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**Direct Transcriptional Regulation of Human Hepatic Cytochrome P450 3A4
(CYP3A4) by Peroxisome Proliferator-Activated Receptor Alpha (PPAR α)**

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PPAR α , peroxisome proliferator-activated receptor alpha; CYP, cytochrome P450; PBR, PPAR α -binding region; POPC, 16:0/18:1-PC, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; PXR, pregnane X receptor; PPRE, peroxisome proliferator response element; PHH, primary human hepatocytes; ADME, absorption, distribution, metabolism and excretion; CHIP, Chromatin immunoprecipitation; RXR, retinoid X receptor; shRNA, small hairpin RNA; DR, direct repeat; ACOX1, acyl-CoA oxidase 1; CAR, constitutive androstane receptor

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Abstract

The nuclear receptor peroxisome proliferator-activated receptor (PPAR) α is primarily known as a regulator of fatty acid metabolism, energy balance, and inflammation, but there is evidence suggesting a wider role in regulating biotransformation of drugs and other lipophilic chemicals. We investigated whether PPAR α directly regulates the transcription of cytochrome P450 (CYP) 3A4, the major human drug metabolizing enzyme. Using chromatin immunoprecipitation in human primary hepatocytes as well as electrophoretic mobility shift and luciferase reporter-gene assays we identified three functional PPAR α -binding regions (PBR)-I, II, III within ~12kb of *CYP3A4* upstream sequence. Furthermore, a humanized CYP3A4/3A7 mouse model showed in vivo induction of CYP3A4 mRNA and protein by WY14,643 in liver but not in intestine, while hepatic occupancy of PBRs by PPAR α was ligand-independent. Using lentiviral gene knock-down and treatment with WY14,643 in primary human hepatocytes, PPAR α was further shown to affect the expression of a distinct set of CYPs including 1A1, 1A2, 2B6, 2C8, 3A4, and 7A1, but not 2C9, 2C19, 2D6 or 2E1. Interestingly, the common phospholipid 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC, 16:0/18:1-PC), previously proposed to reflect nutritional status and shown to be a specific endogenous ligand of PPAR α , induced *CYP3A4* (up to 4-fold) and other biotransformation genes in hepatocytes with similar selectivity and potency as WY14,643. These data establish PPAR α as a direct transcriptional regulator of hepatic CYP3A4. This finding warrants investigation of known and newly developed PPAR α -targeted drugs for their drug-drug interaction potential. Furthermore, our data suggest that nutritional status can influence drug biotransformation capacity via endogenous phospholipid signalling.

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Introduction

Genes encoding enzymes and transporters that protect higher animals from toxic effects of lipophilic xenobiotics have evolved not only to optimize their substrate selectivity and catalytic properties but also their regulation in order to allow flexible responses to environmental changes. In particular the lipid- and xenobiotics-sensing nuclear receptors pregnane X-receptor PXR (NR1I2), the constitutive androstane receptor CAR (NR1I3), and the aryl hydrocarbon receptor AhR, together with liver-enriched transcription factors HNF4 α (NR2A1), C/EBP and others form networks of transcription factors that coordinately regulate hepatic expression of the majority of drug metabolizing cytochromes P450 (CYP), phase II enzymes and transporters in response to xenobiotic exposure (Handschin and Meyer, 2005; Pascussi et al., 2008; Pelkonen et al., 2008). Recent research elucidated extensive crosstalk between these receptors and other nuclear receptors and transcription factors, linking xenobiotic metabolism to the homeostasis of lipids, bile acids, glucose and other endogenous processes (Moreau et al., 2008; Gao and Xie, 2012). Such insight into the connections between xenobiotic metabolism and endogenous homeostasis can therefore enhance our mechanistic understanding of drug toxicity as well as that of pathophysiological conditions of the liver such as dyslipidemia, nonalcoholic fatty liver disease and others (Anderson and Borlak, 2008; Buechler and Weiss, 2011).

The most important class of nuclear receptors for lipid homeostasis are the peroxisome proliferator-activated receptors (PPAR) α (NR1C1), β/δ (NR1C2), and γ (NR1C3), which act as lipid sensors to control the expression of gene networks involved in lipid and energy homeostasis, adipocyte differentiation, and inflammatory responses (Lalloyer and Staels, 2010; Wahli and Michalik, 2012). PPAR α expression is high in tissues with active fatty acid catabolism, including liver, intestine, heart, muscle and others, where it primarily regulates intracellular trafficking and metabolism of lipids including mitochondrial and peroxisomal β -

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oxidation, microsomal ω -hydroxylation, and also cholesterol and bile acid biosynthesis (Pyper et al., 2010). Activating ligands include synthetic fibrates used to treat dyslipidemia and the investigative specific agonist [4-chloro-6-(2,3-xylylidino)-2-pyrimidinylthio]acetic acid (WY14,643), as well as various endogenous lipids (Forman et al., 1997; Kliewer et al., 1997; Chakravarthy et al., 2009). Elucidation of PPAR α target genes by treatment of human and mouse hepatocytes with WY14,643 revealed that regulation of hepatic lipid metabolism is generally well-conserved, while other regulated genes were largely divergent (Rakhshandehroo et al., 2009, 2010). For example, CYP4 family members, which function as microsomal fatty acid omega-hydroxylases, are more inducible in rodents compared to humans (Waxman 1999; Richert et al., 2003; Hsu et al., 2007), while drug metabolizing CYPs of families 1 to 3, including CYP3A4, CYP2B6, CYP2C8, and CYP1A2, appeared to be inducible by fibrates in humans only (Richert et al., 2003; Prueksaritanont et al., 2005; Rakhshandehroo et al., 2009). Although functional PPREs were identified in *CYP4* genes (Johnson et al., 1996) as well as in several phase II enzymes, including members of the UDP-glucuronosyltransferases (Barbier et al., 2003; Senekeo-Effenberger et al., 2007), the basis for PPAR α -dependent hepatic expression of human CYPs of families 1 to 3 remained unclear, in particular whether regulation occurs directly or indirectly, e.g. by involvement of other nuclear receptors such as PXR (Aouabdi et al., 2006). Further evidence suggesting an involvement of PPAR α as a regulator of *CYP3A4*, the most important drug metabolizing P450 of human liver and intestine (Zanger et al., 2008), includes a systems biology approach which analyzed time-dependent expression profiles of primary human hepatocytes (PHH) treated with statins (Schröder et al., 2011) and a pharmacogenomics study, which identified PPAR α polymorphisms as a predictive marker of hepatic CYP3A4 expression and function (Klein et al., 2012).

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Here we used PHH and a CYP3A4/3A7-humanized mouse model to investigate whether PPAR α directly regulates transcription of CYP3A4. Our data suggest a previously unrecognized role of PPAR α as a direct transcriptional regulator of drug metabolizing CYPs of families CYP1-3. We also tested whether a specific phospholipid, that has recently been shown to act as endogenous ligand of PPAR α and that is believed to serve as an indicator of nutritional status (Chakravarthy et al., 2009), is able to induce CYP3A4 and other ADME (absorption, distribution, metabolism and excretion) genes. Our data suggest intricate interactions between lipid homeostasis, nutritional status and drug biotransformation capacity that may be of relevance under diseased conditions such as obesity and hepatic steatosis. Furthermore, our findings suggest a largely uninvestigated potential for PPAR α -dependent drug-drug interactions.

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Materials and Methods

Primary human hepatocytes and treatments. The use of human hepatocytes for research was approved by the local ethics committees of Berlin and Regensburg, and written informed consent was obtained from all patients. Hepatocytes were isolated and cultured essentially as described (Klein et al., 2012) except that 12-well plastic dishes precoated with collagen were used (OMNILAB, Bremen, Germany). Cells were treated for 24h with the indicated substances in 0.1% DMSO (Dimethyl Sulfoxide), Rifampicin (5,6,9,17,19,21-Hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-8-[*N*-(4-methyl-1-piperazinyl)formimidoyl]-2,7-(epoxypentadeca-[1,11,13]trienimino)-naphtho[2,1-*b*]furan-1,11(2*H*)-dion-21-acetat (R3501), CITCO (6-(4-Chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime) (C6240), or WY14,643 ([4-chloro-6-(2,3-xylylidino)-2-pyrimidinylthio]acetic acid) (C7081) from Sigma Aldrich, Steinheim, Germany or in 4% BSA in PBS (for phospholipids) purchased from Avanti Polar Lipids, Hamburg, Germany.

Chromatin immunoprecipitation (ChIP) assay. ChIP was performed using MAGnify Chromatin Immunoprecipitation Kit (Invitrogen, Darmstadt, Germany) according to the manufacturer's description. DNA was sheared by sonication to an average length of ~300bp using Bioruptor (diagenode, Liege, Belgium) and incubated with 10µg of anti-PPAR α antibody (PP-H0723-00; R&D Systems, Wiesbaden, Germany), previously bound to 10µl of magnetic beads at 4°C for 2 hours and DNA was purified using DNA purification beads and eluted in 150µl of elution buffer. Promoter occupation was analyzed with 10µl of immunoprecipitated DNA by Sybr-Green PCR. Primers are listed in the Supplemental Table 1. The results were normalized to HMGCR (3-hydroxy-3-methyl-glutaryl-CoA reductase) promoter region (-1970/-2200bp) used as positive control (van der Meer et al., 2010) and the untranscribed region Untr-5 as negative control (Hariparsad et al., 2009). For ChIP analysis of fresh frozen mouse livers we applied the same protocol using specific manufacturer

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recommendations for tissue handling. Binding to Gys2 (glycogen synthase 2) promoter was assessed in parallel as a positive control for binding of mouse Ppar α (Mandard et al., 2007).

Plasmids. Previously described plasmids: human PPAR α expression plasmid pcDNA3-hPPAR α , kindly provided by T. Tanaka, University of Tokyo, Japan (Tanaka et al., 2002); pcDhsRXRA, encoding human RXR α (Mathäs et al., 2012); CYP3A4 luciferase reporter gene constructs (Tegude et al., 2007). PPREX3-TK-luc construct was purchased from Addgene (#1015) (Kim et al., 1998). Mutations in the identified PPREs were generated using QuickChange™ II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The wild-type and contextual mutation constructs (12-17) were generated from self-designed gene fragments which were synthesized by GeneArt (Life Technologies, Darmstadt, Germany) and cloned into construct 1.

Electrophoretic mobility shift assays (EMSA). Human PPAR α and RXR α proteins were synthesized using expression plasmids and TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI). Complementary single stranded oligonucleotides (Supplemental Table 2) were annealed and radiolabeled as described (Tegude et al., 2007). Binding reactions and gel electrophoresis were performed as described (Geick et al., 2001). Retarded complexes were quantified using BAS1800 II phosphor-storage scanner (Fuji, Kanagawa, Japan) and AIDA software, version 4.19 (Raytest, Straubenhardt, Germany).

In silico analysis. For predictions of potential PPAR α binding sites, NUBIScan version 2.0 (cut-off Z-score 7; Podvinec et al., 2002) at www.nubiscan.unibas.ch and PPRESearch (defaults settings; Venkatachalam et al., 2009) at www.classicus.com/PPRE were used.

Luciferase reporter gene assays. Human hepatocarcinoma HepG2 cells were transfected with 250 ng of reporter plasmid and 50 ng of pcDNA3-hPPAR α expression vector using Turbofect (Fermentas, St. Leon-Rot, Germany). pcDNA3 plasmid DNA was added to adjust the total amount of transfected DNA to 300 ng. To control for transfection efficiency, 25 ng

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of pGL3-TK-Renilla (Promega, Mannheim, Germany) was used. Cells were harvested in reporter “passive” lysis buffer (Promega) after 24h of incubation, and cell extracts were analyzed for firefly and renilla luciferase activities using Enspire® Multimode Plate-Reader (Perkin Elmer, Rodgau, Germany) and Luciferase Assay System from P.J.K. GmbH (Kleinblittersdorf, Germany).

Animal experiment. Experiments with humanized huCYP3A4/3A7 mice (Hasegawa et al., 2011) were performed at TaconicArtemis GmbH (Cologne, Germany). Animals were maintained in a temperature-controlled room (23±1°C) with a 12h light/dark cycle. All experiments were approved by the Local Ethical Review Committee and conducted in compliance with the United Kingdom “Animals (Scientific Procedures) Act” (1986). Fifty mg of WY14,643 in corn oil per kilogram of body weight was orally administered (with syringe) to five male huCYP3A4/3A7 mice at approximately 3 months of age daily for four days. Control animals received corn oil vehicle only. Mice were then anesthetized, exsanguinated, and tissues removed and flash-frozen in liquid nitrogen.

Quantitative RT-PCR Analysis. Isolation of total RNA, quantification, and complementary DNA synthesis were performed as described (Klein et al., 2012). The assays for the detection of PPAR α mRNA (Klein et al., 2012), CYP1A1 (400nM of fw:5'-TGG TCA AGG AGC ACT ACA AAA CC-3', and rev:5'-TCA TCT GAC AGC TGG ACA TTG G-3' primers, and 200nM of probe MGB: 6-FAM-GAT GTG GCC CTT CTC-MGB), and CYP3A4 (Wolbold et al., 2003) were self-designed and oligonucleotides were from Applied Biosystems (Darmstadt, Germany). For all other genes pre-designed Taqman assays were purchased from Applied Biosystems. For comprehensive qPCR profiling we used Fluidigm's Biomark high-throughput qPCR chip platform (Fluidigm Corporation, San Francisco, CA) with 96 gene expression assays (Applied Biosystems; list available on request) assays following

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manufacturer's instructions (Spurgeon et al., 2008). For final evaluation of fold changes, ribosomal gene RPLP0 (human) and beta-actin (mouse) were used for normalization.

Western blot analysis. CYP3A4 protein was detected in mouse liver homogenates using 50 μ g of protein per lane as described earlier (Wolbold et al., 2003). Beta-actin was detected using mouse monoclonal antibody from Sigma-Aldrich (Steinheim, Germany) #A5441 in 1:5000 dilutions.

Lentivirus-mediated RNAi in human primary hepatocytes. Treatment of PHH with lentivirus encoding PPAR α -specific small hairpin RNAs (shRNA) was performed as described (Klein et al., 2012). Further details can be found at <http://seek.virtuelle-leber.de/sops/13>.

Measurement of CYP enzyme activities. CYP enzyme activities were determined in culture supernatants using an LC/MS/MS-based substrate cocktail assay as reported previously (Feidt et al., 2010).

Statistical Methods. For pairwise analysis of statistical significance, the Student's paired t-test (2-tailed) was applied and statistical significance was defined as $P < 0.05$. To identify statistically significant differences, one-way ANOVA with Student-Newman-Keuls posttest was performed with the mean values of at least three independent experiments done in triplicates. For correlations, the Spearman rank correlation coefficient (r_s) was calculated. All calculations were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA).

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Results

ChIP reveals occupation of *CYP3A4* promoter by PPAR α in PHH. In silico analysis of the *CYP3A4* promoter and upstream region (20kb) identified a vast number of putative DR1 and DR2 motifs with different degree of homology to the consensus AGGTCA nuclear receptor half site however did not identify any 100% consensus motif. Thus, we systematically screened ~12kb of upstream region by ChIP of isolated chromatin from PHH using a total of 40 primer pairs. As evident from figure 1, three regions, designated as PPAR α -binding regions (PBR-I, -2600/-3100bp; PBR-II, -7200/-7800bp; PBR-III, -8500/-8900bp) showed significant enrichment of promoter binding by PPAR α as compared to *HMGCR* and unoccupied intermediary gene regions. Pre-treatment of hepatocytes with WY14,643 did not change enrichment profiles (data not shown). Region PBR-II overlapped partially with the well-described XREM (xenobiotic-responsive enhancer module), which includes binding sites for several transcriptional regulators of *CYP3A4* including PXR, HNF (hepatocyte nuclear factor) 4 α , and FXR (farnesoid X receptor).

PPAR α directly binds to distinct motifs within the identified PPAR α binding regions.

We next investigated whether PPAR α can directly bind to PPREs in the identified PBRs. PPAR α is known to bind as PPAR α /RXR α heterodimer to DR1 and DR2 motifs and in silico analysis of PBRs I to III identified several high-score putative binding motifs (Table 1). PPAR α /RXR α specifically bound to three DR1-type motifs and to one DR1/DR2 motif within the identified PBRs (Fig. 2A). Competition EMSA experiments determined relative in vitro binding affinities of PPAR α /RXR α in the order DR1-B>DR1-D \geq DR1/DR2-C>DR1-A (Fig. 2B).

Functional redundancy and cooperativity of *CYP3A4* PPREs in transcriptional regulation by PPAR α .

Results of HepG2 cell cotransfection experiments performed in the absence and presence of WY14,643 are shown in figure 3. Deletion analysis of the *CYP3A4*

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5' upstream promoter region showed that only constructs comprising the region between -2.0 kb and -8.8 kb were transcriptionally activated by PPAR α (Fig. 3A). The -8.8kb construct showed 1.9-fold induction of PPAR α activation by WY14,643. Presence of the PBR-I region comprising motifs DR1-A and DR1-B was sufficient for PPAR α -dependent transactivation (construct #4), which was further enhanced if regions PBR-II and -III with motifs DR1/DR2-C and DR1-D were also present (construct #5). All three distal upstream PBRs acted as PPAR α -dependent enhancers as they distance-independently conferred activation by the receptor to the proximal *CYP3A4* promoter (Fig. 3A, constructs #6-11). To dissect the contribution of individual PPRES, contextual mutational analysis was performed. Because construct #5 was too large for manipulation by mutagenesis, an artificial construct #12 was created, which comprises the three PBRs and 360 bp of proximal promoter sequence (Fig. 3B). While mutation of DR1-A and DR1-D did not show any significant effect, mutation of motifs DR1-B and DR1/DR2-C completely abolished constitutive and inducible PPAR α -dependent transactivation.

PPAR α regulates CYP3A4 in vivo in a humanized CYP3A4/CYP3A7 mouse model.

Since it is was shown that mouse *cyp3a* genes were not responsive to the WY14,643 treatment (Rakhshandehroo et al., 2009), we used a CYP3A4-humanized mouse model to investigate whether CYP3A4 is regulated by mouse Ppar α in vivo. Indeed, as shown in figure 4, oral administration of WY14,643 for 4 days resulted in the significant induction of the mouse *Acox1* (4-fold) and *Cyp4a10* (27-fold) genes, as well as approximately 5-fold induction of the human CYP3A4 in the liver (Fig. 4A). Western blot analysis confirmed about 2-fold induction of CYP3A4 protein (Fig. 4C). However, no induction was seen in the intestine, while the mouse Ppar α target genes were still induced, although at lower fold changes (Fig. 4B).

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Because induction of CYP3A4 by WY14,643 treatment of the humanized mice could also be due to indirect mechanisms or to mouse regulatory sequences further upstream of the human *CYP3A4* promoter, we analyzed promoter occupancy by ChIP assay in the resected liver tissues of the humanized mice. As shown in figure 4D, screening of the 5'-flanking sequence confirmed human *CYP3A4* promoter occupancy by mouse Ppar α at binding regions PBR-III and PBR-II, while PBR-I did not demonstrate binding. As already observed with human hepatocytes, the enrichment profile was essentially identical between untreated and WY14,643-treated animals.

PPAR α regulates a selective set of CYP genes in human primary hepatocytes. To investigate whether regulation of CYP3A4 by PPAR α was characteristic of a broader scheme, we applied lentiviral vectors for shRNA-mediated downregulation of PPAR α to PHH. As shown in figure 5A, knock-down of PPAR α with two different PPAR α -directed shRNA vectors strongly decreased mRNA expression not only of ACOX and CYP3A4 but also of CYPs 1A1, 1A2, 2B6, 2C8, and 7A1, whereas CYPs 2A6, 2C9, 2C19, 2E1, and 3A5 were only marginally influenced or remained unchanged. Measurement of corresponding enzyme activities following PPAR α gene silencing or treatment with WY14,643 resulted in consistent changes of enzyme activities (Fig. 5B).

Inducibility of ADME genes by endogenous PPAR α phospholipid-ligands. To investigate whether the lipid sensor function of PPAR α is of relevance for the regulation of ADME genes, we compared several phospholipids with WY14,643 for inducing potency (Fig. 6A). Indeed, treatment of PHH from three donors with 16:0/18:1PC but not several other phospholipids tested resulted in significant induction of *ACOX1* (1.2 to 1.7-fold), *CYP3A4* (1.8 to 3.1-fold), *CYP2C8* (3.1 to 4.2-fold), *CYP1A2* (0.8 to 2.15-fold) and *CYP2B6* (0.95 to 2.2-fold) genes, whereas the PPAR α target genes *IL1- β* and *CYP7A1* were strongly downregulated by both, synthetic and natural ligands. The PPAR α activating properties of

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16:0/18:1PC thus closely resembled those of WY14,643 in terms of target gene selectivity and potency of induction. This was also shown with a broader selection of target genes (Fig. 6B, Supplement Table 2). The most highly induced genes were as expected metabolic genes (*CPT1A*, *HMGCS2*, *PK4*, up to 5-fold), while several CYPs and other ADME genes (*SULT*, *PXR*) were induced by 86% (*CYP3A5*) up to 280% (*CYP2C8*). The high similarity in WY14,643 or 16:0/18:1PC-induced gene expression changes was reflected in a strong correlation ($r_s=0.83$, $p<0.0001$, $n=91$). WY14,643 treatment of PHH further closely mirrored the gene silencing effects as reflected in a negative correlation ($r_s=-0.56$, $p<0.0001$, $n=91$).

Comparative transcriptional profiling of PPAR α and the xenosensors PXR and CAR.

The broad effects of PPAR α activation on ADME gene expression, which resembled those of the classical xenosensors pregnane X receptor (PXR) and constitutive androstane receptor (CAR), prompted us to further compare these three nuclear receptors. We treated PHH from three different donors with the most specific available agonists (CITCO for CAR, rifampicin for PXR, and WY14,643 for PPAR α), to analyze their gene regulation patterns in a wider selection of target genes. Figure 7 presents a gene expression heatmap of the three donors. Induction of PXR and CAR resulted in the expected distinct upregulation of ADME genes and only slight changes in genes of energy metabolism. Activation of PPAR α revealed a similar pattern of ADME gene upregulation and stronger influence on metabolic-relevant gene expression.

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Discussion

Direct regulation of *CYP3A4* expression by PPAR α is supported by three lines of evidence: first, by ChIP of PHH we elucidated three PBRs of 400~600bp length; second, four PPREs were identified by EMSA; and third, reporter gene analysis demonstrated that two of the PPREs (DR1-B and DR1/DR2-C) are essential for transcriptional activation by PPAR α . The DR1-A and DR1-B sites are located closely together in a previously unremarkable region at approximately -3kb. The DR1-B site displayed the strongest binding affinity of all four sites, in agreement with its strong functional role as demonstrated by mutational analysis. It is of interest that the PBR-I region was less occupied in huCYP3A4/3A7 mouse liver as well as in PHHs from two male donors diagnosed with adiposity and diabetes in comparison to one nondiabetic female donor (data not shown). As we can exclude DNA sequence variants at the identified PPAR α binding sites (data not shown), the basis for this difference remains unclear. The DR1/DR2-C site located within the well-documented XREM partially overlaps with an HNF4 α -binding site, which cooperatively interacts with adjacent PXR sites to promote inducibility by PXR ligands (Tirona et al., 2003). While the DR1/DR2-C site appeared to be transcriptionally highly active and inducible, its affinity by EMSA was lower compared to the DR1-B site. The presence of several nearby and overlapping transcription factor binding sites may lead to protein-protein interactions that could explain the apparent discrepancy. The DR1-D site is located in a previously undescribed region of the *CYP3A4* 5'upstream region and apparently functions independently, although in the context of all PPREs it was not essential. Taken together, our experiments suggest redundancy as well as cooperativity of at least three functional PPAR α -dependent enhancers in *CYP3A4*, which mediate both constitutive and inducible transactivation. Interestingly, pre-treatment of hepatocytes with WY14,643 did not change enrichment profiles of PPAR α binding within the upstream regions of *CYP3A4*. Based on our data, we hypothesize that upstream binding regions of *CYP3A4* are

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continuously occupied by PPAR α and the co-factor environment determines repression or activation of CYP3A4 transcription following binding of a ligand. Therefore, addition of a chemical ligand does not lead to the increased binding but rather to the reinforced release of co-repressors and thereby increase in transcription.

We investigated a possible involvement of PPAR α in the regulation of other drug biotransformation genes by gene silencing experiments. Our data show a pattern of selectively regulated CYPs including 1A1, 1A2, 2B6, 2C8, 3A4, and 7A1 but not CYPs 2C9, 2C19, 2D6 or 2E1 that is well in agreement with previous studies using PPAR α agonists such as WY14,643 (Rakhshandehroo et al., 2009) or fibrates (Prueksaritanont et al., 2005). Beyond that, our data imply an involvement of PPAR α in the expression of these genes not only by induction but also at the constitutive level, although we have not formally addressed direct regulation of these genes. Of note, two functional PPREs were found to be responsible for the regulation of *CYP1A1* in intestinal CaCo2 cells (Villard et al., 2011). It is possible that these PPREs are involved in the regulation of both, CYP1A1 and CYP1A2, because they are located within the bidirectional promoter between the two CYPs. Preliminary bioinformatic analyses suggest the presence of potential high-score DR1/DR2-type binding sites in the genes regulated by PPAR α compared to unregulated genes.

Direct transcriptional activation by PPAR α of other drug biotransformation genes has already been reported for several UDP-glucuronosyltransferases (Barbier et al., 2003; Senekeo-Effenberger et al., 2007), some drug transporters (Cheng et al., 2005; Moffit et al., 2006), as well as the rate-limiting enzyme of heme biosynthesis, aminolevulinic acid synthase (34). The regulation of hepatic CYPs by PPAR α can thus be regarded as a “missing link” within the drug biotransformation network. Our direct comparison of ligand-induced gene expression changes mediated by PPAR α and the two prototypic xenosensors PXR and CAR further

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emphasize the similar target profiles of these three nuclear receptors with respect to the regulation of drug biotransformation genes in the liver.

With respect to the pronounced species difference concerning the regulation of human *CYP1-3* and orthologous mouse genes by PPAR α (Rakhshandehroo et al., 2009), our experiment with huCYP3A4/3A7 mice suggests that this difference must be due to the lack of functional PPRE sites within the mouse Cyp genes, because mouse Ppara was able to bind to the CYP3A4 PBRs II and III and activate hepatic transcription. Therefore, the huCYP3A4/3A7 mouse appears to be a useful model to study CYP3A4 regulation by PPAR α and other factors (Hasegawa et al., 2011). The lack of Ppara-dependent *CYP3A4* induction in mouse intestine is probably related to the similar observation in regard to the XREM HNF4 α -binding site that regulates CYP3A4 in human liver (Tirona et al., 2003) but not in intestinal cells, where a specific additional factor was proposed to be missing (Tegude et al., 2007). Lower inducibility of CYP3A4 in the duodenum compared to liver has been described in this and another humanized mouse model before (Hasegawa et al., 2011). Interestingly, rat *CYP3A* and *2C* genes were found to be inducible by the fibrate gemfibrozil (Liu et al., 2011).

The current data on direct CYP induction through PPAR α suggest a link between endogenous lipids and the regulation of drug biotransformation in human liver. We tested this hypothesis by treating PHH with phospholipids, because a recent study identified POPC as an authentic endogenous ligand bound to PPAR α (Chakravarthy et al., 2009). Indeed, we could show induction of CYP3A4 and numerous other CYPs and drug biotransformation enzymes specifically by POPC but not by related phospholipid species. To our knowledge, this is the first observation that a phospholipid acts as a selective inducer of drug biotransformation genes in human hepatocytes. As described by Chakravarthy and colleagues (Chakravarthy et al., 2009), intracellular availability of POPC as a PPAR α ligand is limited by fatty acid synthase (FAS) and choline-ethanolamine phosphotransferase-1 (CEPT1), suggesting that

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metabolic perturbations in this system, e.g. in obesity and hepatic steatosis, may not only affect hepatic lipid metabolism but also drug biotransformation. The fact that externally administered POPC leads to enzyme induction in PHH suggests that dietary phospholipid components may also influence drug biotransformation gene expression. As a variety of dietary and endogenous lipids including saturated and unsaturated fatty acids, phospholipids, eicosanoids, and many derivatives and metabolites have been implicated in PPAR α activation, our findings suggest an intricate interplay between intermediary metabolism, nutritional status and biotransformation.

In conclusion, we have elucidated the mechanistic basis for constitutive and inducible transcriptional regulation of *CYP3A4* by PPAR α and we provided evidence for a broader range of similarly regulated drug metabolizing CYPs. Since fibrate hypolipidemic drugs are used worldwide in the treatment of dyslipidemia, there is clearly a need to readdress the potential for drug-drug interactions, which may depend on nutritional status. This is of particular importance for newly developed PPAR α ligands to target obesity, insulin resistance and diabetes (Lalloyer and Staels, 2010). Our results furthermore demonstrate the direct influence of phospholipids on CYP regulation, indicating a physiological link between lipid homeostasis and drug biotransformation in the liver, which is likely of importance in diseased states such as obesity and hepatic steatosis.

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Authorship Contributions

Participated in research design: Thomas, Burk, Kandel, Damm, Weiss, Klein, Zanger

Conducted experiments: Thomas, Burk, Kandel, Klumpp

Performed data analysis: Thomas, Burk, Kandel, Klumpp

Wrote or contributed to the writing of the manuscript: Thomas, Burk, Schwab, Zanger

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Footnotes

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Figure Legends

Fig. 1. Binding of PPAR α to the CYP3A4 promoter *in vivo*. Human primary hepatocytes were fixed with formaldehyde in suspension and DNA was sheared by sonication. Sheared chromatin was incubated with PPAR α antibody coupled to magnetic beads. Protein-DNA complexes were precipitated and bound protein was digested by proteinase K. Precipitated DNA was purified and was used, together with input DNA, as template for Sybr-Green PCR using a total of 40 primer pairs spanning approximately 10 kb of the CYP3A4 promoter region. Raw Ct values were normalized to input DNA to calculate the percentage of DNA immunoprecipitated. Primers encompassing the PPRE of the human HMGCR gene were used as positive control. Means relative to intergenic binding (n.c.) \pm standard deviations (SD) of three independent donors (two male, one female) are shown. Significant enrichment was assessed using ANOVA analysis and is indicated by hashes. IR, intermediary region; PBR, PPAR binding region; n.c., negative control.

Fig. 2. PPAR α specifically binds to distinct DR1/DR2 motifs with differential affinities. A. Electrophoretic mobility shift assays using *in vitro* translated proteins bound to radiolabeled doublestranded oligonucleotide probes corresponding to the indicated wild type or mutated (mut) motifs of CYP3A4. As a positive control for binding, the known PPARA binding site of the rat ACOX1 gene (36), here indicated as PPRE, was used. Binding reactions contained (+) or lacked (-) the indicated proteins. Complexes of PPAR α /RXR α heterodimers with the oligonucleotides are marked by an arrow. B. Competition electrophoretic mobility shift assays with radiolabeled wild type motif DR1-B as probe and n-fold molar excess of indicated unlabeled motifs as competitors were performed. Retarded complexes were quantified and expressed as percentage of the complex obtained in the absence of competitors. Data represent means of two independent experiments for each competitor \pm S.D.

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Fig. 3. The PPAR α binding regions in the CYP3A4 promoter show differential and redundant functions. Luciferase reporter gene constructs (A, 1-11; B, 12-17) containing the indicated sequences of the CYP3A4 promoter (in kilobase pairs, kb) were cotransfected with a PPAR α expression plasmid and renilla-luciferase expression vector into HepG2 cells. Cells were treated with either vehicle (DMSO, grey bars) or 50 μ M WY14,643 (black bars) for 24h prior to measurement of firefly/renilla luciferase activities. Firefly luciferase activities were normalized to renilla luciferase activities. Data are means \pm SD of three independent experiments, each performed in triplicates; •, mutated sites; #, statistically significant ($p < 0.05$) compared to construct 1; *, statistically significant ($p < 0.05$) compared to untreated. The TK gene promoter fused to 3 copies of the ACOX1-PPRE was used as positive control.

Fig. 4. Induction of CYP3A4 by WY 14,643 in humanized huCYP3A4/3A7 mice. Humanized CYP3A4/3A7 mice were fed with WY14,643, or vehicle (corn oil) for 4 days and mRNA expression of target genes was analyzed by real-time RT-PCR in liver (A) and intestine (B) tissue lysates normalized to beta-actin. The bars indicate means \pm SD relative to vehicle control set at 1, with 5 animals in each group. # indicate significant changes in the expression of target genes. C. Western blot analysis of liver lysates of the huCYP3A4/3A7 mice treated with vehicle (Oil, n=5) or WY14,643 (WY, n=5). An exemplary Western blot is shown. CYP3A4 protein was quantitated and normalized against β -actin. D. ChIP analysis of Ppara binding to the CYP3A4 promoter in huCYP3A4/3A7 mice. ChIP assay was performed with liver tissues (n=3 in each group) as described in Fig. 1. The promoter of glycogen synthase 2 (Gys2) was analyzed as a positive control for binding of mouse Ppara. Statistical significance ($p < 0.05$, ANOVA) was assessed for expression changes of WY-treated group vs oil (A-C) or for Ppara binding enrichment of PBRs vs negative control (n.c.).

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Fig. 5. PPAR α gene silencing and activation regulates expression and activity of a selective set of CYPs. A. Two shRNA-encoding lentiviral vectors targeted to PPAR α were used to silence PPAR α gene expression in primary human hepatocytes obtained from three donors (light bars, shRNA1; dark bars, shRNA2). mRNA levels were measured by qPCR five days after infection and compared to mRNA levels measured in cells treated with non-targeting shRNA control vector set at 1.0. Data of three independent donors (all male) are shown. B. Enzyme activities measured by LC-MS/MS cocktail assay in the culture supernatants of human hepatocytes following PPAR α gene silencing (means of both shRNAs) (dark bars) or treatment with WY14,643 (light bars); CYP activities measured: CYP1A2, phenacetin O-deethylation; CYP2B6, bupropion 4-hydroxylation; CYP2C8, amodiaquine N-desethylation; CYP2C9, tolbutamide 4'-hydroxylation; CYP2C19, S-mephenytoin 4'-hydroxylation; CYP2D6, propafenone 5-hydroxylation; CYP3A4, atorvastatin o-hydroxylation. Data are means of 3 independent donors (A) and of two donors (B). Statistical significance for RNAi experiments in comparison to control shRNA is indicated by # ($p < 0.05$, paired t-test). Two independent experiments were made for CYP activity measurements; error bars indicate the two measurements.

Fig. 6. Endogenous phospholipid ligands of PPAR α induce ADME gene expression. Primary human hepatocytes of two independent donors (male) were treated for 24 h with WY14,643 (50 μ M) or with different phospholipids (50 μ M): 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (16:0/18:1 GPC); 1-hexadecanoyl-sn-glycero-3-phosphocholine (16:0 GPC); 1-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (18:1 GPC); 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (18:0/18:1 GPC). A. Expression of selected genes determined by qRT-PCR and expressed relative to vehicle (DMSO) control, which was set as 1. Data

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represent means±S.D. of two independent experiments measured in triplicates. Statistical significance ($p < 0.05$, paired t-test) is indicated by #. B. Heatmap showing mRNA expression changes (fold) in human hepatocytes from three independent donors following PPAR α gene silencing or following treatment with WY14,643 (50 μ M) or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (16:0/18:1 GPC; 50 μ M). Fold-changes were calculated in relation to control shRNA (RNAi, means of both shRNAs shown) or vehicle treatment (WY14,643, 16:0/18:1PC). The colour scale from blue (downregulation) to red (upregulation) spans from -2.5 to 2.5 (log₂-scale). Right panel, corresponding Spearman rank correlation analysis of expression profiles of 91 genes analyzed (see Supplemental Table 3).

Fig. 7. Comparison of CAR, PXR, PPAR α transcriptional regulation. Heatmap showing color-coded mRNA expression differences in human hepatocytes from three donors (2 male, 1 female) following induction of CAR (CITCO, 1 μ M), PXR (Rifampicin, 10 μ M) and PPAR α (WY14,643, 50 μ M). The individual donor gene expression patterns are shown on the left (1,2,3), the mean values are represented on the right (1-3). The upper panel presents selected ADME genes, the lower panel selected genes of energy metabolism. The colour scale from blue (downregulation) to red (upregulation) spans from -2.5 to 2.5 (log₂-scale).

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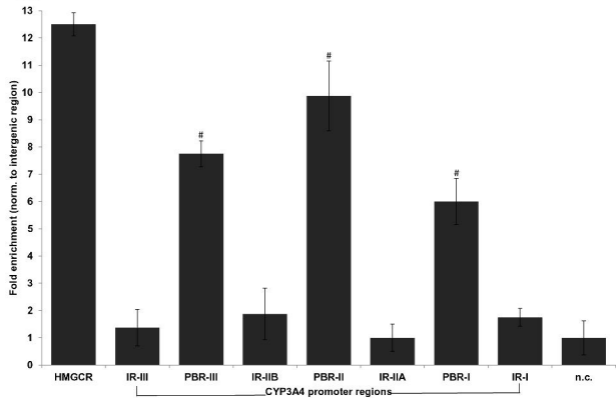
Tables

Table 1 PPREs identified in the CYP3A4 upstream region

Designation	PBR	Position ^a	Sequence (strand)
DR1-A	I	-2915/-2903	GGTACA A AGTTGA (+)
DR1-B	I	-3062/-3050	GGATCA A AGTTCA (-)
DR1/DR2-C	II	-7784/-7764	<i>CAATTA</i> A AGGTCA TA AAGCCC (+)
DR1-D	III	-8816/-8804	AGGGCA A AGGACA (+)

^awith respect to transcriptional start site; PBR, PPAR α binding region; PPRE, PPAR α response element; DR1, DR2, direct repeat with 1 or 2 bases distance; deviations in base changes from the consensus shown in italicized type.

Figure 1



A

probe	PPRE		DR1 A		mut DR1 A		DR1 B		mut DR1 B		DR1/2 C		mut DR1/2 C		DR1 D		mut DR1 D	
PPAR α	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
RXR α	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

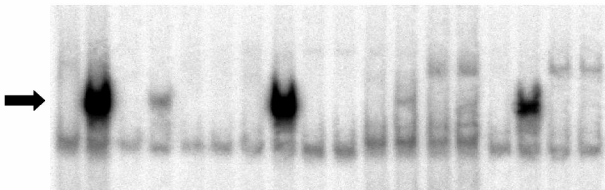
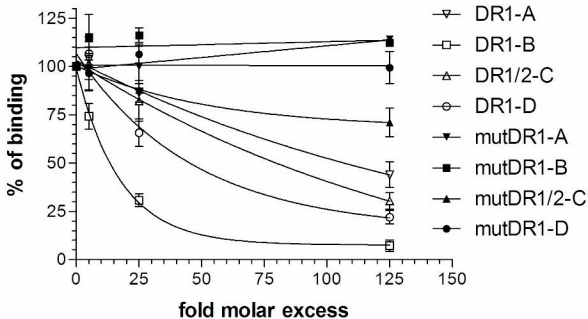
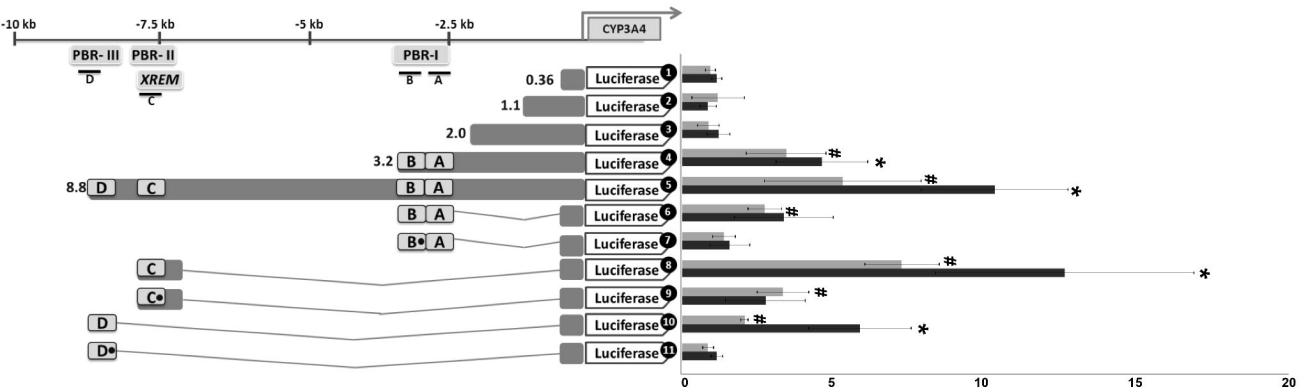
**B**

Figura 3



B

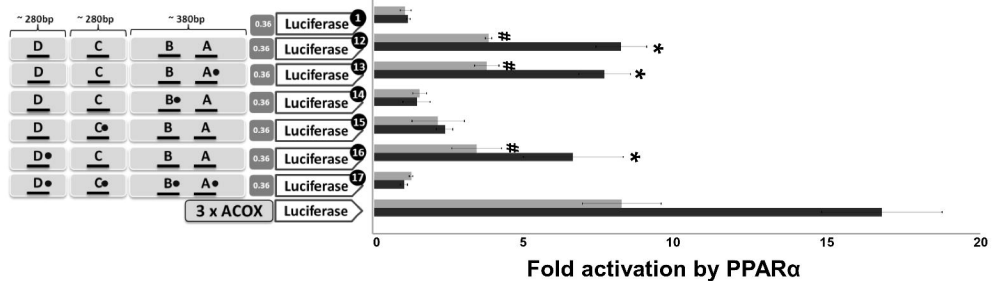
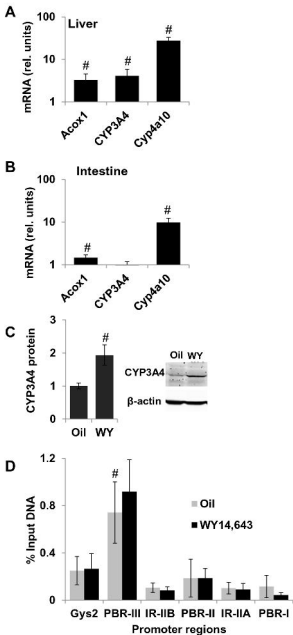
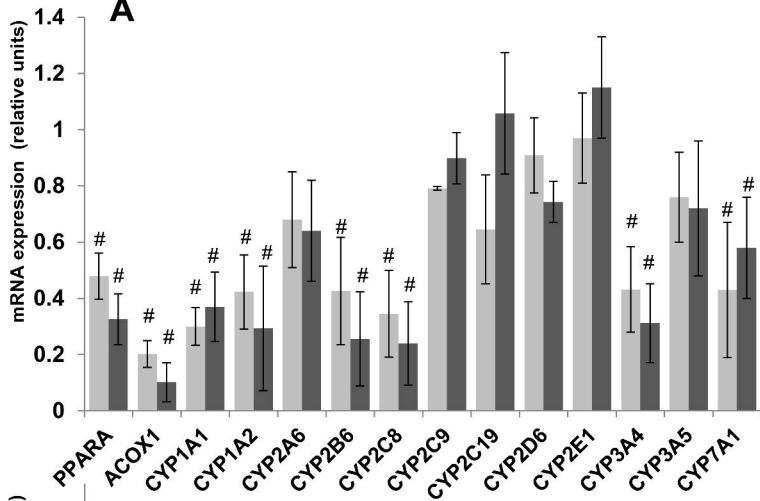
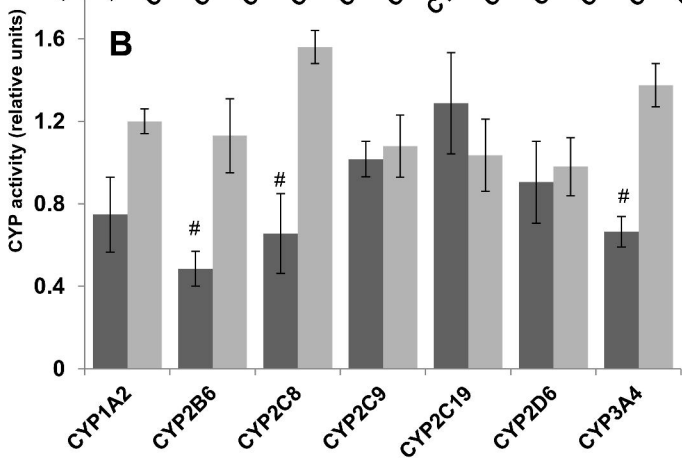
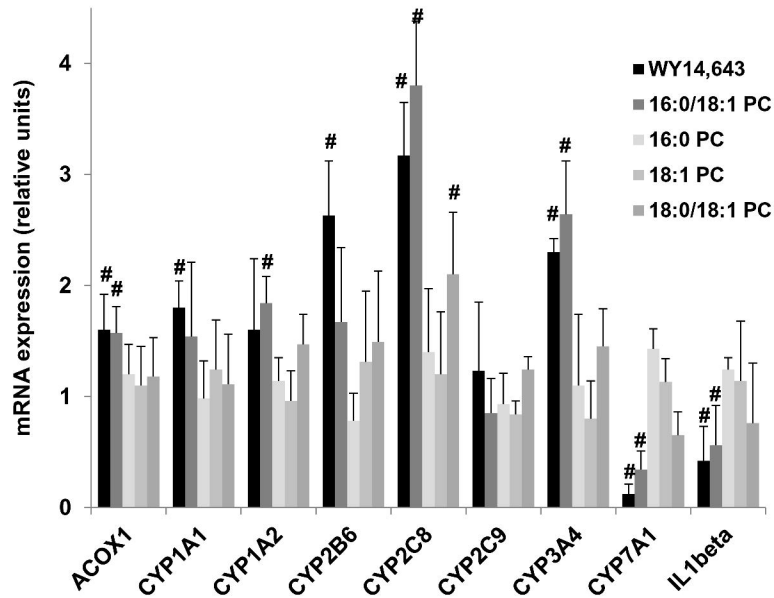


Figure 4



A**B**

A



B

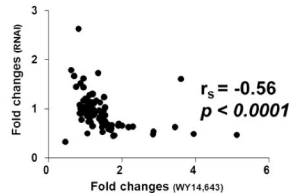
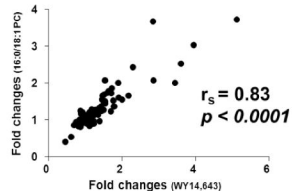
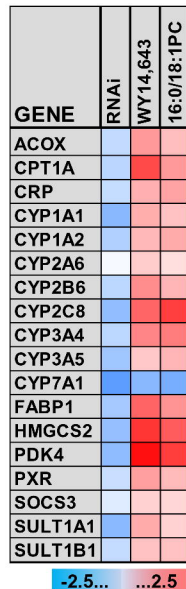
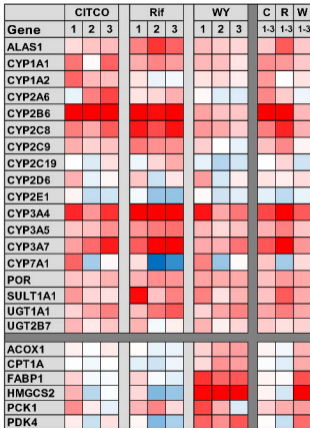


Figure 7



-2.5...

...2.5

Direct Transcriptional Regulation of Human Hepatic Cytochrome P450 3A4 (CYP3A4) by Peroxisome Proliferator-Activated Receptor Alpha (PPAR α)

Maria Thomas, Oliver Burk, Britta Klumpp, Benjamin A. Kandel, Georg Damm, Thomas S. Weiss, Kathrin Klein, Matthias Schwab, Ulrich M. Zanger

Molecular Pharmacology

Supplemental Table 1. SYBR-Green primers for chromatin immunoprecipitation

Name (original)	sequence 5'→3'		position (upstream, starting from TSS)	Name (original)	sequence 5'→3'		position (upstream, starting from TSS)
CYP3A4_1	tggaaatgctccctccctt	fw	499-517	CYP3A4_1	cacatcctaccaagttgaa	rev	693-712
CYP3A4_2	tcattgattttctctattgc	fw	921-940	CYP3A4_2	ctagaatttaataaact	rev	1102-1120
CYP3A4_3	aatctttattattaccctta	fw	974-992	CYP3A4_3	taaagagcataccctgtgag	rev	1131-1150
CYP3A4_4	catttctgcatttccaact	fw	1431-1449	CYP3A4_4	ttccagggtccctctgtca	rev	1604-1622
CYP3A4_5	gttcatctcactggggatt	fw	1460-1478	CYP3A4_5	aagcccgatttagggag	rev	1637-1654
CYP3A4_6	tgagatctgagaacagacag	fw	2014-2033	CYP3A4_6	cagcagcagaggctgcaga	rev	2181-2199
CYP3A4_7	gacgatcaactactccaa	fw	2511-2529	CYP3A4_7	cacatagttctctgcat	rev	2654-2672
CYP3A4_8	actaacagctgatctctggc	fw	3111-3130	CYP3A4_8	tctctcagcattgtgtct	rev	3261-3281
CYP3A4_9	ctgtctctcagatcacaatgc	fw	4121-4141	CYP3A4_9	cccagagattctggtatgtg	rev	4281-4301
CYP3A4_10	tgggatgcaaggctggttcaac	fw	5138-5159	CYP3A4_10	agtgggtttgtcatagatagctc	rev	5326-5348
CYP3A4_11	gtgttagtatagctactcctt	fw	6921-6941	CYP3A4_11	ataaagacattgattgccaga	rev	7071-7091
CYP3A4_12	gcacatatacactgtggaat	fw	9016-9035	CYP3A4_12	atgagtgacaacatcgctg	rev	9140-9159
CYP3A4_13	gttcagtaaaactggaact	fw	10080-10098	CYP3A4_13	ccttcataaacgtttgc	rev	10207-10224
CYP3A4_14	gccaacaccagccagactt	fw	10280-10298	CYP3A4_14	cttcctcagcctcagttgc	rev	10407-10425
CYP3A4_15	cagacagggcctgcacgtca	fw	10437-10456	CYP3A4_15	tccgctcccaaaagtctg	rev	10570-10588
CYP3A4_16	cgagatgtctctggagttca	fw	10501-10520	CYP3A4_16	tggggtttgccatgtgccc	rev	10631-10650
CYP3A4_17	gcacgggtgctctcctcctg	fw	10545-10563	CYP3A4_17	agaggcatgcaccaccac	rev	10683-10700
CYP3A4_18	gaattgctgagcccagg	fw	10732-10749	CYP3A4_18	tccgctcactgcaactctg	rev	10792-10812
CYP3A4_19	gcatctctcagaggttctcta	fw	10706-10727	CYP3A4_19	gaaatggaatctgtctgtt	rev	10801-10821
CYP3A4_20	cagctacttgggaggctgaggc	fw	10729-10745	CYP3A4_20	acgcaggtgctccctgggc	rev	10863-10881
CYP3A4_21	attaccacttcaccccac	fw	11061-11070	CYP3A4_21	agaattccaatttctccatg	rev	11171-11190
CYP3A4_22	gttatagatctgagaggat	fw	11121-11139	CYP3A4_22	ctgggtcacattgaaattc	rev	11188-11208
CYP3A4_23	tggatgaacatgtctacag	fw	11399-11418	CYP3A4_23	cttcattcattatggcc	rev	11460-11488
CYP3A4_24	cactttactatgaagtgaag	fw	11431-11450	CYP3A4_24	agcctgtaccagttgagcag	rev	11570-11589
CYP3A4_25	gtctatcatcaaatggga	fw	11313-11331	CYP3A4_25	ctgttctgcaactactac	rev	11630-11649
CYP3A4_26	tcctgagattggataatcat	fw	11501-11520	CYP3A4_26	atcagtggaattcagtagat	rev	11650-11670
CYP3A4_27	ggaacaggtcactgtggtgc	fw	11801-11820	CYP3A4_27	cttaaggctggccggggac	rev	11848-11866
CYP3A4_28	cttgctgaccgggtgggggc	fw	11870-11889	CYP3A4_28	gccaggaatcagccagcat	rev	11921-11940
CYP3A4_29	gactcaacaatgataataag	fw	12011-12030	CYP3A4_29	gataaaatgttatgtcc	rev	12128-12145
CYP3A4_30	ggtaagctgggctttctc	fw	12031-12050	CYP3A4_30	tgcaattctcttgataaa	rev	12140-12159
CYP3A4_31	gaaatattcaacctgttca	fw	12175-12194	CYP3A4_31	catgcatcaccatgctggc	rev	12346-12366
CYP3A4_32	gagcctgggaggtctcggt	fw	12412-12430	CYP3A4_32	cttttaactctgtgattgatac	rev	12539-12562
CYP3A4_33	gcaaacagctcactctttagg	fw	12673-12693	CYP3A4_33	ctaggtagggtactccaccac	rev	12773-12793
CYP3A4_34	atggctctgtctgaaaggt	fw	12803-12822	CYP3A4_34	cttccaaacccagtgactt	rev	13012-13031
CYP3A4_35	cttctgaccctctgctttc	fw	13207-13226	CYP3A4_35	gtgattcacctgggtcaac	rev	13326-13345
CYP3A4_36	aagaaaattccctgcttatta	fw	13568-13587	CYP3A4_36	acagagaaatagataaata	rev	13738-13757
CYP3A4_37	caggccaattctgtcagtt	fw	16675-16694	CYP3A4_37	acaccaaacagccacacaaa	rev	16910-16929
CYP3A4_38	ccaggaggtggaacaacag	fw	17386-17405	CYP3A4_38	attttgcatgttgggtgac	rev	17509-17528
CYP3A4_39	tgcttctctgaattgtgagat	fw	17853-17873	CYP3A4_39	caaccaaagttgcttctgtc	rev	18032-18052
CYP3A4_40	tcctttgcagaacaacacca	fw	18278-18297	CYP3A4_40	tcacctaaccagagaccttt	rev	18519-18539
CYP3A4_41	ccaatcagaccactctca	fw	18379-18398	CYP3A4_41	ttacagatgagaactg	rev	18947-18964
CYP3A4_42	atagtgatgctgtagcagg	fw	18814-18831	CYP3A4_42	aattatcagctcattatc	rev	19588-19607
CYP3A4_43	ggaggcttagggccagagg	fw	19368-19387	CYP3A4_43	caggagctgtagagatgag	rev	19688-19707
CYP3A4_44	agcgaacagcagacgcttaa	fw	19468-19487	CYP3A4_44	gcaatgatgggctggtgact	rev	19835-19857
CYP3A4_45	gcgaacaagcagacgtttaa	fw	19469-19488	CYP3A4_45	ctggaatactgcatgtctg	rev	19928-19947
CYP3A4_46	ttcacacctatcactgag	fw	19608-19627	CYP3A4_46	accctaaaggagagacagg	rev	20078-20097
CYP3A4_47	ggcctgccctctccatt	fw	19708-19727	CYP3A4_47	ggtcattaatacagccagag	rev	20168-20187
CYP3A4_48	tggctgaggtggtggggtc	fw	19858-19877	CYP3A4_48	caagggcatagctagtcttatt	rev	20320-20342
CYP3A4_49	agagaaggggccaactcttg	fw	19948-19967	CYP3A4_49	catcccttcatgcaatcct	rev	20411-20430
CYP3A4_50	ccccttccaacagaatcac	fw	20098-20117	CYP3A4_50	acacctacagatctttacct	rev	20568-20587
CYP3A4_51	taagaagtcaccagaagtc	fw	20188-20207	CYP3A4_51	gtggagcattggcataaaa	rev	20668-20687
CYP3A4_52	aggagctcactctgtctcag	fw	20343-20362	CYP3A4_52	tcaccttggaaagttggcaa	rev	20808-20827
CYP3A4_53	ggcttgtgggatgaatttc	fw	20588-20607	CYP3A4_53	gaggctgtggatgtttat	rev	20908-20927
CYP3A4_54	ttgagtttctgataagaacc	fw	20688-20707	CYP3A4_54	tccttcagctctgtgttgc	rev	20983-21002
CYP3A4_55	gaagccttctcaactcag	fw	20828-20847	CYP3A4_55	cctgattagcaccacaagtc	rev	21148-21167
CYP3A4_56	gactcagaggagagagata	fw	21004-21022	CYP3A4_56	ctgacaccatgcccagc	rev	21348-21367

List of the primers which were used for the screening of upstream CYP3A4 gene region in chromatin immunoprecipitation analysis using SYBR-Green technology.

Direct Transcriptional Regulation of Human Hepatic Cytochrome P450 3A4 (CYP3A4) by Peroxisome Proliferator-Activated Receptor Alpha (PPAR α)

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Matthias Schwab, Ulrich M. Zanger
Molecular Pharmacology

Supplemental Table 2: Oligonucleotides used in EMSA

Motif	+/- strand oligonucleotides
PPRE (rat acyl CoA oxidase)	GAT CTG AAC GTG ACC TTT GTC CTG GTC CG GAT CCG GAC <u>CAG GAC AAA GGT CAC</u> GTT CA
DR1-A	GAT CCT TAG <u>GGT ACA AAG TTG ACC</u> AAG A GAT CTC TTG GTC AAC TTT GTA CCC TAA G
mut DR1-A	GAT CCT TAG <i>GCC</i> <u>ACA AAC CTG ACC</u> AAG A GAT CTC TTG GTC AGG TTT GTG GCC TAA G
DR1-B	GAT CCG AGA TTG AAC TTT GAT CCA ATG A GAT CTC ATT <u>GGA TCA AAG TTC AAT</u> CTC G
mut DR1-B	GAT CCG ATT GAA GGA AGA TCC AAT A GAT CTA TTG <u>GAT <i>CTT CCT</i> TCA</u> ATC G
DR1/DR2-C	GAT CCT GTC <u>CCA ATT AAA GGT CAT AAA GCC CAG</u> TTT A GAT CTA AAC TGG GCT TTA TGA CCT TTA ATT GGG ACA G
mut DR1/DR2-C	GAT CCT GTC <u>CCA ATT AAA <i>CAA</i> CAT AAA GCC CAG</u> TTT A GAT CTA AAC TGG GCT TTA TGT TGT TTA ATT GGG ACA G
DR1-D	GAT CCT CAC <u>AAG GGC AAA GGA CAT</u> GAA CA GAT CTG TTC ATG TCC TTT GCC CTT GTG AG
mut DR1-D	GAT CCT CAC <u>AAC <i>CGC</i> AAA <i>CCA</i> CAT</u> GAA CA GAT CTG TTC ATG TGG TTT GCG GTT GTG AG

Within the respective strand showing the AGGTCA consensus motif, DR1/DR2 motifs are underlined and mutated bases are indicated in bold and italics.

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Supplemental Table 3: Gene expression analysis (fold changes) by qRT-PCR

GENE	RNAI/1	RNAI/2	RNAI/3	RNAI	StDev	WY14,643/1	WY14,643/2	WY14,643/3	WY14,643	StDev	POPC1	POPC2	POPC3	POPC	StDev
ABC1	1.15	1.02	0.86	1.01	0.12	1.16	1.27	0.19	0.87	0.49	1.11	0.67	1.21	1.00	0.24
ABC2	0.96	0.85	1.13	0.98	0.12	1.13	1.24	1.18	1.18	0.04	0.96	0.67	1.25	0.96	0.24
ABCG2	1.23	1.28	1.13	1.21	0.06	1.20	0.78	1.02	1.00	0.17	1.09	1.12	0.86	1.02	0.12
ACOX1	0.79	0.57	0.63	0.66	0.09	1.45	2.67	1.84	1.99	0.51	1.48	1.51	1.68	1.56	0.09
ADH1A	1.00	0.86	1.03	0.96	0.07	1.12	1.06	1.17	1.12	0.05	0.92	1.12	1.08	1.04	0.09
AHR	1.09	1.23	0.86	1.06	0.15	0.90	1.24	1.31	1.15	0.18	0.86	0.97	1.02	0.95	0.07
ALAS1	0.77	0.68	0.53	0.66	0.10	1.67	1.86	2.17	1.90	0.21	1.82	2.53	1.67	2.01	0.38
ALDH2	0.86	0.75	1.26	0.96	0.22	0.87	1.13	1.57	1.19	0.29	0.95	1.12	1.37	1.15	0.17
ARNT	0.89	1.21	0.86	0.99	0.16	0.91	1.12	1.02	1.02	0.08	0.93	1.18	1.24	1.12	0.14
CCL2	0.64	0.72	0.84	0.73	0.08	1.15	1.34	0.95	1.15	0.16	0.72	0.97	1.12	0.94	0.16
CEBP	1.27	0.94	1.12	1.11	0.13	1.14	1.21	1.07	1.14	0.06	1.03	1.27	1.11	1.14	0.10
CPT1A	0.62	0.73	0.53	0.63	0.08	3.28	4.24	2.78	3.43	0.61	2.49	1.86	1.67	2.01	0.35
CREBBP	1.29	1.11	0.86	1.09	0.17	1.05	0.98	1.12	1.05	0.06	1.01	0.94	1.27	1.07	0.14
CRP	0.66	0.82	0.54	0.67	0.11	1.86	1.54	1.77	1.72	0.13	1.89	1.92	1.77	1.86	0.06
CYP1A1	0.31	0.61	0.43	0.45	0.12	1.84	1.54	1.89	1.76	0.15	1.37	1.54	1.67	1.53	0.12
CYP1A2	0.78	0.56	0.42	0.59	0.15	1.34	1.67	1.89	1.63	0.23	1.89	1.57	1.87	1.78	0.15
CYP2A6	1.04	0.86	0.91	0.94	0.08	1.70	1.34	1.24	1.43	0.20	1.67	1.31	1.11	1.36	0.23
CYP2B6	0.76	0.54	0.59	0.63	0.09	1.70	2.15	2.67	2.17	0.39	1.74	1.57	1.67	1.66	0.07
CYP2C19	1.07	0.96	1.21	1.08	0.10	1.21	1.24	1.02	1.16	0.10	1.13	0.95	1.15	1.08	0.09
CYP2C8	0.59	0.37	0.48	0.48	0.09	2.19	3.45	2.87	2.84	0.52	3.67	3.89	3.45	3.67	0.18
CYP2C9	0.92	1.09	1.12	1.04	0.09	1.11	0.94	0.68	0.91	0.18	1.21	1.08	1.31	1.20	0.09
CYP2D6	0.92	1.25	0.73	0.97	0.21	1.96	1.51	0.68	1.38	0.53	1.49	1.57	0.86	1.31	0.32
CYP2E1	0.82	1.25	1.34	1.14	0.22	1.07	1.32	1.08	1.16	0.12	0.97	0.67	1.44	1.03	0.32
CYP3A4	0.56	0.72	0.63	0.64	0.07	1.80	2.42	2.65	2.29	0.36	4.12	1.89	1.27	2.43	1.22
CYP3A5	0.54	0.37	0.68	0.53	0.13	1.24	1.78	1.36	1.46	0.23	1.56	1.67	1.74	1.66	0.07
CYP3A7	0.68	0.87	0.51	0.69	0.15	1.30	1.66	1.83	1.60	0.22	1.64	1.81	1.76	1.74	0.07
CYP7A1	0.19	0.24	0.57	0.33	0.17	0.47	0.51	0.37	0.45	0.06	0.37	0.36	0.48	0.40	0.05
DPVD	0.67	0.91	1.24	0.94	0.23	0.85	1.21	1.12	1.06	0.15	0.83	1.18	1.34	1.12	0.21
ELK1	1.01	1.24	1.25	1.17	0.11	0.95	0.89	1.02	0.95	0.05	0.95	1.08	1.17	1.07	0.09
FABP1	0.64	0.43	0.55	0.54	0.08	2.70	3.12	2.74	2.85	0.19	1.88	2.18	2.18	2.08	0.14
FDFIT1	0.92	1.19	1.21	1.11	0.13	1.17	1.12	1.02	1.10	0.06	1.17	1.18	1.27	1.21	0.04
FOS	1.13	1.63	0.34	1.03	0.53	1.77	1.48	1.36	1.54	0.17	1.41	1.37	1.58	1.45	0.09
FOXO1	1.21	0.84	0.67	0.91	0.22	1.00	0.85	0.67	0.84	0.14	0.97	1.08	0.94	1.00	0.06
GGPC	0.95	1.12	0.87	0.98	0.10	1.12	0.97	1.11	1.07	0.07	1.09	1.17	1.18	1.15	0.04
GSTA2	0.62	1.12	1.32	1.02	0.30	0.83	0.92	1.54	1.10	0.32	0.90	1.12	1.27	1.10	0.15
GSTM1	0.87	1.15	0.67	0.90	0.20	0.83	0.56	1.24	0.88	0.28	0.95	0.92	0.94	0.94	0.01
GSTP1	1.01	1.24	0.86	1.04	0.16	0.93	1.08	1.33	1.11	0.16	0.86	1.05	1.21	1.04	0.14
HK2	1.10	0.75	0.36	0.74	0.30	1.09	1.12	1	1.07	0.05	1.04	0.86	1.15	1.02	0.12
HMGCR	0.98	1.05	0.84	0.96	0.09	0.92	1.67	1.88	1.49	0.41	1.00	1.27	1.57	1.28	0.23
HMGCS2	0.68	0.42	0.37	0.49	0.13	4.06	3.12	4.65	3.94	0.63	3.34	2.67	3.12	3.04	0.28
HMOX1	1.29	1.19	1.24	1.24	0.04	1.74	1.57	1.21	1.51	0.22	1.63	1.27	1.54	1.48	0.15
HNF1A	0.77	0.57	0.61	0.65	0.09	0.97	1.34	1.27	1.19	0.16	1.01	1.21	1.08	1.10	0.08
HNF4A	0.72	0.76	0.84	0.77	0.05	0.91	1.34	1.12	1.12	0.18	0.89	0.96	1.08	0.98	0.08
IL1b	1.39	1.58	2.4	1.79	0.44	0.45	0.64	0.75	0.61	0.12	0.66	0.53	0.42	0.54	0.10
IL6	1.57	1.86	1.57	1.67	0.14	0.86	0.48	0.77	0.70	0.16	0.87	0.94	0.76	0.86	0.07
IL8	1.47	1.53	1.56	1.52	0.04	1.08	0.82	0.71	0.87	0.16	1.10	0.48	0.86	0.81	0.25
INSIG1	0.63	0.51	0.37	0.50	0.11	0.97	1.12	1.08	1.06	0.06	1.21	1.12	1.08	1.14	0.05
INSIG2	1.44	1.12	1.02	1.19	0.18	0.90	1.05	1.16	1.04	0.10	0.86	1.28	0.57	0.90	0.29
JUN	1.96	1.24	1.12	1.44	0.37	1.05	0.94	0.86	0.95	0.08	1.02	1.18	0.97	1.06	0.09
NAT1	0.75	0.86	1.04	0.88	0.12	1.10	1.34	1.25	1.23	0.10	0.99	1.02	1.34	1.12	0.16
NAT2	0.80	0.94	1.21	0.98	0.17	0.99	1.27	1.26	1.17	0.13	0.96	1.27	1.64	1.29	0.28
NCOA1	1.14	1.12	1.08	1.11	0.02	1.12	1.35	1.23	1.23	0.09	1.09	0.97	0.84	0.97	0.10
NCOA2	0.93	1.24	1.08	1.08	0.13	0.98	0.86	0.67	0.84	0.13	1.02	0.67	1.12	0.94	0.19
NCOA3	1.30	1.34	1.21	1.28	0.05	1.08	1.21	1.02	1.10	0.08	1.02	0.67	0.98	0.89	0.16
NFKBIA	1.16	1.02	1.32	1.17	0.12	1.41	1.24	1.26	1.30	0.07	1.23	1.32	1.12	1.22	0.08
NOS2	1.95	1.24	1.65	1.61	0.29	4.88	3.24	2.67	3.60	0.94	2.67	2.15	2.77	2.53	0.27
NR0B2	1.78	1.54	1.86	1.73	0.14	1.05	1.53	1.45	1.34	0.21	0.95	1.12	1.34	1.14	0.16
NR1H3	0.76	0.86	0.98	0.87	0.09	1.14	1.23	1.27	1.21	0.05	1.10	1.17	1.11	1.13	0.03
NR1H4	0.84	0.95	1.14	0.98	0.12	0.87	1.18	1.64	1.23	0.32	0.90	0.92	0.87	0.90	0.02
NR1I2	0.89	0.64	0.71	0.75	0.10	1.48	1.34	1.58	1.47	0.10	0.99	1.34	1.54	1.29	0.23
NR1I3	0.79	0.76	0.54	0.70	0.11	1.71	1.67	2.34	1.91	0.31	1.18	1.76	1.87	1.60	0.30
NR2F1	0.82	0.94	1.31	1.02	0.21	0.83	0.89	0.91	0.88	0.03	0.88	1.34	1.28	1.17	0.20
NR2F2	0.97	1.24	0.58	0.93	0.27	0.88	0.94	0.93	0.92	0.03	1.03	1.12	1.38	1.18	0.15
NR3C1	1.22	1.56	1.57	1.45	0.16	0.90	0.76	0.58	0.75	0.13	0.87	0.95	0.99	0.94	0.05
PCK1	1.44	1.57	0.92	1.31	0.28	1.29	1.05	1.23	1.19	0.10	1.13	1.34	1.18	1.22	0.09
PDK4	0.38	0.55	0.48	0.47	0.07	4.35	5.34	5.67	5.12	0.56	3.79	3.75	3.64	3.73	0.06
POLR2A	0.97	1.12	1.33	1.14	0.15	0.98	1.16	1.31	1.15	0.14	0.95	0.96	0.97	0.96	0.01
PON1	0.68	0.57	0.64	0.63	0.04	1.47	1.34	1.54	1.45	0.08	1.44	1.54	1.67	1.55	0.09
POR	1.27	0.7	0.64	0.87	0.28	1.58	1.62	1.43	1.54	0.08	1.58	1.57	1.34	1.50	0.11
PPARA	0.56	0.48	0.67	0.57	0.08	1.68	1.52	1.87	1.69	0.14	1.05	1.36	1.28	1.23	0.13
PPARG	1.75	1.31	0.85	1.30	0.37	1.05	1.24	1.32	1.20	0.11	1.02	1.37	1.38	1.26	0.17
PTGS2	4.61	1.53	1.75	2.63	1.40	1.01	0.68	0.74	0.81	0.14	0.64	0.84	0.97	0.82	0.14
RXRA	0.79	0.83	0.71	0.78	0.05	0.87	0.89	0.91	0.89	0.02	0.96	0.67	1.24	0.96	0.23
SCD	0.79	0.86	0.92	0.86	0.05	1.52	1.34	1.67	1.51	0.13	1.57	1.67	1.84	1.69	0.11
SLC10A1	0.84	1.32	0.87	1.01	0.22	0.81	0.84	0.97	0.87	0.07	0.81	1.34	1.58	1.24	0.32
SLC22A7	0.62	0.75	1.11	0.83	0.21	1.39	1.37	1.42	1.39	0.02	1.09	0.94	0.88	0.97	0.09
SLCO1B1	0.70	0.67	1.11	0.83	0.20	0.96	1.02	1.34	1.11	0.17	1.04	0.67	0.81	0.84	0.15
SOC3	0.90	0.83	0.67	0.80	0.10	1.34	1.12	1.68	1.38	0.23	1.57	1.34	1.08	1.33	0.20
SOD2	1.69	1.54	1.64	1.62	0.06	0.83	0.92	1.11	0.95	0.12	0.87	1.12	1.34	1.11	0.19
SREBF1	0.86	1.41	1.13	1.13	0.22	1.27	1.34	1.58	1.40	0.13	1.24	1.71	1.02	1.32	0.29
SREBF2	1.01	1.08	0.98	1.02	0.04	0.96	0.94	0.76							