Bakker for assisting with the animal experiments. We thank Dr. Mark Leibowitz (Ligand Pharmaceuticals) for the kind gift of Lg100268. This study was supported by the Centre for Human Nutrigenomics, with additional support by the Netherlands Organization for Scientific Research (NWO), the Wageningen Center for Food Sciences, the Dutch Nutrigenomics Consortium, the Swiss National Science Foundation and Etat de Vaud and the National Institutes of Health grant GM46897.

Abbreviations used: PPAR, Peroxisome Proliferator-Activated Receptor; LPS, Lipopolysaccharide; IL-1ra, Interleukin-1 receptor antagonist; PPRE, PPAR Response Elements; RXR, Retinoid X Receptor; Wt, Wildtype; SAA, Serum Amyloid A; STAT3, Signal Transducer and Activator of Transcription 3; IL-1RacP, Interleukin-1 Receptor accessory Protein; LIFR, Leukaemia Inhibitory Factor Receptor; IL-6R, Interleukin 6 Receptor; PMA, Phorbol Myristic Acid; IFN, Interferon.

## References

1. Robinson-Rechavi M, Escriva Garcia H, Laudet V. The nuclear receptor superfamily. *J Cell Sci* 2003;116:585-586.
2. Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev* 1999;20:649-688.
3. Mandard S, Muller M, Kersten S. Peroxisome proliferator-activated receptor alpha target genes. *Cell Mol Life Sci* 2004;61:393-416.
4. Kersten S, Mandard S, Escher P, Gonzalez FJ, Tafuri S, Desvergne B, Wahli W. The peroxisome proliferator-activated receptor alpha regulates amino acid metabolism. *Faseb J* 2001;15:1971-1978.
5. Devchand PR, Keller H, Peters JM, Vazquez M, Gonzalez FJ, Wahli W. The PPARalpha-leukotriene B4 pathway to inflammation control. *Nature* 1996;384:39-43.
6. Delerive P, Fruchart JC, Staels B. Peroxisome proliferator-activated receptors in inflammation control. *J Endocrinol* 2001;169:453-459.
7. Lo Verme J, Fu J, Astarita G, La Rana G, Russo R, Calignano A, Piomelli D. The nuclear receptor peroxisome proliferator-activated receptor-alpha mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol Pharmacol* 2005;67:15-19.
8. Delerive P, De Bosscher K, Besnard S, Vanden Berghe W, Peters JM, Gonzalez FJ, Fruchart JC, et al. Peroxisome proliferator-activated receptor alpha negatively regulates the

vascular inflammatory gene response by negative cross-talk with transcription factors NF-kappaB and AP-1. *J Biol Chem* 1999;274:32048-32054.

9. Delerive P, De Bosscher K, Vanden Berghe W, Fruchart JC, Haegeman G, Staels B. DNA binding-independent induction of IkappaBalpha gene transcription by PPARalpha. *Mol Endocrinol* 2002;16:1029-1039.
10. Dubuquoy L, Dharancy S, Nutten S, Pettersson S, Auwerx J, Desreumaux P. Role of peroxisome proliferator-activated receptor gamma and retinoid X receptor heterodimer in hepatogastroenterological diseases. *Lancet* 2002;360:1410-1418.
11. Welch JS, Ricote M, Akiyama TE, Gonzalez FJ, Glass CK. PPARgamma and PPARdelta negatively regulate specific subsets of lipopolysaccharide and IFN-gamma target genes in macrophages. *Proc Natl Acad Sci U S A* 2003;100:6712-6717.
12. Lee CH, Chawla A, Urbiztondo N, Liao D, Boisvert WA, Evans RM, Curtiss LK. Transcriptional repression of atherogenic inflammation: modulation by PPARdelta. *Science* 2003;302:453-457.
13. Kuipers F, Jong MC, Lin Y, Eck M, Havinga R, Bloks V, et al. Impaired secretion of very low density lipoprotein-triglycerides by apolipoprotein E-deficient mouse hepatocytes. *J Clin Invest* 1997;100:2915-2922.
14. Di Poi N, Tan NS, Michalik L, Wahli W, Desvergne B. Antiapoptotic role of PPARbeta in keratinocytes via transcriptional control of the Akt1 signaling pathway. *Mol.Cell* 2002;10:721-733.
15. Lennard AC. Interleukin-1 receptor antagonist. *Crit Rev Immunol* 1995;15:77-105.
16. Butcher C, Steinkasserer A, Tejura S, Lennard AC. Comparison of two promoters controlling expression of secreted or intracellular IL-1 receptor antagonist. *J Immunol* 1994;153:701-711.

17. Gabay C, Porter B, Guenette D, Billir B, Arend WP. Interleukin-4 (IL-4) and IL-13 enhance the effect of IL-1 $\beta$  on production of IL-1 receptor antagonist by human primary hepatocytes and hepatoma HepG2 cells: differential effect on C-reactive protein production. *Blood* 1999;93:1299-1307.
18. Gabay C, Smith MF, Eidlen D, Arend WP. Interleukin 1 receptor antagonist (IL-1Ra) is an acute-phase protein. *J Clin Invest* 1997;99:2930-2940.
19. Orfila C, Lepert JC, Alric L, Carrera G, Beraud M, Pipy B. Immunohistochemical distribution of activated nuclear factor kappaB and peroxisome proliferator-activated receptors in carbon tetrachloride-induced chronic liver injury in rats. *Histochem Cell Biol* 2005;123:585-593.
20. Kleemann R, Gervois PP, Verschuren L, Staels B, Princen HM, Kooistra T. Fibrates down-regulate IL-1-stimulated C-reactive protein gene expression in hepatocytes by reducing nuclear p50-NFkappa B-C/EBP-beta complex formation. *Blood* 2003;101:545-551.
21. Gabay C, Gigley J, Sipe J, Arend WP, Fantuzzi G. Production of IL-1 receptor antagonist by hepatocytes is regulated as an acute-phase protein in vivo. *Eur J Immunol* 2001;31:490-499.
22. Gabay C, Porter B, Fantuzzi G, Arend WP. Mouse IL-1 receptor antagonist isoforms: complementary DNA cloning and protein expression of intracellular isoform and tissue distribution of secreted and intracellular IL-1 receptor antagonist in vivo. *J Immunol* 1997;159:5905-5913.
23. Gregory SH, Wing EJ. Neutrophil-Kupffer cell interaction: a critical component of host defenses to systemic bacterial infections. *J Leukoc Biol* 2002;72:239-248.
24. Gervois P, Kleemann R, Pilon A, Percevault F, Koenig W, Staels B, Kooistra T. Global suppression of IL-6-induced acute phase response gene expression after chronic in

vivo treatment with the peroxisome proliferator-activated receptor-alpha activator fenofibrate.

J Biol Chem 2004;279:16154-16160.

25. Gervois P, Vu-Dac N, Kleemann R, Kockx M, Dubois G, Laine B, Kosykh V, et al.

Negative regulation of human fibrinogen gene expression by peroxisome proliferator-activated receptor alpha agonists via inhibition of CCAAT box/enhancer-binding protein beta.

J Biol Chem 2001;276:33471-33477.

26. Chikano S, Sawada K, Shimoyama T, Kashiwamura SI, Sugihara A, Sekikawa K,

Terada N, et al. IL-18 and IL-12 induce intestinal inflammation and fatty liver in mice in an

IFN-gamma dependent manner. Gut 2000;47:779-786.

27. Desiderio S, Yoo JY. A genome-wide analysis of the acute-phase response and its

regulation by Stat3beta. Ann N Y Acad Sci 2003;987:280-284.

Figure 1

*Microarray analysis of mouse liver after PPAR $\alpha$  activation and LPS treatment*

Wildtype mice and PPAR $\alpha$ <sup>-/-</sup> mice were exposed to Wy14643 0.1% for 5 days after which RNA was isolated. Pooled liver RNA (n=5) was used for microarray analysis with the Affymetrix Mouse Expression Array 430A (A). Wildtype mice were exposed to LPS for 3h and pooled liver RNA (n=3) was analyzed using microarray (B). SLR= Signal log ratio, the actual fold change can be calculated by using fold change =  $2^{(SLR)}$  and represents the fold change between mice receiving either Wy14643 or LPS and untreated mice. Fold-changes for all genes shown were statistically significant. [This figure appears in colour on the web.]

Figure 2

*Confirmation of gene expression results from the microarray experiment using qPCR*

Gene expression results of LIFR, STAT3, SAA, IL-18, IL-1RacP, and the IL-6 receptor were confirmed by qPCR on liver of Wildtype and PPAR $\alpha$ <sup>-/-</sup> animals fed the PPAR $\alpha$  agonist Wy-14643. Expression of the Wildtype control animals was set at 1. Differences between Wildtype mice treated with Wy-14643 and Wildtype control mice were evaluated by Student's T-test (\*\* =P<0.005, \* =P<0.05). Error bars represent SEM (n=4).

Figure 3

*PPAR $\alpha$  is mainly responsible for the gene expression changes in liver*

Wildtype mice were fed the PPAR $\alpha$  agonist Wy14643, the PPAR $\beta/\delta$  agonist L165041 or the PPAR $\gamma$  agonist Rosiglitazone. Liver RNA was isolated and qPCR was performed for LIFR, STAT3, SAA, IL-18, IL-1RacP and the IL-6 receptor. Gene expression levels from the animals receiving vehicle only were set at 1. \* Significantly different between control and treated animals (Student's T-test, P<0.05). Error bars represent SEM (n=5).

Figure 4

*PPAR $\alpha$  regulates gene expression of sIL-1ra in mouse liver*

Expression of sIL-1ra and icIL-1ra was determined by qPCR in wild-type and PPAR $\alpha$  null mice after feeding with Wy14643 (A) and in fed and 24-hour fasted state (B). (C) Mice were given Wy14643, L165401 and Rosiglitazone and sIL-1ra and icIL-1ra expression was determined in mouse liver. \*\* Significantly different between control and fasted or Wy14643 fed animals (Student's T-test,  $P < 0.005$ ). Error bars represent SEM (n=4).

Figure 5

*Protein levels of IL-1ra are PPAR $\alpha$ -dependently increased in mouse liver*

Equal amounts of total liver cell lysates from Wildtype and PPAR $\alpha$ <sup>-/-</sup> animals fed the PPAR $\alpha$  agonist Wy14643 were analysed by Western Blot using a mouse IL-1ra antibody. Migration of molecular-weight markers is shown. Molecular mass sizes are given in kDa.

Figure 6

*PPAR $\alpha$  is essential for the induction of sIL-1ra in liver during LPS-induced inflammation.*

(A) Wildtype and PPAR $\alpha$ <sup>-/-</sup> animals were exposed to LPS or saline for 16 h. Liver RNA was isolated and expression of sIL-1ra was measured by qPCR. Significant differences for sIL-1ra expression between Wt and PPAR $\alpha$ <sup>-/-</sup> mice treated with LPS were observed (Student's t-test,  $P < 0.01$ ). The sIL-1ra to IL-1b ratio after exposure to LPS was calculated in Wildtype and PPAR $\alpha$ <sup>-/-</sup> mice after LPS treatment. IL-1b expression was determined via qPCR. sIL-1ra to IL-1 $\beta$  ratios of Wildtype and PPAR $\alpha$ <sup>-/-</sup> mice were significantly different (Student's t-test,  $P < 0.002$ ). Error bars represent SEM (n = 4). (B) Primary mouse hepatocytes from Wt and

PPAR $\alpha$   $-/-$  mice were treated with IL-1 $\beta$  (5 ng/ml) or vehicle. After 24 h, expression levels of sIL-1ra were determined *via* qPCR.

#### Figure 7

*Up regulation of sIL-1ra by PPAR $\alpha$  is regulated by a PPRE present in the sIL-1ra promoter*

(A) HepG2 cells were transfected with the wildtype sIL-1ra promoter and expression plasmids for PPAR $\alpha$  and RXR $\alpha$ . Cells were treated with Wy14643 (50  $\mu$ M) for 24h after which luciferase and  $\beta$ -galactosidase activities were determined in the cell lysates. The relative luciferase activity of the DMSO treated cells was set to 1. Error bars represent SEM (n=3) (B) Alignment of the consensus PPRE with the sequence found in the mouse soluble promoter and the mutated PPRE used in the transfection experiment. (C) HepG2 cells were transfected with the mutant sIL-1ra promoter and expression plasmids for PPAR $\alpha$  and RXR $\alpha$  and treated with Wy14643 (50  $\mu$ M) for 24h. Thereafter, relative luciferase activity of the cell lysates were determined. (D) In vivo Chromatin Immunoprecipitation (CHIP) of the PPRE in the sIL-1ra mouse promoter was performed using an antibody against PPAR $\alpha$ . The gene sequence spanning the putative PPRE and a random control sequence were analyzed by PCR in the immunoprecipitated chromatin of mouse liver. Livers of fasted and Wy14643 fed animals were used.

#### Figure 8

*Human sIL-1ra gene expression is affected by PPAR $\alpha$*

(A) HepG2 cells were treated with IL-1 $\beta$  (10 ng/ml), RXR-agonist (5  $\mu$ M) and PPAR $\alpha$ -agonist (100  $\mu$ M Wy14643) for 24h. sIL-1ra gene expression was determined using qPCR. The relative expression from the DMSO treated cells was set on 1. (B) The culture medium was analyzed for IL-1ra protein levels by Western blotting using a human IL-1ra antibody.



Molecular-mass sizes are given in kDa. (C) Undifferentiated THP-1 cells and THP-1 cells differentiated to macrophages using PMA (100  $\mu$ M) were treated with Wy14643 (50  $\mu$ M) for 36h. sIL-1ra gene expressed was measured by qPCR. The relative expression of undifferentiated THP-1 cells treated with DMSO was set at 1.

*Table 1* Primer sequences used for Real Time PCR

Gene	Forward primer	Reverse primer	Product size
<i>Mouse sIL-1ra</i>	AAATCTGCTGGGGACCCTAC	TGAGCTGGTTGTTTCTCAGG	164 bp
<i>Mouse icIL-1ra</i>	CAGTTCCACCCTGGGAAGGT	AGCCATGGGTGAGCTAACAGGACA	128 bp
<i>Mouse STAT3</i>	GACCCGCCAACAAATTAAGA	TCGTGGTAAACTGGACACCA	214 bp
<i>Mouse IL-18</i>	ACAACCTTGGCCGACTTCAC	GGGTTCACTGGCACTTTGAT	127 bp
<i>Mouse IL-1RAcP</i>	TTGCCACCCAGATCTATTC	CCAGACCTCATTGTGGGAGT	122 bp
<i>Mouse SAA</i>	GCGAGCCTACTGACATGA	TTTTCTCAGCAGCCCAGACT	121 bp
<i>Mouse LIF-receptor</i>	AGGAATGCCACAATCAGAGG	AACCCGGAAAGTGTATGCAG	90 bp
<i>Mouse IL-6 receptor</i>	CAGCGACACTGGGGACTATT	AGTCACTCTTCCCGTTGGTG	220 bp
<i>Mouse <math>\beta</math>-actin</i>	GATCTGGCACCACACCTTCT	GGGGTGTGAAGGTCTCAA	139 bp
<i>Human sIL-1ra</i>	GCCTCCGCAGTCACCTAAT	TCCCAGATTCTGAAGGCTTG	108 bp
<i>Human <math>\beta</math>-actin</i>	AACACCCCGCCATGTACG	ATGTCACGCACGATTTCCC	254 bp

Figure 1

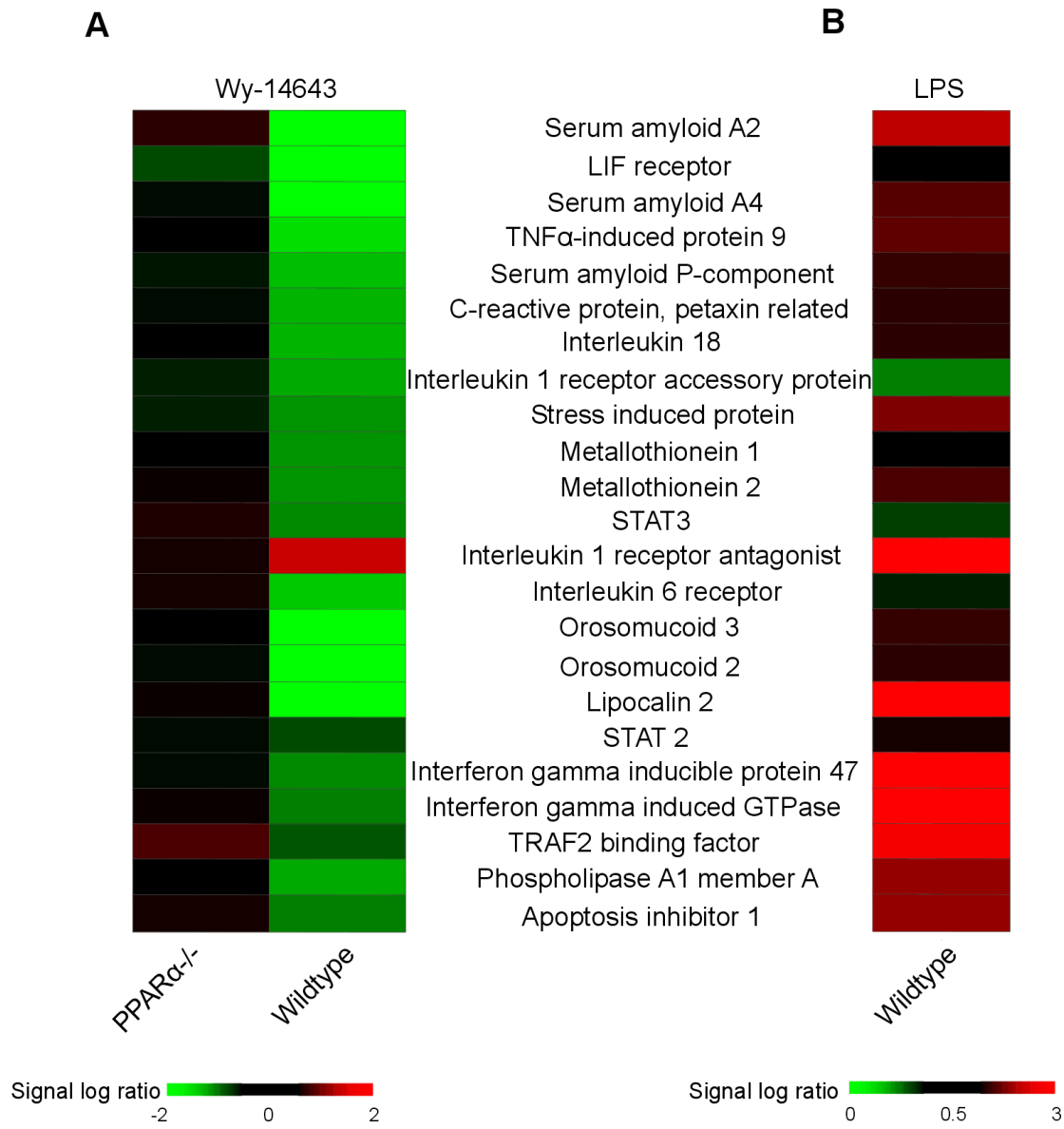


Figure 2

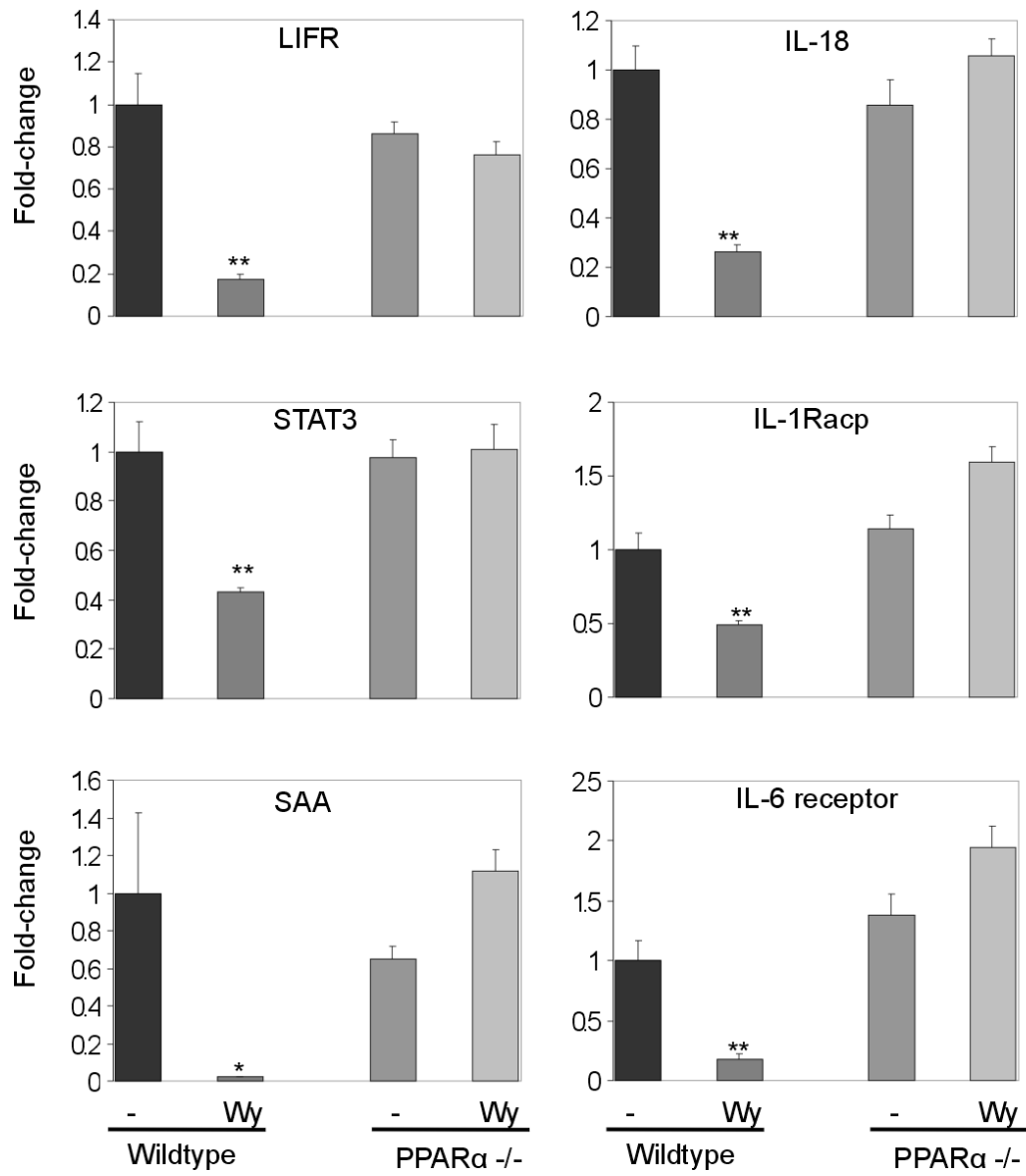


Figure 3

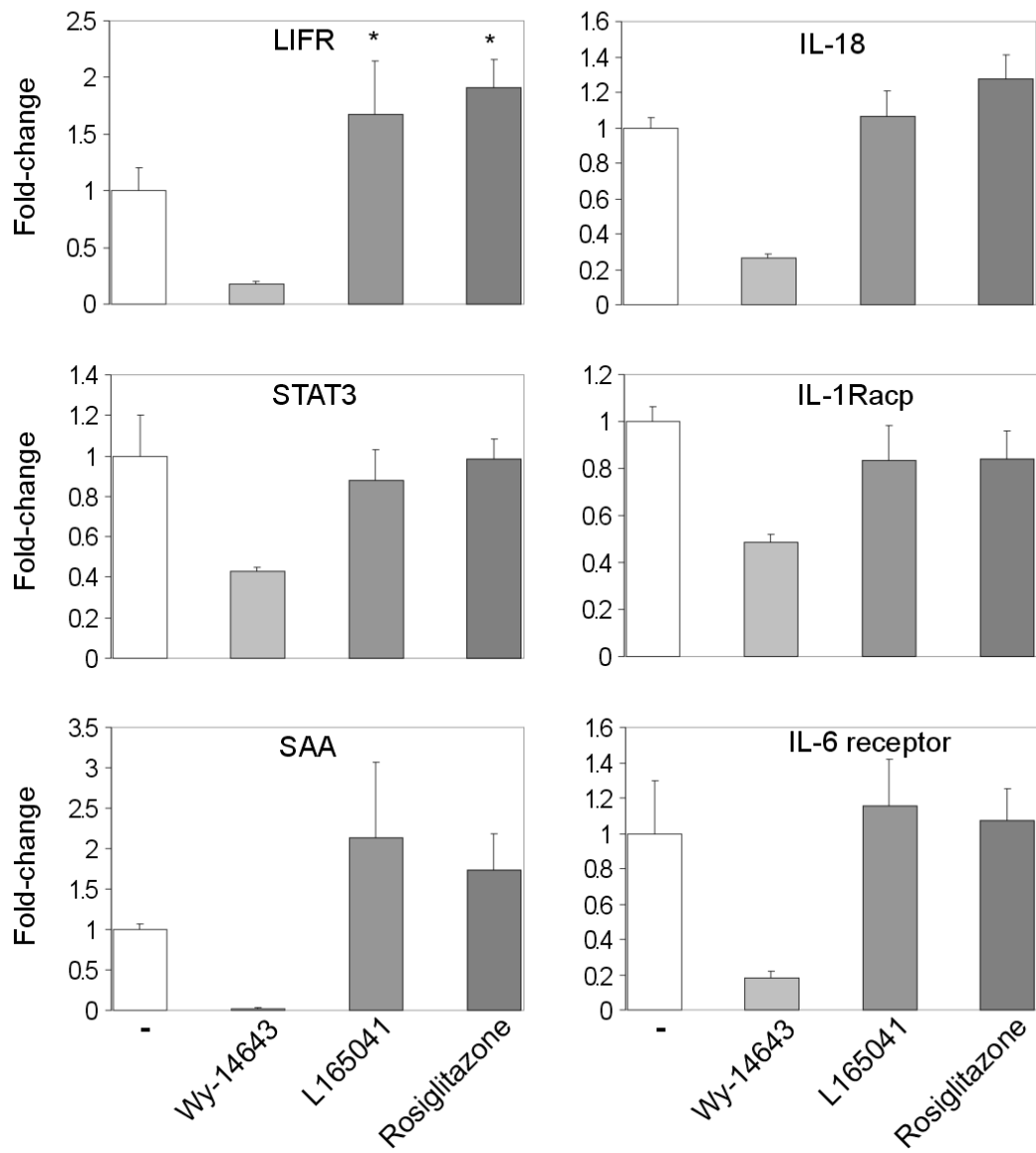
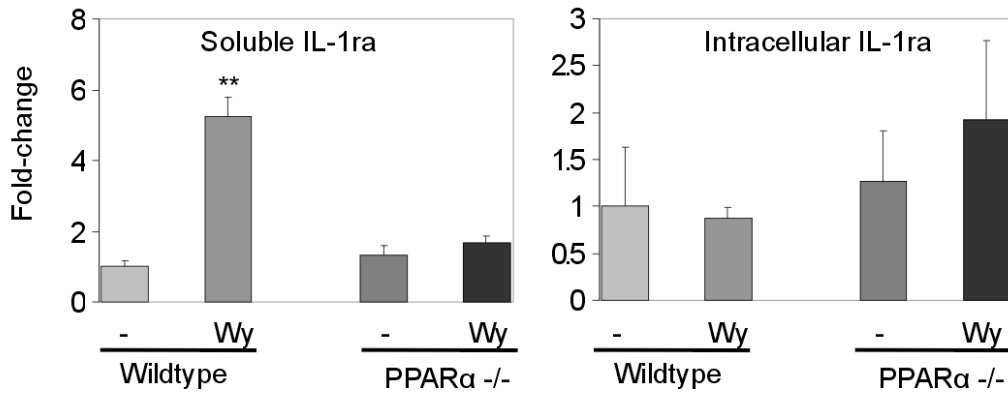
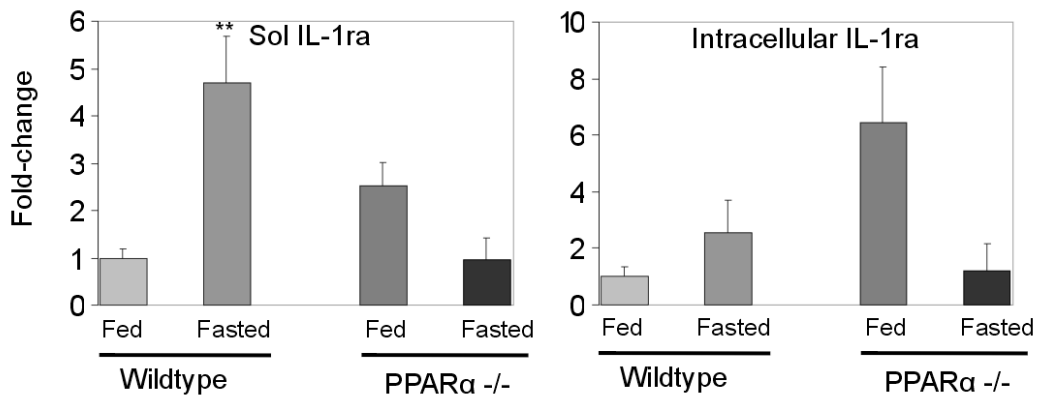


Figure 4

**A**



**B**



**C**

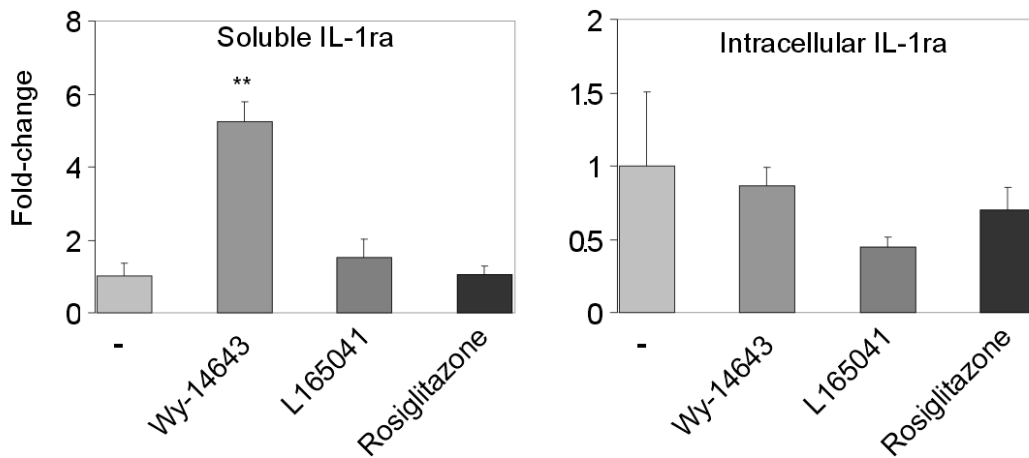


Figure 5



Figure 6

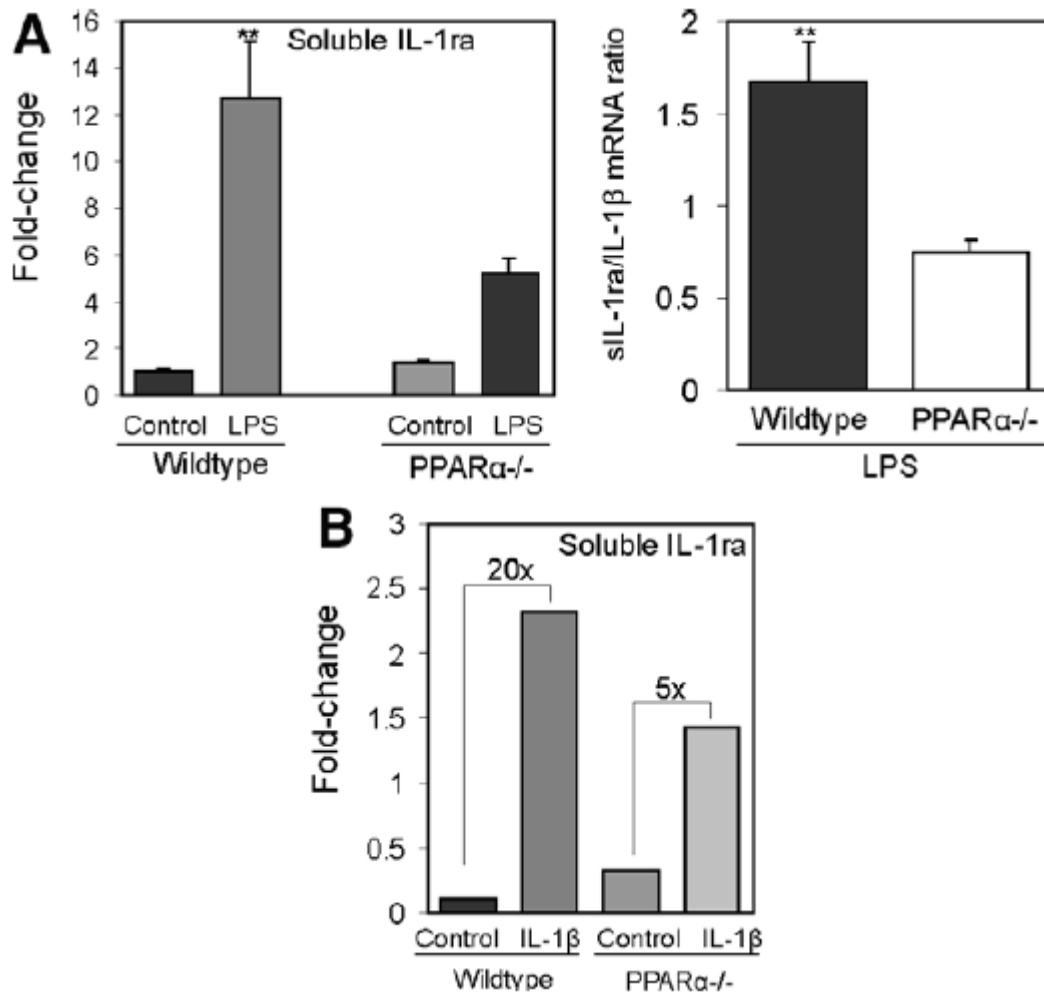






Figure 8

