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Stienstra et al., PPARα controls the expression of the sIL-1ra in liver

*The Interleukin 1 receptor antagonist is a direct target gene of PPARα in liver*

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Short title: PPARα controls the expression of the sIL-1ra in liver

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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, PPARα, PPARβ/δ and PPARγ, 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 PPARα 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 PPARα on inflammation are mainly achieved by suppressing gene expression. Since the initial observation that PPARα-/- 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 PPARα-
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-κB pathway. Physical interaction of PPARα with NF-κB prevents its activation and downstream pro-inflammatory effects (8). Moreover, PPARα has been shown to up-regulate the expression of IkB, the natural NF-κB 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 PPARγ 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 PPARβ/δ has also revealed a specific role for PPARβ/δ in regulating inflammatory processes (12).

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

2.1. Chemicals

Wy-14643 was obtained from ChemSyn Labarotories. 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-1β 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 PPARα-/- 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 μg of total
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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, Sommerville, 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 μg 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) 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 β-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’CCGCTCGAGCGGAGCAATAGAATAGTC 3’ and the reverse primer sIL-1ra-Rprom 5’ CCCAAGCTTGGGACAGAGAAGGATSAGAAGGA 3’ including restrictions sites for both XhoI and HindIII were used to PCR-amplify 1900 bp of the sIL-ra 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’ TTTCTCTAGGGCTGGAGGACAGCAAACTTCT 3’ combined with primer sIL-1ra-Rprom and the reverse primer sIL-1ra-mutR 5’ AGAAGTTTTGCTGTCCAGCAATTCTTCTCAGGACAAGAGAAA 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 mPPARα was cloned into pSG5 (Stratagene). After cloning, fragments were sequenced to confirm the integrity of the constructs. The RXRα 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% O2/5% CO2. 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 x 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-1β (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 β-galactosidase reporter was co-transfected to normalize for differences in transfection efficiency. After transfection, cells were treated with the PPARα ligand Wy14643 at 50 μM or vehicle (DMSO) for 24 h prior to lysis. Promega luciferase assay (Promega) and standard β-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 μM.

2.9. Chromatin Immunoprecipitation (ChIP)

Wt or PPARα -/- 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 PPARα-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’-CTCCCTTTCCCCTTCTGTCCCTCATT-3’ for the forward primer and 5’-TTCCCAAACCTCCCCACCACCACCCCATCC-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 PPARα, but not PPARβ/δ and PPARγ, is involved in the negative regulation of inflammatory related-genes in liver

To screen for potential novel target genes of PPARα involved in inflammation in liver we performed micro-array analysis. A comparison was made between liver RNA from Wt and PPARα-/- mice treated or not with the synthetic PPARα ligand Wy14643. The expression of a large number of genes involved in inflammation was decreased by Wy14643 in Wt but not PPARα-/- 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, orosumucoid, 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 PPARα activation. Real-time quantitative PCR (qPCR) confirmed the marked suppression of the selected genes involved in inflammation by Wy14643 in Wt but not PPARα-/- mice (Fig. 2).

To evaluate whether the down-regulation of inflammatory genes in liver is specific to PPARα activation, we studied the effect of synthetic PPARβ/δ and PPARγ 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. **PPARα 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 PPARα 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 PPARα 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 PPARα-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 PPARα 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 **PPARα is essential to induce expression of sIL-1ra during inflammatory conditions in liver**

To investigate whether PPARα is involved in the regulation of sIL-1ra during inflammatory conditions, Wt and PPARα -/- 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 PPARα -/- mice (Fig. 6A). Induction of IL-1β expression was similar between Wt and PPARα -/- mice after exposure to LPS (data not shown). Subsequently, the ratio of IL-1ra to IL-1β, which is often used to determine the activation of IL-1 signaling pathways,
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was drastically improved in Wt mice compared to PPARα mice after exposure to LPS, suggesting a shift towards a more anti inflammatory phenotype (Fig. 6A). Treatment of Wt and PPARα -/- primary mouse hepatocytes with IL-1β to induce sIL-1ra gene expression gave comparable results, as shown by a more pronounced increase in sIL-1ra gene expression in hepatocytes expressing PPARα compared to PPARα -/- hepatocytes (Fig. 6B).

3.4. sIL-1ra is a true target gene of PPARα in mouse liver

To determine whether the sIL-1ra promoter is directly regulated by PPARα, 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 PPARα and RXRα expression plasmids revealed that while RXRα alone did not have any effect on the promoter activity (data not shown), transfection of both PPARα and RXRα expression plasmids increased the activity of the sIL1-ra 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 PPARα-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 PPARα and Wy14643 was completely abolished, suggesting that the effects of PPARα are mediated by the PPRE identified (Fig. 6C).

Finally, to investigate if PPARα is bound to the PPRE identified in mouse liver, in vivo chromatin immunoprecipitation (ChIP) was performed using an anti-PPARα antibody. In mice, treatment with Wy-14643 and fasting increased the binding of PPARα to this PPRE in Wt but not PPARα -/- mice. No binding was observed to a control sequence located 1800
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base pairs upstream from the PPRE (Fig. 6D). Taken together, these data indicate that the PPRE identified mediates the effects of PPARα on sIL-1ra gene expression.

3.5. sIL-1ra is also regulated by PPARα 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-1β, an inducer of sIL-1ra expression, resulted in the expected increase in gene expression levels (Fig. 7A). Incubation with RXRα and PPARα agonists alone did not result in major changes in gene transcription. However, combined treatment with both IL-1β and PPARα/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 PPARα localization shifts from hepatocytes towards Kupffer cells during hepatic inflammation (19), it was of interest to test the ability of PPARα 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 PPARα in human hepatocytes and macrophages.
4. Discussion

Since the initial discovery of a possible role for PPARα in controlling the inflammatory response (5), considerable progress has been made towards resolving the function of PPARα during inflammation (8, 9, 20). It is now clear that PPARα 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 PPARα with a functional PPRE in its promoter, suggesting that PPARα 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-κB activation (21). Under non-inflammatory conditions, PPARα activation by Wy14643 or fasting markedly increased sIL-1ra gene expression in liver, which was not observed in PPARα −/− mice. Thus, PPARα 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 PPARα regulates sIL-1ra expression via a PPRE located around 700 bp upstream of the transcription start site.

In human HepG2 cells PPARα activation was able to increase sIL-1ra gene expression when cells were co-treated with IL-1β which is known to induce expression of sIL-ra by NF-κB and C/EBP binding to the sIL-1ra promoter (22). In THP-1 cells differentiated towards macrophages, PPARα was also capable of inducing sIL-1ra expression. Since macrophage/monocyte type cells including Kupffer cells have an important role in controlling
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hepatic inflammation (23), the PPARα-dependent regulation of the IL-1ra gene in these cells might contribute to the immune-modulating functions of PPARα 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-1β to its receptor via increased expression of its natural antagonist, PPARα might prevent or counteract the activation of the IL-1β-signalling cascade including the pro-inflammatory NF-κB pathway.

The availability of microarray techniques offers the opportunity to screen for new PPARα controlled genes and pathways with the aim of elucidating the effects of PPARα on inflammation. Using this approach, our initially analysis focused on inflammation-related genes that are regulated by PPARα 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 PPARα 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 PPARα activation. Furthermore, the LIF-receptor, which belongs to the IL-6 receptor family, was strongly decreased after PPARα-agonist treatment in mouse liver. Downstream of this pathway, PPARα is also ably to suppress the expression of STAT3, a transcription factor implicated in the regulation of inflammatory signaling in liver (27). Finally, PPARα seems to have a role in regulating IFNγ-activated genes which is illustrated by the suppression of IFNγ-inducible genes by PPARα.
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Our analysis clearly demonstrates an important immunosuppressive effect by PPARα in liver. One limitation of the present study is that we did not address the effect of PPARα 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 PPARα agonists prior to IL-6 treatment, the subsequent acute phase responses in liver were diminished (24). Although future experiments should aim at combining both PPARα activation and inflammation in liver to appraise the importance of PPARα in regulating inflammation, our data reveal several candidate genes by which PPARα 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, PPARα activation may render the liver less susceptible to the effects of inflammation. Up-regulation of soluble IL-1ra by PPARα may contribute to its immune-modulating effects.

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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


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


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Figure 1

*Microarray analysis of mouse liver after PPARα activation and LPS treatment*

Wildtype mice and PPARα-/- 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-1RαcP, and the IL-6 receptor were confirmed by qPCR on liver of Wildtype and PPARα-/- animals fed the PPARα 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

*PPARα is mainly responsible for the gene expression changes in liver*

Wildtype mice were fed the PPARα agonist Wy14643, the PPARβ/δ agonist L165041 or the PPARγ agonist Rosiglitazone. Liver RNA was isolated and qPCR was performed for LIFR, STAT3, SAA, IL-18, IL-1RαcP 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).
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Figure 4

**PPARα regulates gene expression of sIL-1ra in mouse liver**

Expression of sIL-1ra and icIL-1ra was determined by qPCR in wild-type and PPARα 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 PPARα-dependently increased in mouse liver**

Equal amounts of total liver cell lysates from Wildtype and PPARα-/- animals fed the PPARα 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

**PPARα is essential for the induction of sIL-1ra in liver during LPS-induced inflammation.**

(A) Wildtype and PPARα -/- 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 PPARα -/- 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 PPARα -/- mice after LPS treatment. IL-1b expression was determined via qPCR. sIL-1ra to IL-1β ratios of Wildtype and PPARα -/- mice were significantly different (Student’s t-test, P < 0.002). Error bars represent SEM (n = 4). (B) Primary mouse hepatocytes from Wt and
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PPARα-/- mice were treated with IL-1β (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 PPARα 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 PPARα and RXRα. Cells were treated with Wy14643 (50 μM) for 24h after which luciferase and β-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 PPARα and RXRα and treated with Wy14643 (50 μM) 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 PPARα. 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 PPARα

(A) HepG2 cells were treated with IL-1β (10 ng/ml), RXR-agonist (5 μM) and PPARα-agonist (100 μM Wy14643) for 24h. sIL-ra 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 μM) were treated with Wy14643 (50 μM) for 36h. sIL-ra 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

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<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size</th>
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<tr>
<td>Mouse icIL-1ra</td>
<td>CAGTTCCACCCTGGGAAGGT</td>
<td>AGCCATGGGTGAGCTAAACAGGACA</td>
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<td>Mouse STAT3</td>
<td>GACCCGCAAACAAATTAAGA</td>
<td>TCGTGTTAAAACCTGGACACCA</td>
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<td>Mouse IL-18</td>
<td>ACAACTTTGGCCGACTTCAC</td>
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<td>Mouse IL-1RAcP</td>
<td>TTGCCACCCCAGATCTATTTC</td>
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<td>Mouse SAA</td>
<td>GCGAGCCTACACTGACATGA</td>
<td>TTTTCTCAGCAGCCCAACTGACT</td>
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<td>Mouse LIF-receptor</td>
<td>AGGAATGCCACAATCAGAGG</td>
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<td>Mouse IL-6 receptor</td>
<td>CAGCGACACTGGGGACTATT</td>
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Stienstra et al., PPARα controls the expression of the sIL-1ra in liver
Stienstra et al., PPARα controls the expression of the sIL-1ra in liver

**Figure 2**

- LIFR
- IL-18
- STAT3
- IL-1Racp
- SAA
- IL-6 receptor

Fold-change

- Wildtype
- PPARα -/-
- Wy

**""""** indicates significant difference.
Figure 3

Stienstra et al., PPARα controls the expression of the sIL-1ra in liver
Stienstra et al., PPARα controls the expression of the sIL-1ra in liver

Figure 4

A

B

C
Figure 5

Stienstra et al., PPARα controls the expression of the sIL-1ra in liver.
Stienstra et al., PPARα controls the expression of the sIL-1ra in liver

Figure 6
Stienstra et al., PPARα controls the expression of the sIL-1rα in liver

Figure 7

A

![Graph showing fold-induction of Wy-14643 and PPARα+RXRα](image)

B

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C

![Graph showing fold-induction of Wy-14643 and PPARα+RXRα](image)

D

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Stienstra et al., PPARα controls the expression of the sIL-1ra in liver

**Figure 8**

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Protein marker

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