Peroxisome Proliferator-Activated Receptor β/δ (PPAR β/δ) but Not PPAR α Serves as a Plasma Free Fatty Acid Sensor in Liver[†]

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Peroxisome proliferator-activated receptor α (PPAR α) is an important transcription factor in liver that can be activated physiologically by fasting or pharmacologically by using high-affinity synthetic agonists. Here we initially set out to elucidate the similarities in gene induction between Wy14643 and fasting. Numerous genes were commonly regulated in liver between the two treatments, including many classical PPAR α target genes, such as *Aldh3a2* and *Cpt2*. Remarkably, several genes induced by Wy14643 were upregulated by fasting independently of PPAR α , including *Lpin2* and *St3gal5*, suggesting involvement of another transcription factor. Using chromatin immunoprecipitation, *Lpin2* and *St3gal5* were shown to be direct targets of PPAR β/δ during fasting, whereas *Aldh3a2* and *Cpt2* were exclusive targets of PPAR α . Binding of PPAR β/δ to the *Lpin2* and *St3gal5* genes followed the plasma free fatty acid (FFA) concentration, consistent with activation of PPAR β/δ by plasma FFAs. Subsequent experiments using transgenic and knockout mice for *Angptl4*, a potent stimulant of adipose tissue lipolysis, confirmed the stimulatory effect of plasma FFAs on *Lpin2* and *St3gal5* expression levels via PPAR β/δ . In contrast, the data did not support activation of PPAR α by plasma FFAs. The results identify *Lpin2* and *St3gal5* as novel PPAR β/δ target genes and show that upregulation of gene expression by PPAR β/δ is sensitive to plasma FFA levels. In contrast, this is not the case for PPAR α , revealing a novel mechanism for functional differentiation between PPARs.

Hepatic lipid metabolism is governed by a complex interplay between hormones, transcription factors, and energy substrates, allowing for rapid adaptations to changes in metabolic needs (21). According to the traditional view, energy substrates such as fatty acids influence lipid metabolism by promoting flux through a particular pathway via mass action. However, it has become clear that energy substrates can also directly govern the transcription of enzymes involved in lipid metabolism via mechanisms analogous to those of many hormones. Indeed, it is now evident that glucose and fatty acids play a major regulatory role in hepatic lipid metabolism via direct activation or inhibition of specific transcription factors, including carbohydrate response element binding protein (6, 63), sterol response element binding protein 1 (SREBP1) (2, 41, 58, 61, 62), and peroxisome proliferator-activated receptor α (PPAR α) (38).

Although numerous transcription factors have been shown to be activated by fatty acids *in vitro*, recent data suggest that PPAR α is dominant in mediating the effects of dietary fatty acids on gene expression in liver (48). PPAR α is a member of the superfamily of nuclear receptors and is closely related to

the other PPAR isoforms, β/δ and γ (32). Similar to several other nuclear receptors, PPARs function as heterodimers with the retinoid X receptor and bind to specific sequences on the DNA referred to as PPAR response elements (PPREs) (8, 11, 26). Numerous studies have shown that fatty acids can directly bind to PPARs and activate DNA transcription (12, 17, 24, 28, 31, 50). Binding of fatty acids changes the conformation of the PPAR protein (13, 23, 37, 60) and leads to recruitment of coactivator proteins (31, 48). Besides fatty acids and their derivatives, PPARs bind synthetic agonists, including the thiazolidinediones, which serve as agonists for PPAR γ , and the fibrates, which are PPAR α agonists (51).

Most of the information about the function of PPAR α in liver and its impact on target genes is based on studies that have used high-affinity synthetic PPAR α agonists. These pharmacological studies have shown that PPAR α regulates a remarkably large number of genes, many of which are involved in hepatic lipid metabolism, thereby explaining the positive effect of synthetic PPAR α agonists on plasma lipid parameters (9, 38). However, PPAR α did not evolve as a receptor for fibrates but rather as a fatty acid sensor. Accordingly, the question arises to what extent results from pharmacological studies reflect the physiological function of PPAR α .

Physiological experiments using PPAR $\alpha^{-/-}$ mice have shown that PPAR α is especially important for the adaptive response to fasting. During fasting, the absence of PPAR α elicits a complex phenotype characterized by fatty liver, hypo-

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ketonemia, hypoglycemia, hypothermia, and elevated plasma free fatty acid (FFA) levels (1, 19, 27, 34). Furthermore, the hepatic induction of numerous metabolic genes during fasting is abolished in PPAR α ^{-/-} mice. While both pharmacological and physiological studies thus support a major role for PPAR α in hepatic lipid metabolism, evidence suggests that there is only partial overlap between genes upregulated by PPAR α during fasting and genes upregulated by synthetic PPAR α agonists (45). One possible explanation is that PPAR α responds differently to pharmacological compared to physiological activation. Additionally, there may be a role for other PPAR subtypes. Besides PPAR α , PPAR β/δ has been shown to be well expressed in hepatocytes (10, 22). However, the functional role of PPAR β/δ in hepatocytes and its physiological mechanisms of activation remain unknown.

Here we initially set out to elucidate the similarities and discrepancies in gene regulation in liver between pharmacological PPAR α activation by Wy14643 and physiological PPAR α activation by fasting. While our data reveal major overlap between the effects of Wy14643 and fasting, the data also indicate that a number of pharmacological PPAR α target genes are induced by fasting independently of PPAR α . Subsequent analysis uncovered a role for PPAR β/δ in hepatic gene regulation and revealed different mechanisms of activation of PPAR α versus PPAR β/δ in mouse liver. Specifically, we found that upregulation of gene expression by PPAR β/δ is sensitive to plasma FFAs, while this is not the case for PPAR α .

MATERIALS AND METHODS

Materials. Tridocosahexaenoin was obtained from Nu-Chek-Prep, Inc. (Elysian, MN). SYBR green was purchased from Eurogentec (Seraing, Belgium). Protease inhibitor cocktail was purchased from Roche Diagnostics (Almere, The Netherlands), sonicated salmon sperm DNA was from Invitrogen (Breda, The Netherlands), and proteinase K was from Fermentas (St. Leon-Rot, Germany). All other chemicals were from Sigma (Zwijndrecht, The Netherlands).

Animals. Purebred Sv129 PPAR α ^{-/-} mice (129S4/SvJae) and corresponding wild-type mice (129S1/SvImJ) were purchased from Jackson Laboratory (Bar Harbor, ME). The PPAR β/δ ^{-/-} mice were on a mixed background (Sv129/C57BL/6) and have been previously described (42). The *Angptl4*^{-/-}, *Angptl4*^{+/-}, and transgenic mice were on a C57BL/6 background and have been previously described (30, 36, 39). *Angptl4*-transgenic mice overexpress *Angptl4* in numerous tissues, including adipose tissue (36, 39), while the *Angptl4*^{-/-} mice lack *Angptl4* expression in all tissues (30). Only male mice were used, with 4 to 10 mice per group. For the fasting experiment, food was withdrawn for 24 h starting at the onset of the light cycle.

PPAR α ligand testing. Wild-type and PPAR α ^{-/-} mice were fasted for 4 h and thereafter given an intragastric gavage of 400 μ l Wy14643 (10 mg/ml in 0.5% carboxymethyl cellulose; 160 mg/kg of body weight). The control treatment was 400 μ l of 0.5% carboxymethyl cellulose. Livers were collected after 6 h.

Oral lipid load testing. Wild-type and PPAR α ^{-/-} mice were given an intragastric gavage of 400 μ l synthetic triglyceride (tridocosahexaenoin) after a 4-hour fast. The control treatment was 0.5% carboxymethyl cellulose (400 μ l). Livers were collected 6 h after gavage.

PPAR β/δ ligand testing. Wild-type mice were given a single oral gavage of 150 μ g GW501516 (6 mg/kg). Alternatively, PPAR α ^{-/-} mice were fed 0.025% (wt/wt) L165041 mixed in food for 5 days (40 mg/kg).

Mice were anesthetized with a mixture of isoflurane (1.5%), nitrous oxide (70%), and oxygen (30%). Blood was collected by orbital puncture, after which the mice were sacrificed by cervical dislocation. Livers were dissected and directly frozen in liquid nitrogen. For RNA analyses, tissue from the same part of the liver lobe was used.

The animal studies were approved by the Local Committee for Care and Use of Laboratory Animals at Wageningen University, The Netherlands, and the University of Lausanne, Switzerland.

Affymetrix microarray. Total RNA from mouse liver was extracted with TRIzol reagent, purified, and DNase treated using the SV total RNA isolation

system (Promega, Leiden, The Netherlands). RNA quality measurements were performed on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands) using 6000 Nano Chips in combination with the eukaryote total RNA Nano assay. RNA was judged as suitable for array hybridization only if samples showed intact bands corresponding to the 18S and 28S rRNA subunits, displayed no chromosomal peaks or RNA degradation products, and had an RNA integrity number above 8.0. Five micrograms of RNA was used for the cRNA synthesis mixture for one cycle (Affymetrix, Santa Clara, CA). Hybridization, washing, and scanning of Affymetrix mouse genome 430 2.0 arrays/Affymetrix Nugo mouse arrays were carried out according to standard Affymetrix protocols.

Packages from the Bioconductor Project were used for analyzing the scanned arrays (14). Arrays were normalized using quantile normalization, and expression estimates were compiled using GC-RMA (GeneChip robust multiarray average), applying the empirical Bayes approach (59). A nonspecific filtering step was applied to remove genes with low variation and included only those genes that had an interquartile range across the samples of at least 0.25 on a log₂ scale (55).

Gene set enrichment analysis (GSEA) was used to relate changes in gene expression to functional changes between mice treated with the PPAR α agonist Wy14643 for 6 h and mice fasted for 24 h. GSEA takes into account a broad context of physically interacting networks in which gene products function, including biochemical, metabolic, and signal transduction routes (53). Gene sets with a false discovery rate *P* value of <0.1 were considered significantly over-represented.

Plasma metabolites. Plasma was obtained from blood by centrifugation for 10 min at 10,000 $\times$ g. Plasma triglycerides and the glycerol concentration in cell culture medium were determined using kits from Instruchemie (Delfzijl, The Netherlands). Plasma free fatty acids were determined using a kit from WAKO Chemicals (Instruchemie, Delfzijl, The Netherlands).

Fat explants. Epididymal adipose tissue was excised and cut into 0.2- to 0.3-cm³ pieces. The explants were subsequently incubated for 15 min at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 1% lipid-free bovine serum albumin (BSA) and 1 mg/ml collagenase type 1. Fat cells were liberated by gentle stirring followed by centrifugation of the cell suspension for 1 min at 400 $\times$ g. Fat cells were isolated from the surface and washed once in phosphate-buffered saline (PBS). Subsequently, fat cells were incubated in DMEM containing 1% lipid-free BSA and 1 μ M isoproterenol at 37°C with 5% CO₂. Medium was collected at various time points and frozen for measurement of free fatty acids and glycerol (kits from Instruchemie, Delfzijl, The Netherlands).

RNA isolation and quantitative reverse transcription-PCR. Total liver RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. A NanoDrop ND-1000 spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands) was used to determine RNA concentrations. One microgram of total RNA was reverse transcribed using iScript (Bio-Rad, Veenendaal, The Netherlands). cDNA was amplified on a Bio-Rad MyIQ or iCycler PCR machine using Platinum *Taq* DNA polymerase (Invitrogen). PCR primer sequences were taken from the PrimerBank (56) and ordered from Eurogentec. Sequences of the primers used are available upon request.

Transactivation assay. Conserved PPREs were identified at 1,291 or 23,333 nucleotides downstream of the transcriptional start site (TSS) of the mouse *Lpin2* or *St3gal5* gene, respectively, using a published algorithm (20). A 201-nucleotide and 183-nucleotide fragment surrounding the putative PPRE within the *Lpin2* and *St3gal5* genes, respectively, was PCR amplified from mouse genomic DNA (strain C57BL/6) and subcloned into the KpnI and BglII sites of the pGL3 promoter vector (Promega, Leiden, The Netherlands). The reporter vector (PPRE)₃-TK-luciferase was included as a positive control. Reporter vectors were transfected into human hepatoma HepG2 cells together with an expression vector (pSG5) for mouse PPAR β/δ , in the presence or absence of GW501516 (1 μ M). A β -galactosidase reporter vector was cotransfected to normalize for differences in transfection efficiencies. Transfections were carried out using Nanjuice (Novagen, Nottingham, United Kingdom). Luciferase activity was measured 24 h posttransfection using the Promega luciferase assay kit on a Fluoroskan Ascent FL apparatus (Thermo Labsystems, Breda, The Netherlands). β -Galactosidase activity was measured in the cell lysate by a standard assay using 2-nitrophenyl- β -D-galactopyranoside as a substrate.

For the GAL4 transactivation assay, the human osteosarcoma cell line U2OS was maintained in DMEM Glutamax containing 10% fetal calf serum (Invitrogen), 100 μ g of penicillin/ml, and 100 μ g streptomycin/ml (Invitrogen). For luciferase reporter assays, cells were seeded in 24-well plates and transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Each well was cotransfected with 1 μ g of the 5 $\times$ GAL4-E1BTATApGL3 reporter, 10 ng of pcDNA3-GAL4-hPPAR δ DBD, and 2 ng of pCMV-

Renilla (Promega, Madison, WI). The next day, medium was replaced with medium containing ligands. At 24 h after the incubation, cells were harvested in passive lysis buffer (Promega) and assayed for luciferase activity according to the manufacturer's protocol (Promega dual-luciferase reporter assay system) and for *Renilla* luciferase activity to correct for transfection efficiency. The relative light units were measured by a Centro LB 960 luminometer (Berthold Technologies, Bad Wildbad, Germany). Experiments were performed in triplicate.

Chromatin immunoprecipitation (ChIP) assay. It is becoming increasingly apparent that most nuclear receptor binding sites, including PPREs, are not found in proximity of the annotated TSS of a gene but are often located quite distant (4, 33, 43). Nuclear receptors bound to such distal sites likely contact the basal transcription machinery via DNA looping. Binding of PPAR to distant PPREs can thus be demonstrated by showing cross-linking of PPAR to the TSS (5, 49).

Wild-type and PPAR $\alpha^{-/-}$ mice on an Sv129 background were fed or fasted for 24 h ($n = 3$). Transgenic mice overexpressing *Angptl4* (*Angptl4*-Tg), wild-type mice (*Angptl4* $^{+/+}$), and homozygous knockout mice (*Angptl4* $^{-/-}$) ($n = 3$ /group) were fasted for 24 h. At the end of the fasting period, mice were killed by cervical dislocation and livers were extracted. Livers were cut in smaller pieces and directly put in PBS containing 1% formaldehyde. Cross-linking was stopped after 15 min by adding glycine to a final concentration of 0.125 M for 5 min at room temperature. After a short centrifugation to collect the liver pieces, two washing steps with ice-cold PBS were carried out. Livers were homogenized and thereafter centrifuged. After supernatant was removed, liver homogenate was resuspended in lysis buffer (1% sodium dodecyl sulfate [SDS], 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, protease inhibitors), and the lysates were sonicated with a Bioruptor (Diagenode, Liège, Belgium) to achieve a DNA length of 300 to 1,000 bp. After removal of cellular debris by centrifugation, supernatants were diluted 1:10 in ChIP dilution buffer (150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 7.5, protease inhibitors). Chromatin was incubated overnight at 4°C with 2 μ g antibody, 25 μ l BSA (10 mg/ml), and 2.4 μ l sonicated salmon sperm (10 mg/ml). Antibodies used were anti-PPAR α (sc-9000), anti-PPARGC1 α (sc-13067), and anti-PPAR β/δ (sc-7197), all of which were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Immunocomplexes were collected with 25 μ l MagaCell protein A magnetic beads (Isogen Life Science) for 1 h at room temperature and subsequently washed sequentially with 700 μ l of the following buffers: twice with ChIP wash buffer 1 (150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8, protease inhibitors), once with ChIP wash buffer 2 (500 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1% SDS, 20 mM Tris-HCl, pH 8, protease inhibitors), once with ChIP wash buffer 3 (250 mM LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8), and two times with TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8). Elution of immunocomplexes was carried out in 250 μ l of elution buffer (10 mM EDTA, 0.5% SDS, 25 mM Tris-HCl, pH 7.5) at 64°C for 30 min. After collection of supernatant, elution was repeated with 250 μ l elution buffer at room temperature for 2 min. After combining the supernatants, cross-linking was reversed at 64°C overnight with 2.5 μ l proteinase K (20 mg/ml) for digestion of any remaining proteins. Genomic DNA fragments were recovered by phenol-chloroform extraction with phase lock gel (Eppendorf, Wessling-Berzdorf, Germany), followed by salt-ethanol precipitation. Samples were diluted in sterile H₂O and analyzed by quantitative PCR.

Primers were from Eurogentec and designed to cover the TSS of the following genes: *Aldh3a2* (forward [F], 5'-CAGGTGAGGGAGCAGTAC-3'; reverse [R], 5'-CGCTTGGCTCTTTCTGAAG-3'); *Cpt2* (F, 5'-GCCAGTCACGCAACAGCAG-3'; R, 5'-TAGTTTAGAGACCGCTCCG-3'); *Lpin2* (F, 5'-CCGTCTTGATTGGGAGG-3'; R, 5'-GAAGGAACTCACCAGATCC-3'); *St3gal5* (F, 5'-GCCTTCCACTATCTAATCAG-3'; R, 5'-GTGTCCGCTCTGCGACTG-3'); *Rplp0* (F, 5'-CGAGGACCGCTGGTTCTC-3'; R, 5'-GTCACTGGGAGAGAGAGG-3').

FAO cell culture. Rat hepatoma FaO cells were grown in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were incubated with albumin only (control), albumin-bound oleic or linoleic acid (100 μ M; molar ratio for FA:BSA of 3.5:1), or GW501516 (100 nM) for 4 h, followed by RNA isolation and quantitative reverse transcription-PCR.

Cofactor recruitment assay. Nuclear receptor PamChip arrays (PamGene, s'Hertogenbosch, The Netherlands) were used as described previously (29). Upon binding a ligand, PPAR β/δ undergoes a conformational change which promotes the formation of a cofactor binding pocket, subsequently allowing interaction with the so-called LxxLL motif within some coregulators. The Pam-Chip arrays consist of 53 peptides encompassing the LxxLL motifs of 22 different coregulator proteins. Briefly, the arrays were incubated with glutathione *S*-transferase-tagged PPAR β/δ ligand binding domain (LBD) (Invitrogen, Breda, The Netherlands) in the presence and absence of ligand (GW501516 at 400 nM;

oleic and linoleic acids at 125 μ M). Quantification of the interaction between PPAR β/δ and coregulators was made using Alexa 488-conjugated anti-glutathione *S*-transferase rabbit polyclonal antibody (Invitrogen).

Microarray accession numbers. All microarray experimental results have been deposited into the Gene Expression Omnibus database under accession number GSE 8396 and GSE 17863.

RESULTS

Overlap in gene regulation between pharmacological and physiological PPAR α activation. PPAR α in liver can be activated pharmacologically by using synthetic agonists such as Wy14643 or physiologically by fasting. To assess the similarities and discrepancies in gene regulation between these two stimuli, we compared microarray data from livers of mice treated with the synthetic PPAR α agonist Wy14643 for 6 h and livers of mice subjected to 24 h of fasting. GSEA showed great similarity and overlap in top-regulated pathways between fasting and Wy14643 treatment, almost all of which corresponded to pathways of lipid metabolism (Fig. 1A). Much less overlap was observed at the individual gene level (Fig. 1B). Nevertheless, a substantial number of genes upregulated by Wy14643 were also induced by fasting. Many of these genes represent classical PPAR α target genes involved in fatty acid catabolism, such as *Acox1*, *Cpt2*, *Aldh3a2*, *Acot8*, *Ehhadh*, and *Hmgcs2*. Consistent with an important role of PPAR α , induction of classical PPAR α target genes by fasting was abolished in PPAR $\alpha^{-/-}$ mice (Fig. 1C and D). In contrast, a number of Wy14643-responsive genes could be identified that were more significantly upregulated by fasting in PPAR $\alpha^{-/-}$ mice than in wild-type mice, suggesting PPAR α -independent regulation during fasting (Fig. 1C and D). Overall, these data indicate that targets of pharmacological PPAR α activation exhibit diverse responses following physiological PPAR α activation by fasting, being either up- or downregulated and showing a variable dependence on PPAR α .

To explore the possible mechanism underlying the more significant induction by fasting of a number of pharmacological PPAR α targets in PPAR $\alpha^{-/-}$ mice compared to wild-type mice, two representative genes were investigated in more detail: *Lpin2* and *St3gal5*. Remarkably, in contrast to classical PPAR α targets *Cpt2* and *Aldh3a2* (Fig. 2A and C), induction of *Lpin2* and *St3gal5* by fasting and dietary fatty acids was largely maintained in PPAR $\alpha^{-/-}$ mice (Fig. 2B and D). These results imply that the effects of fasting and dietary fatty acids on hepatic expression of *Lpin2* and *St3gal5* may be partially mediated by a transcription factor other than PPAR α . On the contrary, effects of fasting and dietary fatty acids on *Cpt2* and *Aldh3a2* are entirely mediated by PPAR α . It should be mentioned that the expression profiles of *Cpt2* and *Aldh3a2* are representative of a large set of classical PPAR α targets (Fig. 1D).

PPAR β/δ as an alternative transcription factor to PPAR α in mouse liver during fasting. One obvious candidate alternative transcription factor is PPAR β/δ , which is well expressed in liver (10, 16). Supporting regulation of *Lpin2* and *St3gal5* by PPAR β/δ , the PPAR β/δ agonists GW501516 and L165041 significantly induced *Lpin2* and *St3gal5* mRNA (Fig. 3A). To establish whether *Lpin2* and *St3gal5* are direct PPAR target genes, we identified a conserved PPRE within the *Lpin2* and *St3gal5* genes (Fig. 3B) and cloned a genomic region encompassing the PPRE in front of a luciferase reporter to perform

FIG. 1. Hepatic genes activated by Wy14643 and fasting show a variable dependence on PPAR α . Livers from wild-type and PPAR α ^{-/-} mice treated with the PPAR α agonist Wy14643 for 6 h or fasted for 24 h were used for gene expression profiling ($n = 4$ to 5 mice per group). (A) Overlap in top-regulated pathways between Wy14643 treatment and fasting according to gene set enrichment analysis. Gene sets with a false discovery rate P value of <0.1 were considered significant. (B) Overlap of upregulated genes between Wy14643 treatment and fasting (criteria for inclusion: $P < 0.01$ and a change of >1.5 -fold). (C) Scatter plot showing the effects of fasting in genes significantly upregulated by Wy14643. The y axis and x axis show the effects of fasting in wild-type and PPAR α ^{-/-} mice, respectively. Red dots represent classical PPAR α target genes, while blue dots are Wy14643-responsive genes that are more significantly upregulated by fasting in the PPAR α ^{-/-} mouse compared to wild type. (D) Heatmap showing the changes (n -fold) of genes compared to the wild-type control/fed state. (Upper panel) Classical PPAR α target genes, showing a PPAR α -dependent increase in gene expression upon Wy14643 treatment as well as fasting. Genes in the lower panel exhibit a PPAR α -dependent induction upon Wy14643 treatment but are induced independently of PPAR α upon fasting.

transactivation assays. GW501516 significantly increased reporter activity for the *Lpin2* and *St3gal5* genomic regions, which was further enhanced by cotransfection with PPAR β/δ (Fig. 3C). In subsequent ChIP experiments, PPAR β/δ as well as PPAR α could be cross-linked to the TSS of the *Lpin2* and *St3gal5* genes, at least in the fasted state, which provided evidence for the presence of a distant functional PPRE (Fig. 3D). These data suggest that *Lpin2* and *St3gal5* genes represent direct PPAR target genes. Interestingly, while fasting increased binding of both PPAR α and PPAR β/δ to the *Lpin2* and *St3gal5* genes, fasting increased binding of only PPAR α to the *Aldh3a2* and *Cpt2* genes (Fig. 3D). No binding of PPAR α and PPAR β/δ to the negative control gene *Rplp0* was observed. All together these data suggest that *Lpin2* and *St3gal5* are dual targets of PPAR α and PPAR β/δ , whereas *Aldh3a2* and *Cpt2* are exclusive targets of PPAR α . In agreement with this notion, induc-

tion of *Lpin2* and *St3gal5* by fasting was partially abolished in PPAR β/δ ^{-/-} mice (Fig. 3E).

Given the more pronounced induction of *Lpin2* and *St3gal5* by fasting in PPAR α ^{-/-} versus wild-type mice, we speculated that either expression of PPAR β/δ may be upregulated in PPAR α ^{-/-} mice as a compensatory mechanism or that ligand activation of PPAR β/δ is enhanced in PPAR α ^{-/-} mice. While we could not detect a change in PPAR β/δ mRNA in PPAR α ^{-/-} mice (data not shown), consistent with the second scenario, plasma FFA levels were markedly elevated in fasted PPAR α ^{-/-} mice (Fig. 3F), which was associated with marked induction of PPAR β/δ binding to the *Lpin2* and *St3gal5* promoter (Fig. 3D). These data suggest that in the absence of PPAR α plasma FFAs can induce *Lpin2* and *St3gal5* expression via PPAR β/δ . It should be noted that levels of plasma FFAs during fasting are unaltered in PPAR β/δ ^{-/-} mice (Fig. 3G).

FIG. 2. *Lpin2* and *St3gal5* are induced during fasting independently of PPAR α . Livers from wild-type and PPAR $\alpha^{-/-}$ mice fasted for 24 h or treated with tridocosahexaenoin (DHA) for 6 h were used for gene expression profiling ($n = 4$ to 5 mice per group). (A and B) Gene expression of classical PPAR α targets *Aldh3a2* and *Cpt2* (A) and *Lpin2* and *St3gal5* (B) in fed and fasted wild-type and PPAR $\alpha^{-/-}$ mice. (C and D) Gene expression of *Aldh3a2* and *Cpt2* (C) and *Lpin2* and *St3gal5* (D) after treatment with the dietary fatty acid tridocosahexaenoin. Error bars represent standard errors of the means. *, significantly different according to Student's t test ($P < 0.05$).

Circulating FFAs activate PPAR β/δ but not PPAR α in mouse liver. Importantly, recent evidence suggests that PPAR α in liver cannot be (ligand) activated by plasma FFAs, while it can be activated by fatty acids synthesized de novo (3). To study the activation of PPAR α and PPAR β/δ by plasma FFAs, we modulated fasting plasma FFA levels by taking advantage of a unique transgenic model system based on whole-body overexpression or inactivation of the mouse *Angptl4* gene, which encodes a prolipolytic factor involved in lipid metabolism (39, 52). Previously, intravenous injection of Angptl4 was shown to cause an immediate increase in plasma FFA (65). In support, Angptl4 increased release of glycerol from 3T3-L1 adipocytes (Fig. 4A).

Consistent with a prolipolytic effect of Angptl4, Angptl4 overexpression in mice was associated with a significant increase in release of fatty acids and glycerol from adipose tissue, whereas the opposite was observed in Angptl4 $^{-/-}$ mice (Fig. 4B). In agreement with these data, fasting plasma FFA levels were increased or decreased upon Angptl4 overexpression or inactivation, respectively (Fig. 4C). In fact, the fasting-induced increase in plasma FFA was entirely blunted in Angptl4 $^{-/-}$ mice. Plasma triglyceride levels were increased or decreased upon Angptl4 overexpression or inactivation (Fig. 4D), respectively, reflecting the well-documented inhibitory effect of Angptl4 on lipoprotein lipase activity (25). Finally, the defective lipolysis in Angptl4 $^{+/-}$ and Angptl4 $^{-/-}$ mice was supported by the absence of changes in adipocyte cell size upon fasting, in contrast to Angptl4-Tg and wild-type mice (Fig. 4E). These results corroborate the stimulatory effect of Angptl4 on adipose tissue lipolysis, which we exploited to study the effect of plasma FFAs on hepatic gene expression.

If hepatic PPAR α is activated by plasma FFAs, expression of classical PPAR α target genes during fasting would be expected to be proportional to the plasma FFA level throughout the various Angptl4 mouse models. Remarkably, rather than going down,

gene expression of classical PPAR α targets *Aldh3a2*, *Cpt2*, and others was stable or went up as plasma FFAs declined (Fig. 5A; see also Fig. S1A in the supplemental material). Expression of *Ppar α* itself, which is auto-regulated, followed a very similar pattern (Fig. 5B), suggesting that regulation of classical PPAR α targets is determined by PPAR α expression level. Supporting the use of the Angptl4 mouse models to study hepatic gene regulation by FFA, hepatic expression of *Srebp1*, which is known to be suppressed by fatty acids, negatively correlated with plasma FFA concentration (data not shown). In combination with previously published data (3), these data strongly suggest that PPAR α is not activated by plasma FFA in mouse liver.

While plasma FFAs seemingly do not activate hepatic PPAR α , data presented above suggested that plasma FFAs induce *Lpin2* and *St3gal5* expression by activating PPAR β/δ , at least when PPAR α is absent. In order to explore activation of hepatic PPAR β/δ by plasma FFA, we first determined that fatty acids are able to bind and activate PPAR β/δ in vitro. In a transactivation assay using the GAL4-LBD_{PPAR β/δ} fusion, oleic and linoleic acids markedly induced luciferase activity, indicating activation of PPAR β/δ (Fig. 5C). Next we studied the effects of oleic and linoleic acids on the interaction between PPAR β/δ and peptides corresponding to specific coregulator-nuclear receptor binding regions (29). Both fatty acids and GW501516 promoted the interaction between PPAR β/δ and numerous coregulator peptides, which demonstrated their binding to PPAR β/δ (Fig. 5D). Finally, oleic and linoleic acids significantly induced expression of the PPAR β/δ marker gene *Adfp* in hepatoma cells (Fig. 5E). These data demonstrate that fatty acids directly activate PPAR β/δ . To assess activation of PPAR β/δ in vivo by circulating FFAs in the presence of PPAR α , we determined binding of PPAR β/δ to the *Lpin2* and *St3gal5* genes in the Angptl4 mouse models by using ChIP. Importantly, independent of PPAR β/δ gene expression levels,

FIG. 4. Angptl4 stimulates adipose tissue lipolysis. (A) Glycerol concentration in medium of 3T3-L1 cells treated for 30 min with isoproterenol or with concentrated conditioned medium of HEK293 cells transfected with mAngptl4. Control cells were treated with conditioned medium of nontransfected HEK293 cells. Error bars represent standard errors of the means. *, significantly different according to Student's *t* test ($P < 0.05$). (B) Increase in fatty acid and glycerol concentrations in medium of adipose tissue explants from transgenic mice overexpressing *Angptl4* (Tg), wild-type (+/+), and homozygous knockout (-/-) mice. Values are corrected for weight of explants. (C and D) Plasma FFAs (C) and triglycerides (D) in transgenic mice overexpressing *Angptl4* (*Angptl4*-Tg) and wild-type (*Angptl4*^{+/+}), heterozygous (*Angptl4*^{+/-}), and homozygous (*Angptl4*^{-/-}) mice fed or in a 24-h-fasted state ($n = 5$). Gray bars, fed state; black bars, 24-h-fasted state. Error bars represent standard errors of the means. Different letters indicate statistically significant differences (Student's *t* test; $P < 0.05$). (E) Eosin and hematoxylin staining of epididymal adipose tissue.

vious data (64), expression of *Pgc1a* went up significantly during fasting (Fig. 6A). Importantly, fasting markedly enhanced binding of PGC1 α to the TSS of the PPAR α target genes *Aldh3a2* and *Cpt2*, which was abolished in PPAR α ^{-/-} mice (Fig. 6B). No binding of PGC1 α to *Rplp0* was observed. These data suggest that upregulation of *Pgc1a* mRNA may contribute to induction of classical PPAR α target genes during fasting via increased PPAR α -dependent binding of PGC1 α to gene promoters.

DISCUSSION

It has been clearly established that PPAR α governs the fasting-induced upregulation of numerous genes involved in hepatic fatty acid oxidation, many of which are direct PPAR α target genes (19, 27, 34). However, it has remained unclear whether elevated plasma FFAs themselves are responsible for the induction of hepatic fatty acid catabolism via enhanced ligand activation of PPAR α (19, 27, 34). Recently, using mice

FIG. 5. Plasma FFAs do not activate hepatic PPAR α . Transgenic mice overexpressing *Angptl4* (*Angptl4*^{Tg}) or wild-type (*Angptl4*^{+/+}), heterozygous (*Angptl4*^{+/-}), and homozygous knockout (*Angptl4*^{-/-}) mice were sacrificed in the fed state or after a 24-h fast ($n = 5$). Results show hepatic gene expression of classical PPAR α targets *Aldh3a2* and *Cpt2* (A), *PPARα* (B), and *PPARβ/δ* (F). Gray bars, fed state; black bars, 24-h-fasted state. Error bars represent standard errors of the means. *, $P < 0.05$. (C) Fatty acids activate PPAR $\beta/δ$ in a transactivation assay using a GAL4-LBD_{PPAR $\beta/δ$} fusion. Fatty acids were used at 125 μ M or 250 μ M (normal or bold plus sign). (D) A nuclear receptor PamChipH assay was used to measure the interaction between PPAR $\beta/δ$ and immobilized peptides corresponding to specific coregulator-nuclear binding regions in the presence and absence of fatty acids (125 μ M). (E) Fatty acids (100 μ M) upregulated expression of PPAR $\beta/δ$ target *Adfp* in rat FaO hepatoma cells. (G) Chromatin was extracted from livers of 24-h-fasted transgenic mice overexpressing *Angptl4* (Tg) and wild-type (+/+) and homozygous *Angptl4* knockout (-/-) mice ($n = 3$ per group). ChIP was performed with antibodies against PPAR α and PPAR $\beta/δ$ on the TSS of *Lpin2*, *St3gal5*, and *Rplp0*. Rabbit IgG was used as a specificity control. Error bars represent standard deviations. Different lowercase letters indicate statistically significant differences (Student's t test; $P < 0.05$). (H) Hepatic gene expression of *Lpin2* and *St3gal5*. Gray bars, fed state; black bars, 24-h-fasted state. Error bars represent standard errors of the means. Different lowercase letters indicate statistically significant differences (Student's t test; $P < 0.05$).

FIG. 6. PPAR α activation during fasting may be mediated by PGC1 α upregulation. (A) Expression of *Pgc1 α* in livers of fed or fasted wild-type mice ($n = 5$). Fasting statistically significantly induced gene expression of *Pgc1 α* ($P < 0.05$). Error bars represent standard errors of the means. (B) Chromatin was extracted from livers of fed and 24-h-fasted wild-type and PPAR $\alpha^{-/-}$ mice ($n = 3$ per group). ChIP was performed with antibodies against PGC1 α on the TSS of *Aldh3a2* and *Cpt2* and the negative control gene *Rplp0*. Rabbit IgG was used as a specificity control. Gray bars, fed state; black bars, 24-h-fasted state. Error bars represent standard deviations. Fasting significantly induced binding of PGC1 α in wild-type but not PPAR $\alpha^{-/-}$ mice ($P < 0.05$).

with liver-specific inactivation of the *Fasn* gene, Chakravarthy et al. showed that unlike dietary fatty acids and de novo-synthesized fatty acids, circulating FFAs fail to activate hepatic PPAR α (3). In the present study, by using mice differentially expressing *Angptl4* we arrived at essentially the same conclusion.

Importantly, our data also suggest that in contrast to PPAR α , hepatic PPAR β/δ can be activated by plasma FFA, which accounts for the plasma FFA- and fasting-dependent upregulation of several genes in wild-type and PPAR $\alpha^{-/-}$ mice, including *Lpin2* and *St3gal5*. Indeed, we demonstrated that *Lpin2* and *St3gal5* expression and binding of PPAR β/δ to the *Lpin2* and *St3gal5* promoter closely mirror plasma FFA levels. The role of PPAR β/δ in gene regulation by plasma FFA during fasting was substantiated by the observation that induction of *Lpin2* and *St3gal5* by fasting is reduced in PPAR $\beta/\delta^{-/-}$ mice.

In contrast to plasma FFAs, evidence abounds indicating that dietary fatty acids are able to activate PPAR α (40, 44, 46). Recently, it was shown that the effects of dietary fatty acids on hepatic gene expression are quantitatively almost entirely mediated by PPAR α (48). Additionally, the present data suggest that dietary fatty acids can also activate PPAR β/δ , as induction of *Lpin2* and *St3gal5* by dietary fat was entirely or partially maintained in PPAR $\alpha^{-/-}$ mice.

It may be argued that the lack of effect of declining plasma FFAs on hepatic PPAR α activation may be because PPAR α , in contrast to PPAR β/δ , is already saturated with fatty acids at low plasma FFA levels, thus allowing no further activation. Previously, it has been shown that fatty acids bind to PPAR β/δ with an about 5- to 10-fold-lower affinity than to PPAR α (60). However, as treatment with synthetic agonists clearly results in more pronounced PPAR α activation compared to fasting (44), the argument of PPAR α saturation is only tenable if we assume fatty acids act as partial agonists that do not elicit full PPAR α activity compared to synthetic agonists. Saturation of PPAR α is also not supported by the findings reported by Chakravarthy et al. (3).

Alternatively, it is conceivable that fatty acids are present in hepatocytes in distinct pools which have different activities toward PPAR α and PPAR β/δ . In this context, it should be realized that dietary fatty acids present in chylomicron remnants are internalized differently compared to plasma FFAs. Whereas the former are liberated after endosomal and lysoso-

mal degradation of cholesteryl esters and triglycerides, plasma FFAs are likely internalized via diffusion as well as via specific fatty acid transport proteins, including CD36. The third contributor to the hepatic fatty acid pool is de novo lipogenesis, a process which occurs in the cytosol. It is presently unclear to what extent these three sources of fatty acids undergo similar metabolic fates. Recent studies support the existence of distinct hepatic fatty acid pools that are differentially shuttled into various metabolic pathways, including oxidation and incorporation into very-low-density lipoprotein (VLDL) triglycerides (67). For example, there is evidence that fatty acids generated by de novo lipogenesis only marginally contribute to VLDL triglycerides, in contrast to plasma FFAs (15). Our present and previous data suggest that in terms of gene regulation, a similar type of segregation occurs between the three sources of fatty acids (3). The mechanism underlying the differential activity of fatty acids from distinct pools toward PPAR α and PPAR β/δ remains unknown. One could hypothesize a role for fatty acid binding proteins (FABPs). It can be speculated that FABP1, which has been shown to interact with PPAR α (57), picks up lipoprotein-derived fatty acids and shuttles them to PPAR α , whereas another FABP expressed in liver such as FABP2 may selectively bind free fatty acids coming from plasma and shuttle them to PPAR β/δ .

An important lingering question is that if plasma FFAs do not activate hepatic PPAR α during fasting, what mechanism accounts for activation of PPAR α -dependent gene regulation during fasting? Previously, a role for PGC1 α in fasting-dependent upregulation of hepatic mitochondrial fatty acid oxidation and ketogenesis was shown (47, 54). Our ChIP analysis indicates enhanced recruitment of PGC1 α , which itself is upregulated by fasting, to classical PPAR α target genes during fasting. Accordingly, activation of PPAR α by fasting may be driven by the increase in PGC1 α expression, although an important role for other coactivators cannot be excluded. Recently, it was shown that PGC1 α cooperates with BAF60a (SMARCD1) to activate transcription of PPAR α target genes involved in peroxisomal and mitochondrial fatty acid oxidation genes (35).

While our data suggest that PPAR β/δ mediates the effects of plasma FFAs on a small set of genes in the liver, the overall importance of PPAR β/δ in hepatic gene regulation by FFAs remains unclear. The same is true for the actual functional role of PPAR β/δ in liver. Presently, combined transcriptom-

ics and metabolomics analyses of livers of PPAR α ^{-/-} and PPAR β/δ ^{-/-} mice are under way to gain more understanding about the role of PPAR β/δ in the liver and to determine the extent to which PPAR α and PPAR β/δ regulate distinct sets of genes and govern distinct metabolic pathways, especially under physiological circumstances.

In this study we have used variable expression of the *Angptl4* gene to create variations in fasting plasma FFA levels in mice. *Angptl4* is a potent inhibitor of lipoprotein lipase and hepatic lipase and decreases uptake of lipoprotein remnants by the liver, thereby decreasing hepatic uptake of dietary fatty acids (36). In addition, it stimulates adipose tissue lipolysis, as shown by the acute increase in plasma FFA upon injection of recombinant *Angptl4* (65) and by elevated plasma FFAs and glycerol levels in mice overexpressing *Angptl4* (39). The prolipolytic effect of *Angptl4* is supported by recent data in humans (52). In the present paper, *Angptl4* markedly induced glycerol release from 3T3-L1 adipocytes. Activation of lipolysis by *Angptl4* was further substantiated by the altered release of fatty acids and glycerol from adipose tissue explants from *Angptl4*-Tg and *Angptl4*^{-/-} mice, as well as by the lack of an increase in FFA during fasting in *Angptl4*^{-/-} mice. As a consequence, hepatic VLDL production is reduced in *Angptl4*^{-/-} mice (7). By inhibiting lipoprotein lipase and stimulating adipose tissue lipolysis, *Angptl4* promotes switching of hepatic fatty acid uptake from remnant-derived fatty acids toward plasma FFAs (39). Importantly, the variations in plasma FFAs in the *Angptl4* mouse models are specifically elicited by fasting, permitting study of the impact of differential plasma FFAs on hepatic gene expression during fasting.

While in vivo and in vitro studies using synthetic PPAR α agonists are extremely relevant to assess the toxicological and pharmacological impacts and significance of PPAR α , it is unclear to what extent they report on the physiological role of PPAR α in liver. Our results reveal that several genes upregulated following pharmacological PPAR α activation are not induced by PPAR α under physiological conditions such as fasting, or are induced by fasting independently of PPAR α but are dependent on PPAR β/δ . It is well known that in reporter assays PPAR α and PPAR β/δ (and PPAR γ) can activate the same genes, suggesting that all PPARs share an intrinsic ability to transactivate any given PPAR target gene. The present data on *Lpin2* and *St3gal5* are consistent with the notion that in vivo the dominant receptor in the regulation of a particular PPAR target is context dependent and importantly may differ between pharmacological and physiological stimuli. Genes other than *Lpin2* and *St3gal5* that have been shown to be activated by both PPAR α and PPAR β/δ include *Adfp* (18), *G0s2* (66), and *Pdk4* (5). Overall, the data imply that studies using high-affinity synthetic PPAR agonists are not perfectly suited to assess the functions of PPARs during normal physiology.

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