PPARα activators and fasting induce the expression of adipose differentiation-related protein in liver[S]

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Abstract The adipose differentiation-related protein (ADFP)/adipophilin belongs to a family of PAT (for perilipin, ADFP, and TIP47) proteins that associate on the surface of lipid droplets (LDs). Except for LD association, a clear role for ADFP has not been found. We demonstrate that ADFP is transcriptionally regulated by peroxisome proliferator-activated receptor α (PPARα) in mouse liver and rat and human hepatoma cells through a highly conserved direct repeat-1 (DR-1) element. Although the ADFP mRNA is highly increased by a synthetic PPARα agonist, the ADFP protein is only substantially increased in cells containing LDs, such as hepatocytes incubated with fatty acids, and in livers of fasted mice. ADFP is induced by fasting even in the absence of a functional PPARα, in marked contrast to the PPARα target gene acyl-coenzyme A oxidase-1. Activation of LXRs, which stimulates LD formation through the activation of lipogenesis, does not affect ADFP mRNA levels. TIP47, another PAT member known to be expressed in liver, was unaffected by all treatments. This constitutively expressed PAT member seems to be less transcriptionally regulated than ADFP. These observations suggest that ADFP is primarily a fasting-induced protein in liver that coats the newly synthesized triacylglycerol-containing LDs formed during fasting.—Dalen, K. T., S. M. Ulven, B. M. Arntsen, K. Solaas, and H. I. Nebb. **PPARα activators and fasting induce the expression of adipose differentiation-related protein in liver. *J. Lipid Res.* 2006.** 47: **931–943.**

Supplementary key words adipose differentiation-related protein • adipophilin • tail-interacting protein of 47 kDa • fatty liver • direct repeat-1 • peroxisome proliferator-activated receptor • peroxisome proliferator-activated receptor response element • promoter • triacylglycerol • lipid droplet

Most tissues are able to store triacylglycerol (TAG), cholesteryl esters (CEs), or lipids in relatively small (<1 μm diameter) lipid droplets (LDs) that can be used as an energy source or for membrane biogenesis (1). Although the interior of those LDs consists largely of neutral lipids, numerous proteins have recently been extracted together with the LD fractions isolated from two different cell lines (2, 3), suggesting that LDs should be viewed more as active intracellular compartments.

Vimentin (4), perilipin (5, 6), and mouse adipose differentiation-related protein (ADFP; adipophilin in humans) (7) were the first proteins experimentally demonstrated to associate with the LD surface. This cellular location was more recently also experimentally confirmed for tail-interacting protein of 47 kDa (TIP47) (8, 9) and S3-12 (10). Perilipin, ADFP, and TIP47 exhibit high sequence identity within an N-terminal motif termed PAT-1 (for perilipin, ADFP, and TIP47) and a more distally located PAT-2 domain (9, 11). The N-terminus of S3-12 shares limited identity with the PAT-1 motif, but the protein shares sequence homology with ADFP and TIP47 in the PAT2 domain and the C terminus (12). S3-12 and the PAT proteins have amphipathic 11-mer helical repeats in common. These repeats are also found in other lipid binding proteins, such as synucleins, apolipoproteins, phosphate cytidyltransferases, and dehydrins (13). The PAT proteins and S3-12 are expressed in distinct tissues, suggesting that they have evolved to fine-tune lipid metabolism according to the particular needs of these tissues (12).

At present, the LD-associating properties have only been thoroughly studied for ADFP and perilipin (14, 15). Perilipin expression is confined to adipose tissue and steroidogenic cells, where the protein associates with TAG and CE droplets, respectively (5, 16, 17). Perilipin has been found to have an important role in the hormonal control of basal and stimulated lipolytic activity within adipose tissue (18, 19). Except for the well-established targeting to LDs (1, 7, 9, 15), less is known about the function of ADFP. Initially, when cloned, ADFP was thought to be preferentially expressed in adipocytes, with increasing mRNA expression during adipocyte differentiation (20).

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Abbreviations: ACO-1, acyl-coenzyme A oxidase-1; ADFP, adipose differentiation-related protein; CE, cholesterol ester; CMC, carboxymethyl cellulose; DR-1, direct repeat-1; LD, lipid droplet; ME-1, cytosolic malic enzyme-1; OA, oleic acid; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-activated receptor response element; RXR, retinoid X receptor; SREBP-1, sterol-regulatory element binding protein-1; TAG, triacylglycerol; TIP47, tail-interacting protein of 47 kDa; TTA, tetradecylthioacetic acid.

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[S] The online version of this article (available at http://www.jlr.org) contains additional figures.

Later analyses, however, have demonstrated that ADFP mRNA is more ubiquitously expressed, with tissue enrichment in metabolic organs and tissues (7, 12, 19). The ADFP protein associates with smaller neutral LDs located within most tissues, but rarely in adipose cells that express perilipin (7, 19). Forced expression of ADFP in COS-1 cells stimulates long-chain fatty acid (FA) uptake (21), and stimulation of cells in culture with FAs [i.e., oleic acid (OA)] induces the formation of LDs coated with ADFP (7, 9, 22).

The cellular content of ADFP mRNA is altered by lipids or their metabolites. Incubation of adipogenic cells in vitro by long-chain FAs increases ADFP mRNA content (23). Other lipids, such as oxidized low density lipoprotein (24) and VLDL, increase ADFP mRNA content in macrophages (25). In addition, synthetic ligands for peroxisome proliferator-activated receptor β/δ (PPARβ/δ) have been found to increase ADFP mRNA in macrophages (25–27). The PPAR family consists of PPARβ/δ and two other isotypes, PPARα and PPARγ. They belong to a subfamily of nuclear receptors that heterodimerize with retinoid X receptors (RXRs) and regulate transcription by binding to specific peroxisome proliferator-activated receptor response elements (PPREs) in the promoter region of target genes (28). PPARγ is highly enriched in white adipose tissue and macrophages (29). PPARα is enriched in liver and other metabolic tissues, such as muscle, heart, and kidney (30). PPARβ/δ is more ubiquitously expressed (31). The PPAR isotypes play distinct roles in fatty acid transport and metabolism. PPARα is irreplaceable for a proper response to fasting in liver (32) and stimulates the transcription of numerous genes encoding for proteins in the FA oxidation pathways (33).

We have previously demonstrated that transcription of S3-12 and perilipin in adipocytes is stimulated by PPARγ (12). Several independent studies suggest that the expression of ADFP is similarly stimulated by lipid metabolites that are believed to activate members of the PPAR family (23, 24, 34, 35). However, to date, we have not been able to demonstrate a direct regulation of ADFP by PPARγ in adipose tissue (12). Although highly transcribed in adipose cells, the ADFP protein is almost undetectable in the cytosol or at the surface of LDs when perilipin is expressed (7, 19). Therefore, the presence of ADFP might be more relevant in liver, where ADFP is expressed at high levels and perilipin is not normally expressed (5, 7, 12). By the use of animal and cell culture experiments, we demonstrate that although ADFP mRNA is highly induced by PPARα activators, the increased mRNA does not necessarily result in more cellular ADFP protein. ADFP mRNA and protein levels, however, are highly induced by fasting, a physiological condition that also favors the accumulation of TAG-containing LDs. This accumulation of TAGs seems to be a major regulator of cellular ADFP protein content.

MATERIALS AND METHODS

Materials

Restriction enzymes were obtained from Promega (Madison, WI). Cell culture reagents, 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid (WY-14643), fatty acids, Oil Red O, oligonucleotides, and chemicals were purchased from Sigma (St. Louis, MO). Cell culture plasticware was obtained from Corning, Inc. (Corning, NY). T0901317 and MK-886 were obtained from Alexis (Lausen, Switzerland). The pSG5-mPPARα, pSG5-mRXRα, and pSG5 (Stratagene, La Jolla, CA) expression vectors were provided by Jan-Åke Gustafsson (Department of Bioscience and Medical Nutrition, Novum, Huddinge, Sweden). LG100268 and GW501516 were kindly provided by J. Lehmann (CareX, Strasbourg, France). Tetradecylthioacetic acid (TTA; C14-*S*-acetic acid:0) (36) was kindly provided by Jon Bremer (University of Oslo, Norway).

BSA binding of fatty acids

Stock solutions of OA (#O1008), α-linolenic acid (1,12,15-octadecatrienoic acid; #L2376), *cis*-4,7,10,13,16,19-docosahexaenoic acid (#D2534), or arachidonic acid (#A9673) were made by adding ethanol to 25 mg/ml final concentration. To make FA (6 mM)/BSA (2.4 mM) stock solutions, ethanol was removed by evaporation under nitrogen, and the dried FAs were dissolved in 100 μl of 0.1 M NaOH before BSA binding in 1.5 ml of 2.4 mM fat-free BSA (Sigma; #A6003) at 50°C for 5 min. TTA was dissolved in 1 M NaOH before binding to BSA. FA/BSA stock solutions were sterile-filtered through 0.22 μm μStar LB filters (Costar; #8110) and stored at −20°C for no longer than 3 weeks before use.

Culturing, transfection, and lipid staining of cells

COS-1 cells (American Type Culture Collection) were cultured in high-glucose-containing DMEM (Sigma; #6546). Human hepatoma HepG2 cells (American Type Culture Collection) were cultured in minimum essential medium Eagle (Oh Biocoat (BD Biosciences, Bedford, MA) Sigma; #M2279). Rat hepatoma 7800-C1 cells (37) were cultured in low-glucose-containing DMEM (Sigma; #D6046). All culture media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml), and cells were incubated at 37°C in 5% CO_2. For experiments, 7800-C1 cells were seeded in 12-well dishes (2×10^5 cells/well) and cultured for 2–3 days before the start of experiments. All stimulations were performed in 2 ml medium/well.

In transient transfection experiments, COS-1 cells were seeded in six-well dishes with reporter (5 μg) and cotransfected with pSV-β-galactosidase (3 μg, as internal control) and pSG5, pSG5-mRXRα, and/or pSG5-mPPARα expression vectors (1 μg each) with calcium phosphate precipitation (38). HepG2 cells were seeded at 70% confluency in 12-well dishes in antibiotic-free medium 24 h before transfection with reporter (400 ng), pSV-β-galactosidase (250 ng), and expression vectors (80 ng of each). DNA and 2 μl of Lipofectamine2000 (Invitrogen Life Technologies, Carlsbad, CA) were dissolved in 2×100 μl of OptiMEM-I (Invitrogen Life Technologies), mixed, and incubated for 20 min before transfection of cells in 2 ml of OptiMEM-I for 6 h. After transfection, culture medium was replaced with growth medium containing FAs and synthetic ligands for 24 h. Cells were harvested in 250 μl (COS-1) or 100 μl (HepG2) of reporter lysis buffer (Promega), and luciferase activities were measured with a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). Relative luciferase activity was normalized against β-galactosidase activity (control = 1). All transfections are representative of at least three individual experiments performed in triplicate. Error bars represent standard deviations.

Oil Red O (0.5%) was dissolved in isopropanol and filtered to remove unsolved particles. Before use, Oil Red O solution was mixed with MilliQ water, (60:40) to make Oil Red O coloring

reagent. For lipid staining, cells were fixed in 4% paraformaldehyde/PBS for 15 min, washed with PBS, incubated with Oil Red O coloring reagent for 15 min, and finally washed in PBS to remove unincorporated stain.

Preparation and analysis of RNA

Total RNA was extracted with TRIZOL® reagent (Invitrogen). RNA (10 or 20 μg) was separated on a 1% agarose formaldehyde/MOPS gel and blotted and hybridized as described (38). 36B4 (acidic ribosomal phosphoprotein P0) mRNA (39) was used as a loading control. Probes were generated by radiolabeling of cDNAs with [α-^{32}P]dCTP (Amersham Biosciences, Buckinghamshire, UK) by use of the Megaprime DNA Labeling System (Amersham Biosciences). Northern blots were visualized by Hyperfilm MP, scanned with Personal Densitometer SI, and analyzed using ImageQuant™ software (Amersham Biosciences).

Full-length or partial cDNA sequences used to generate radiolabeled probes have been described elsewhere [m-ADFP, m-S3-12, h-perilipin, m-aFABP/aP2 (12), m-SREBP-1, h-LXRα (38)] or were cloned by PCR with the following primers: r-ACO-1-s, 5′-CACGCAATAGTTCTGGCTCA-3′; r-ACO-1-a, 5′-GCCATTCGACATTCTTCGAT-3′; m-ACO-1-s, 5′-AAGCCTGACGGCACGTATGTAAA-3′; m-ACO-1-a, 5′-CAGAAGTCAAGTTCCACGCCACT-3′; m-ME-1-s, 5′-GGATGTCGTCAAGGCTATTGTGG -3′; and m-ME-1-a, 5′-GCTGGTCGGACTACTCAAAGCAA-3′.

Electrophoretic mobility-shift assays

Proteins were synthesized from pSG5-mPPARα, pSG5-mRXRα, and pSG5 expression vectors using the TNT T7-coupled in vitro transcription/translation system (Promega). Oligonucleotide probes were labeled using T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP (Amersham Biosciences). Binding and separation of protein-DNA complexes from free probe was performed as described (38). Oligos used are as follows (only upper primer is shown): m-ADFP-DR-1-s, 5′-GGCCTTTTGTAGGTGAAAGGGCAAAGAAAGAGG-3′; m-ADFP-DR-1-s-mut, 5′-GGCCTTTTGTAccGAcAcGCAAAGAAAGAGG-3′; h-adipophilin-DR-1-s, 5′-ATTT-TGTAGGTGAAAGGGCGAGAGTCT-3′; and h-adipophilin-DR-1-s-mut,5′-ATTTTGTAcTGAcAcGCGAGAGTCT-3′.

Identification, cloning, and mutagenesis of promoters

The complete mouse ADFP gene and a short 5′ upstream promoter sequence have previously been cloned and partially characterized (40). Full-length cDNA sequences were used to search nonredundant and high-throughput genomic sequence databases by BLAST (41) to obtain longer promoter sequences [human adipophilin mRNA (accession number BC005127) annealed to htgs: #AC024502.2, and mouse ADFP mRNA (accession number L09734.1) annealed to htgs: #AL824707.5]. The human and mouse ADFP promoters were scanned with a consensus PPRE (RGGBSAAAGGTCA) with the use of the GCG program package (Wisconsin Package version 10.0; Genetics Computer Group) and compared by BLAST2 alignment (42).

Full-length promoters were amplified with PfuTurbo (Stratagene) with mouse (m; Clontech; #6650-1) or human (h; Clontech; #6550-1) genomic DNA as template with PCR settings as described (38). Primers used were as follows: 5-h-adipophilin-promoter (*Kpn*I), 5′-TAGGTACCAAAAACGTCTCCTTTGTCCTCTGGA-3′; 3-h-adipophilin-promoter (*Kpn*I), 5′-TAGGTACCTCTAACGCGTTTCCCTTTCGATAAT-3′; 5-m-ADFP-promoter (*Nhe*I), 5′-ATGCTAGCATCGCCTTGGGATCTAATCTTGGT-3′; and 3-m-ADFP-promoter (*Nhe*I), 5′-ATGCTAGCCTAACAGGAGAGCTGAGGGACGAG-3′. The deletion construct for the human adipophilin reporter was generated by restriction digestion with *Pvu*II followed by religation of the vector. Site-directed mutagenesis of the DR-1 element was performed with PCR as described (38) with the oligonucleotides used in the electrophoretic mobility-shift assays as targeting primers.

Protein measurements and Western blot analysis

Frozen mouse liver tissue (25 mg) was homogenized in 1 ml of lysis buffer [1× PBS, 1% Nonidet P-40, 0.1% SDS, and 1× Compete (Boehringer Mannheim)] using a Mini-Beadbeater™ (BioSpec Products, Inc., Bartlesville, OK) for 20 s at 4,200 rpm with 0.5 mm glass beads (BioSpec Products). Cell homogenate from 7800-C1 cells (100 μl) was homogenized by sonication for 2 × 2 s. Protein concentrations were measured with the BC assay method (Interchim; #FT-40840).

Proteins (10 μg from tissue/50 μg from cells) were denatured by boiling for 5 min in loading buffer (final concentration, 40 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 0.002% bromphenol blue, and 0.1 M mercaptoethanol), resolved on a 10% Tris-HCl SDS gel (Criterion™ Precast Gel; Bio-Rad), and transferred to a polyvinylidene difluoride membrane (Hybond-P; Amersham Biosciences; #RPN 303F) by electrotransfer (2.5 mM Tris-HCl, 19 mM glycine, and 20% methanol). Five microliters of Precision Plus Protein™ Standard (Bio-Rad; #161-0374) was used as molecular weight marker. The membranes were blocked (PBS, 5% nonfat dry milk, and 0.1% Tween) overnight at 4°C before incubation with primary antibody [guinea pig anti-adipophilin diluted 1:2,000 (Research Diagnostic; #RDI-PROGP40) against mouse samples, and mouse anti-adipophilin diluted 1:200 (#RDI-PRO610102) against rat samples] for 1 h at room temperature. The membrane was washed three times (PBS containing 0.1% Tween) followed by incubation with secondary antibody [horseradish peroxidase-labeled goat anti-guinea pig antibody (Southern Biotech; #6090-05) or anti-mouse antibody (#1031-05)] diluted 1:8,000 for 1 h. Finally, the membranes were washed again and developed using enhanced chemiluminescence (ECL-plus; Amersham Biosciences; RPN 2132) and visualized with Hyperfilm MP (Amersham Biosciences). To ensure equal loading of protein, the membranes were stripped and incubated with primary antibody against β-actin (diluted 1:10,000; Sigma; #A5441).

Measurement of lipid composition in liver and 7800-C1 cells

Liver tissue samples (20–50 mg) were homogenized in PBS (1.5 ml) as described for Western blot analysis. Lipids were extracted from the liver homogenate (200 μg of protein) and separated by high-performance thin-layer chromatography essentially as described (43, 44). In brief, lipids were extracted from the homogenate by organic phase extraction using methanol-chloroform and dried under nitrogen. Cholesterol acetate (2 μg) was added during homogenization as an internal standard. Samples were resolved in 90 μl of chloroform and spotted to an activated high-performance thin-layer chromatography plate [Nano-sil-20 (10 cm × 20 cm); Alltech Associates, Inc.], and lipid fractions were separated in 87% hexane, 10% diethyl ether, and 3% isopropanol in a CAMAG Horizontal Developing chamber (Camag, Switzerland). The lipid fractions were visualized with acidic Mg solution and quantified by scanning the plates using the Gel Logic 100 Imaging system (Eastman Kodak Co., Rochester, NY) and integrating the density areas using Kodak 1D version 3.6.1 software.

7800-C1 cells were harvested in dH_2O, frozen to disrupt the cell membrane, and homogenized by ultrasound treatment (2 × 5 s at a working frequency of 47 kHz ± 6%) before TAG measurement using the Triglycerides Enzymatique PAP 150 kit (Bio-

Mérieux; #61236) essentially as described. TAG concentration was related to protein concentration.

Animal experiments

All animal use was approved and registered by the Norwegian Animal Research authority. Mice were maintained in a temperature-controlled (22°C) facility with a strict 12 h light/dark cycle and given free access to normal chow diet (Special Diets Services No. 3, containing 64% carbohydrates, 31.5% proteins, and 2.0% fat; Scanbur BK AS) and water. WY-14643 and T0901317 were dissolved in vehicle [1% (w/v) carboxymethyl cellulose (CMC) dissolved in water]. Each mouse was gavaged with 10 μl vehicle/g body weight. Mice were euthanized by cervical dislocation, and tissues were rapidly dissected, quickly frozen in liquid nitrogen, and stored at −80°C.

C57Bl/6J mice (B&K Universal, Ltd., Sollentuna, Sweden; 10 weeks, 25 g) were fed twice (24 and 8 h before they were euthanized) with vehicle (CMC) or WY-14643 (2 × 50 mg/kg). The PPARα-deficient (PPARα$^{-/-}$) mice (Sv/129, Jae substrain), obtained from the Jackson Laboratory (Bar Harbor, ME), have been described (33). 129/SvEvTac@Bom (Bomholtgård, Denmark) was used as a wild-type control (PPARα$^{+/+}$). In the 7 day feeding experiment, PPARα$^{+/+}$ and PPARα$^{-/-}$ mice (12–18 weeks, 25–35 g) were fed daily with vehicle (CMC) or WY-14643 (10 mg/kg), with the last dose given 4 h before mice were euthanized.

In the 24 h feeding experiment, PPARα$^{+/+}$ and PPARα$^{-/-}$ mice (12–13 weeks, 25–30 g) were fed twice (24 and 8 h before they were euthanized) with vehicle (CMC), T0901317 (2 × 10 mg/kg), or WY-14643 (2 × 50 mg/kg).

In the fasting/refeeding experiment, male PPARα$^{+/+}$ (7–9 weeks, 20–25 g) and PPARα$^{-/-}$ mice (6–8 weeks, 20–25 g) were divided into three groups: nonfasted (control), fasted, and refed. The control group was fed ad libitum, the fasted group was fasted for 24 h, and the refed group was fasted for 24 h and then refed for 12 h. The experiment was staggered to ensure that all mice were euthanized at the same time of the day at the end of the dark period.

RESULTS

Expression of ADFP mRNA is induced by a synthetic PPARα agonist in rat hepatoma 7800-C1 cells

ADFP is highly expressed in liver (7, 12) but has been poorly studied in that organ. Observations in other tissues and cells suggest that the expression of ADFP is stimulated by lipid metabolites that are believed to activate members of the PPAR family (23, 24, 34, 35). PPARα is the most active PPAR isotype in liver (31), and to test if activation of PPARα affects ADFP expression in liver, we stimulated rat 7800-C1 hepatoma cells (37) with increasing concentrations of a potent synthetic PPARα agonist (WY-14643) for 24 h. A robust induction of the ADFP transcript was observed at 10 μM concentration (**Fig. 1A**). The same was observed for the two characterized PPARα target genes acyl-coenzyme A oxidase-1 (ACO-1) (45) and cytosolic malic enzyme-1 (ME-1) (46, 47). The induction of ADFP mRNA by WY-14643 (10 μM) was initiated already at 4–6 h, similar to ACO-1 and ME-1, suggesting a direct effect of PPARα activation on ADFP transcription (Fig. 1B). Stimulation with the RXR agonist LG100268 (1 μM), which activates the heterodimeric partner of PPARα, increased the transcription of ACO-1 and ME-1 similar to what was observed for WY-14643, but LG100268 had less effect on ADFP mRNA transcription (Fig. 1C). TIP47, another PAT member expressed at significant levels in liver (12), was not regulated by any of these treatments.

WY-14643 induces the expression of ADFP mRNA in liver

The existing findings on ADFP expression have mainly been obtained from in vitro cell culture experiments. To determine whether activation of PPARα regulates ADFP expression in vivo, we treated C57Bl/6J mice with vehicle

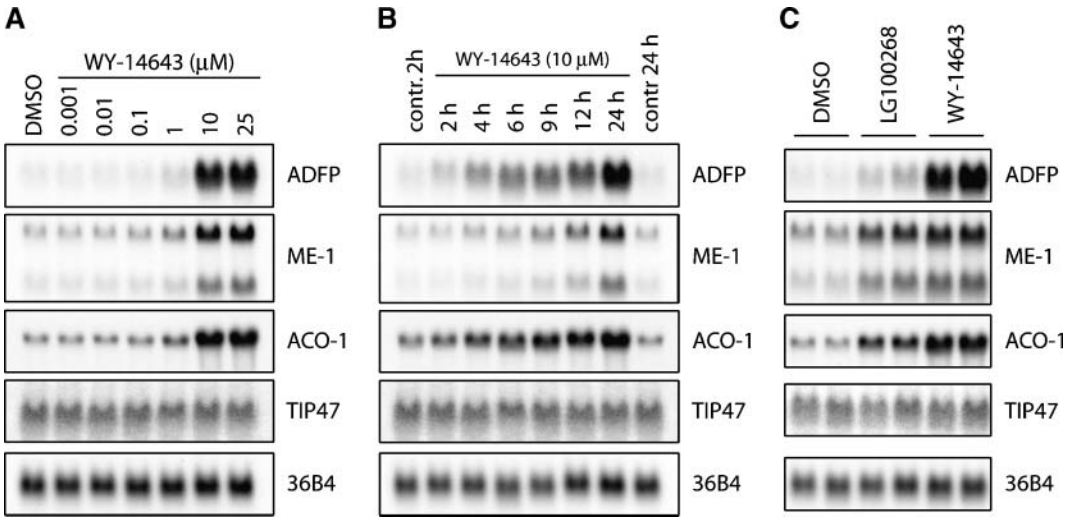

Fig. 1. Transcription of adipose differentiation-related protein (ADFP) is increased in rat hepatoma 7800-C1 cells by activation of peroxisome proliferator-activated receptor α (PPARα). Expression of ADFP, cytosolic malic enzyme-1 (ME-1), acyl-coenzyme A oxidase-1 (ACO-1), and tail-interacting protein of 47 kDa (TIP47) mRNAs after stimulation of rat hepatoma 7800-C1 cells by increasing the concentration of WY-14643 for 24 h (A) or by treating with WY-14643 (10 μM) for 2 to 24 h (B). For both experiments, each lane contains RNA pooled from two independent stimulations. C: Treatment with LG100268 (1 μM) or WY-14643 (10 μM) for 24 h.

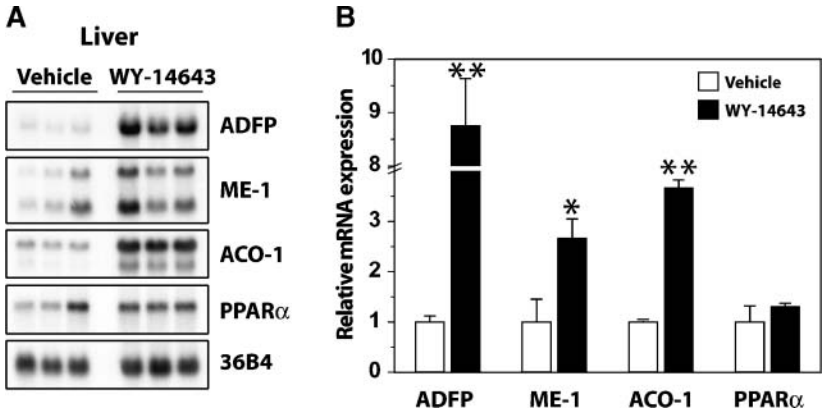

Fig. 2. Expression of ADFP mRNA is induced in liver by treatment with a PPARα agonist. Male C57Bl/6J mice were gavage-fed twice with vehicle [carboxymethyl cellulose (CMC); control] or the PPARα agonist (WY-14643; 2 × 50 mg/kg) for 24 and 8 h before euthanasia. Livers were rapidly taken out and used for RNA isolation and Northern analysis. A: Expression of ADFP, ME-1, ACO-1, and PPARα mRNAs in liver. Each well contains total RNA (15 μg/lane) pooled from two mice. B: Expression correlated against the 36B4 signal. Statistical differences were evaluated with one-way ANOVA (* $P < 0.01$, ** $P < 0.001$). Error bars represent standard deviations.

(CMC) or the synthetic PPARα agonist (WY-14643; 2 × 50 mg/kg) for 24 h. Liver samples were dissected and subjected to RNA analysis. A strong induction of the ADFP, ACO-1, and ME-1 transcripts was observed after administration of the PPARα agonist in liver (**Fig. 2A, B**). As found also in 7800-C1 cells, the induction of ADFP mRNA by WY-14643 treatment was much higher compared with the induction observed for the PPARα target genes ACO-1 and ME-1.

The ADFP promoter contains an evolutionarily conserved PPAR-responsive DR-1 element

These analyses suggest a direct transcriptional regulation of ADFP expression by PPARα. Therefore, we analyzed the human adipophilin and the mouse ADFP promoter for the presence of a typical DR-1-like PPRE. Only one conserved direct repeat-1 (DR-1) element, located ~2,000 bp upstream of the transcription start site, was identified (see supplementary Fig. IA, B). This element was found to bind all three PPAR isotypes in electrophoretic mobility-shift assays (see supplementary Fig. IC; results not shown). We next cloned the mouse ADFP (−2,781 to +50) and the human adipophilin (−3,592 to +415) promoters into the pGL3-basic luciferase reporter vector and transfected these reporters transiently into COS-1 cells to test the functionality of the identified DR-1 element. These analyses established the DR-1 element as a functional PPRE (see supplementary Fig. II).

Transcriptional stimulation of the ADFP gene by long-chain FAs depends on the DR-1 element

Long-chain FAs increase ADFP mRNA levels in adipocyte precursor cells (23), but the molecular mechanism involved has not been investigated. Long-chain FAs are believed to function as physiological PPARα ligands (35); hence, we tested whether selected long-chain FAs, such as OA (C18:1; 100 μM), α-linolenic acid (C18:3; 100 μM), arachidonic acid (C20:4; 50 μM), docosahexaenoic acid (C22:6; 50 μM), and the FA analog TTA (C14-*S*-acetic acid:0; 50 μM) (36), were able to stimulate transcription of the ADFP gene. Wild-type and DR-1-mutated mouse ADFP reporter constructs were transfected into HepG2 cells in the absence or presence of RXRα and PPARα expression vectors and stimulated with WY-14643, TTA, and the FAs listed above (**Fig. 3**). Only the wild-type reporter responded to the added FAs and the synthetic PPARα ligands, suggesting that the FA regulation of ADFP transcription is mediated through the identified DR-1 element.

WY-14643 treatment does not induce ADFP mRNA in PPARα$^{-/-}$ mice

The synthetic WY-14643 compound is selective for PPARα at lower doses (35), but in vitro analyses suggest that it can activate PPARγ at higher doses (≥100 μM) (48). To determine if the effect seen in WY-14643 treatment is mediated exclusively through PPARα, we fed PPARα$^{+/+}$ and PPARα$^{-/-}$ mice with vehicle (control) or WY-14643 (10 mg/kg) daily for 1 week. In agreement with previous results (33), WY-14643 treatment resulted in a marked increase in liver size in PPARα$^{+/+}$ mice but not in PPARα$^{-/-}$ mice (**Table 1**). WY-14643 strongly induced the expression of ADFP, ACO-1, and ME-1 mRNA in the liver of PPARα$^{+/+}$ mice but had no effect in PPARα$^{-/-}$ mice (**Fig. 4A** and results not shown). This demonstrates that the effect obtained by WY-14643 on ADFP mRNA levels is dependent on the presence of an intact PPARα protein. The mRNAs for TIP47 (results not shown) and RXRα remained unchanged by the treatment or disruption of the PPARα gene.

We next analyzed hepatic ADFP protein and lipid content. A small induction of the ADFP protein was observed after WY-14643 feeding in PPARα$^{+/+}$ mice, whereas no change in ADFP protein content was observed in PPARα$^{-/-}$ mice (Fig. 4B). Analysis of the hepatic lipid content revealed

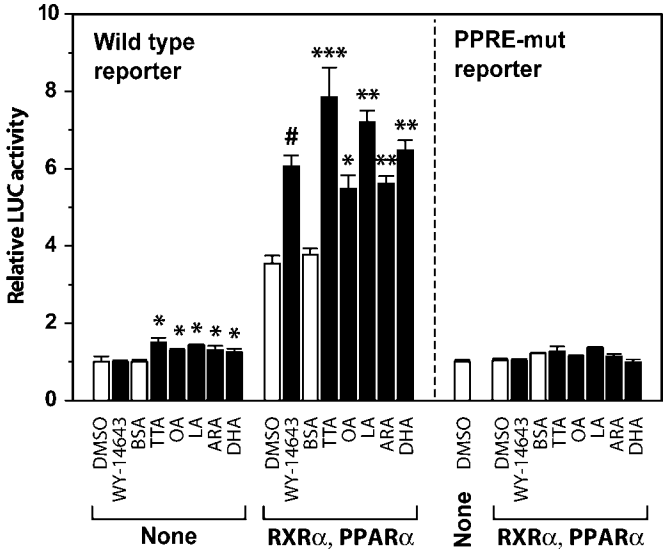

Fig. 3. The fatty acid-mediated stimulation of ADFP expression is dependent on the conserved direct repeat-1 (DR-1) element. Human hepatoma HepG2 cells were transiently transfected with either wild-type or peroxisome proliferator-activated receptor response element (PPRE)-mutated mouse ADFP reporter in the presence of pSG5 or pSG5-retinoid X receptor (RXRα) and pSG5-PPARα expression vectors and stimulated with WY-14643 (10 μM) or FAs [100 μM oleic acid (OA) or α-linolenic acid (LA), 50 μM arachidonic acid (ARA), docosahexaenoic acid (DHA), or tetradecylthioacetic acid (TTA)]. One representative of three experiments performed in triplicate is shown, and values shown are means ± SD. Statistical differences from controls were evaluated using a two-tailed Student's *t*-test. # $P < 0.001$, WY-14643 against DMSO. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, TTA and FAs against BSA. LUC, luciferase.

no evident differences in the lipid classes among the mice (Fig. 4C).

ADFP mRNA and protein content are increased during fasting and refeeding independent of PPARα

PPARα has been ascribed an important role in the adaptive response to fasting (32, 33). Fasting is a powerful stimulator of adipose tissue lipolysis, which gives rise to a profound increase in plasma FFA content. These FFAs are readily taken up by the liver and oxidized, converted to ketone bodies, or incorporated into LDs. Such LDs have been found to accumulate in the liver of both fasted wild-type and PPARα$^{-/-}$ mice (32). Because our previous analyses established ADFP as a direct PPARα target gene, we analyzed the effect of PPARα and fasting/refeeding on ADFP mRNA and protein levels and hepatic lipid content. PPARα$^{+/+}$ and PPARα$^{-/-}$ mice were fed ad libitum (control), fasted for 24 h (fasted), or fasted for 24 h and refed for 12 h (refed). Surprisingly, the ADFP mRNA was highly induced by fasting in both PPARα$^{+/+}$ and PPARα$^{-/-}$ mice, with a prolonged higher expression after refeeding compared with control fed mice (**Fig. 5A**). This result differed from those seen in ACO-1 and PPARα mRNAs, which were only induced by fasting in the PPARα$^{+/+}$ mice. Yet another expression pattern was observed for the ME-1 mRNA, which was relatively unaffected by fasting or removal of PPARα but increased slightly by refeeding. The expression of ME-1, however, was very similar to the expression of RXRα. The TIP47 mRNA remained relatively unchanged by the fasting and refeeding treatments.

The ADFP protein was highly induced by fasting and remained high even after 12 h of refeeding (Fig. 5B). The fasting ADFP protein content was higher in PPARα$^{+/+}$ mice compared with PPARα$^{-/-}$ mice, which suggests that the presence of PPARα favors accumulation of the ADFP protein. Analysis of the liver lipid content showed a considerable increase in accumulated lipids, mainly as TAG, in fasted and refed mice compared with control fed mice. The amount of TAG accumulated in PPARα$^{+/+}$ and PPARα$^{-/-}$ mice was similar, but the net increase was larger in PPARα$^{+/+}$ mice, because of the higher TAG content in control fed PPARα$^{-/-}$ mice. Nevertheless, the similar amount of TAG found in fasted and refed PPARα$^{+/+}$ and PPARα$^{-/-}$ mice suggests that a functional PPARα gene is not required for TAG accumulation during fasting (Fig. 5C).

Stimulation of 7800-C1 cells with long-chain FAs induces ADFP mRNA and protein content and LD formation

The two in vivo experiments described above reveal an interesting difference in ADFP protein levels. Even though both WY-14643 treatment and fasting stimulate ADFP mRNA content to approximately similar levels, fasting seems to favor a much higher increase in ADFP protein content. This suggests that physiological changes that occur during fasting favor accumulation of the ADFP protein. The ADFP protein furthermore seems to be stabilized during the refeeding period, when ADFP protein

TABLE 1. Body and liver weight in mice treated with WY-14643

	PPARα$^{+/+}$		PPARα$^{-/-}$	
Variable	CMC	WY-14643	CMC	WY-14643
Number of mice	6	6	6	6
Weight of mice (g)	26.6 ± 0.8	27.9 ± 1.6	28.0 ± 1.0	28.1 ± 0.9
Weight of liver (g)	1.04 ± 0.04	1.48 ± 0.03[a]	1.06 ± 0.06	0.96 ± 0.03

CMC, carboxymethyl cellulose; PPAR, peroxisome proliferator-activated receptor. Mean body weight (±SEM) and mean liver weight (±SEM) of PPARα$^{+/+}$ and PPARα$^{-/-}$ mice (n = 6) gavaged daily with vehicle (CMC) or WY-14643 (10 mg/kg) for 1 week. The mice were euthanized 4 h after the last gavage. Liver weights were significantly increased by WY-14643 feeding in wild-type mice (PPARα$^{+/+}$) but not in PPARα$^{-/-}$ mice.
[a] $P < 0.001$.

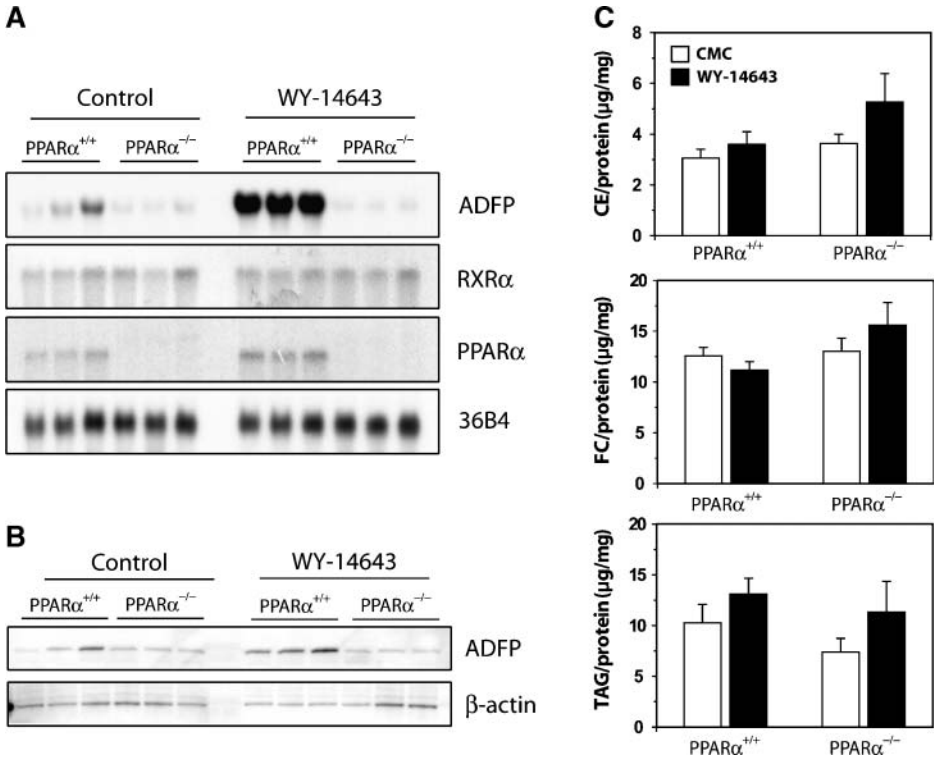

Fig. 4. Induction of ADFP mRNA in vivo by WY-14643 is dependent on PPARα. Male PPARα$^{+/+}$ and PPARα$^{-/-}$ mice (Sv/129 strain) were gavaged with vehicle (CMC) or WY-14643 (2 × 10 mg/kg) for 1 week and euthanized 4 h after the last administration of ligand. Livers were rapidly taken out and subjected to Northern, Western, and lipid analysis. A: Liver content of ADFP, RXRα, and PPARα mRNAs. Each lane contains total RNA (20 μg) pooled from two mice (n = 6). B: Liver content of ADFP protein (10 μg of protein pooled from two mice per lane). C: Liver content of cholesteryl esters (CE), free cholesterol (FC), and triacylglycerols (TAGs) relative to liver protein content (n = 6). Error bars represent standard deviations.

level remains high, even though the ADFP mRNA declines to basal levels.

Fasting and refed states are almost contradictory when it comes to hormonal signaling. This argues against a hormonal regulation of ADFP protein accumulation. However, it is well established that ADFP associates on the surface of LDs (1, 7, 9, 15). TAG-containing LDs are present in the liver during both fasting and refeeding, which suggests that LDs might favor accumulation of the ADFP protein. To test this hypothesis, we stimulated rat 7800-C1 cells in vitro with selected long-chain FAs, such as OA (100 μM), linolenic acid (100 μM), arachidonic acid (50 μM), docosahexaenoic acid (50 μM), and the FA analog TTA (50 μM) (36), and measured ADFP mRNA and protein levels and LD content.

The ADFP mRNA was moderately increased after 8 h of stimulation by most FAs, whereas the same FAs had more effect after 24 h (**Fig. 6A**). The polyunsaturated long-chain FAs (α-linolenic acid, arachidonic acid, and docosahexaenoic acid) and TTA were more potent compared with the monounsaturated FA (OA). This fatty acid-selective effect on induction of the ADFP mRNA is in agreement with the results obtained from the transfection studies (Fig. 3). No alteration of TIP47 mRNA was found with any of the fatty acids. All of the selected FAs increased the ADFP protein content (Fig. 6B). We next analyzed the intracellular lipid pool by coloring the cells with Oil Red O. The cells stimulated with FAs contained larger and more LDs compared with control cells (receiving BSA) or cells stimulated with TTA (Fig. 6C). These LDs are likely formed by an increased uptake of FAs from the medium and incorporation of these into LDs and are not attributable to increased de novo lipogenesis, because the mRNAs for lipogenic genes such as sterol-regulatory element binding protein-1 (SREBP-1) and FAS were not affected by the addition of the FAs (Fig. 6A and results not shown). Interestingly, no evident correlation among ADFP mRNA and ADFP protein levels were found after stimulation with the selected FAs. As examples, OA treatment resulted in the largest increase in ADFP protein levels but barely stimulated ADFP mRNA levels. The opposite effect was found for TTA. A much better correlation was observed among ADFP protein levels and the amount of accumulated Oil Red O-colored lipids.

The presence of OA increases LD formation synergistically with induced cellular ADFP protein content

The experiments described above suggest that the availability of FAs and the formation of LDs favor the accumulation of hepatic ADFP protein. To further test this hypothesis, we stimulated 7800-C1 cells in vitro with either BSA or OA (100 μM) in combination with different ago-

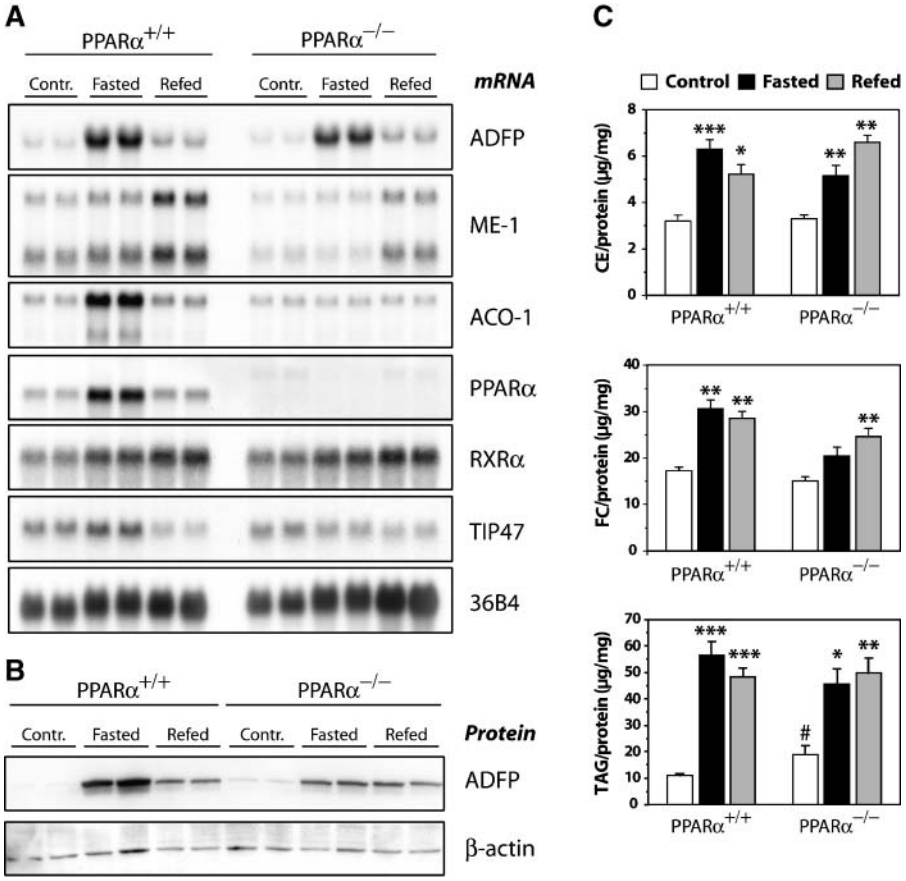

Fig. 5. Induction of ADFP mRNA during fasting occurs independent of PPARα and is correlated to increased hepatic ADFP protein and TAG content. Male PPARα$^{+/+}$ and PPARα$^{-/-}$ mice were fed ad libitum [control (Contr.)], fasted for 24 h (fasted), or fasted (24 h) and refed for 12 h (refed) before being euthanized. Livers were rapidly taken out and subjected to Northern, Western, and lipid analysis. A: Liver content of ADFP, TIP47, ME-1, ACO-1, PPARα, and RXRα mRNAs. Each lane contains total RNA (20 μg) pooled from five mice (PPARα$^{+/+}$) or four mice (PPARα$^{-/-}$) applied in duplicate on the gel. B: Liver content of ADFP protein (10 μg/well, pooled and performed in duplicate). C: Liver content of cholesteryl esters (CE), free cholesterol (FC), and TAGs relative to liver protein content (n = 5, PPARα$^{+/+}$ mice; n = 4, PPARα$^{-/-}$ mice). Statistical differences from controls were evaluated using a two-tailed Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Liver TAG was higher in PPARα$^{-/-}$ mice compared with PPARα$^{+/+}$ mice ($^{\#}$ $P < 0.05$). Error bars represent standard deviations.

nists for nuclear receptors involved in lipid homeostasis. OA was selected because this FA was the strongest inducer of ADFP protein level in 7800-C1 cells, despite the fact that it had only a minor effect on ADFP mRNA levels (Fig. 6). We also used a synthetic LXR agonist (T0901317), which is a strong stimulator of lipogenesis. It is well known that activation of LXRs directly stimulates the transcription of a cascade of lipogenic enzymes in liver, including FAS and SREBP-1c (49). This increased production of FAs also results in liver lipid accumulation. We also treated the cells with a PPARα antagonist (MK-886), a PPARδ agonist (GW501516), and a RXR agonist (LG100268).

After 72 h of stimulation, accumulated lipids were stained with Oil Red O. Cells cultured in BSA and stimulated with WY-14643 (PPARα agonist), LG100268 (RXR agonist), or T0901317 (LXR agonist) increased the lipid content, whereas the lipid content in cells stimulated with GW501516 (PPARβ/δ agonist) or MK-886 (PPARα antagonist) remained unchanged (**Fig. 7A**). The lack of any effect by the PPARβ/δ agonist compared with the PPARα agonist is likely attributable to the low expression of PPARβ/δ compared with PPARα in these cells (results not shown). In agreement with the findings described above (Fig. 6C), supplementation of the culture medium with OA increased the lipid content. Interestingly, stimulation with both OA and WY-14643 resulted in an additional increase in accumulated lipids. Stimulation with LG100268 and T0901317 also increased the lipid content compared with OA alone, but not as much as observed for WY-14643. GW501516 and MK-886 had no clear effect. We expect that the variable degree of Oil Red O coloring is the result of the unequal ability of the cells to take up OA and incorporate it into TAG-containing LDs. Therefore, we measured the TAG content in cells stimulated as described above. The intracellular TAG content corresponded well with the degree of Oil Red O coloring (Fig. 7B).

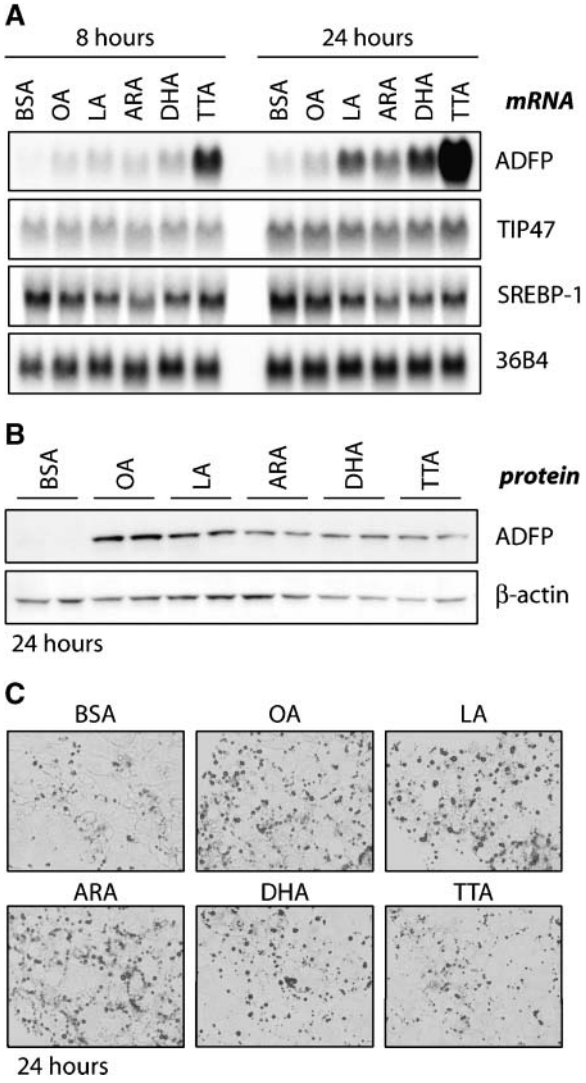

Fig. 6. Culturing of rat 7800-C1 cells in the presence of long-chain FAs induces ADFP mRNA and protein contents and favors lipid accumulation. A: ADFP, TIP47, and sterol-regulatory element binding protein-1 (SREBP-1) mRNA levels after stimulation with selected FAs [100 μM OA and α-linolenic acid (LA), 50 μM arachidonic acid (ARA) and docosahexaenoic acid (DHA)] and the FA analog TTA (50 μM) for 8 and 24 h. B: ADFP and β-actin protein content in cells after 24 h of stimulation with the same FAs. C: Determination of lipid accumulation in cells stimulated as described above for 24 h. Lipids were colored with Oil Red O. All experiments were performed six times. Each lane contains total RNA (20 μg) or protein (50 μg) pooled from six wells.

At the transcriptional level, WY-14643 and LG100268 substantially increased ADFP mRNA, whereas GW501516 had only a minor effect (Fig. 7C). T0901317 and MK-886 had no effect on ADFP transcription. As expected, T0901317 and LG100268 stimulated the induction of SREBP-1 and FAS mRNAs (Fig. 7C and results not shown), which suggests that some of the accumulated lipids in these cells can be ascribed to increased FA synthesis. The mRNA content of TIP47 remained unchanged by all treatments.

At the protein level, treatment with WY-14643 increased ADFP protein content, which was further increased with a combination of WY-14643 and OA treatment (Fig. 7D). Essentially the same effects were observed with LG100268 treatment, but the amount of ADFP protein in the samples was lower. T0901317 treatment had no effect on ADFP protein content in the absence of OA, but a weak increase was observed in combination with OA. MK-888 and GW501516 had no particular effect on ADFP protein levels.

A comparison of the ADFP mRNA, ADFP protein, and TAG accumulation showed a much better correlation among ADFP protein and cellular TAG content than between ADFP protein and ADFP mRNA levels. However, despite the clear accumulation of LDs in T0901317-stimulated cells, the level of ADFP protein was much lower than expected compared with the amount of ADFP protein found in WY-14643- and LG100268-treated cells. Therefore, we examined the level of ADFP protein in 7800-C1 cells incubated with BSA or OA and stimulated with WY-14643 or T0901317 for 24 or 72 h. The amount of ADFP protein remained stable in cells cultured in the presence of either WY-14643 or T0901317, but a reduction in ADFP protein was found in cells treated with OA alone (Fig. 7E). This suggests that activation of the lipogenic pathway through LXRs does not affect the transcription of ADFP, but the increase in FA synthesis and LD formation with prolonged stimulation seems to have a stabilizing effect on the ADFP protein content.

Liver ADFP mRNA is not regulated upon stimulation of de novo lipogenesis by activation of LXRs

It is impossible to study the complex regulations involved in fasting and de novo lipogenesis in a cell culture system. To clarify whether the ADFP gene is transcriptionally regulated by a LXR agonist in vivo, we fed PPARα$^{+/+}$ and PPARα$^{-/-}$ mice with vehicle (control), the LXR agonist (T0901317; 2 × 50 mg/kg), or the PPARα agonist (WY-14643; 2 × 50 mg/kg) for 24 h. As expected, ADFP and ACO-1 were only regulated in wild-type mice by WY-14643 treatment (**Fig. 8A**). Similar to the results obtained in vitro in 7800-C1 cells, treatment with the LXR agonist had no effect on ADFP mRNA in the liver. Treatment with T0901317, however, did increase the transcription of the known LXR target genes SREBP-1 and FAS, even in the absence of a functional PPARα gene. The induction of genes in the lipogenic pathway without any effect on ADFP mRNA argues against ADFP playing a role in LD coating during lipogenesis in the liver. Rather, the increased ADFP expression by externally provided fatty acids, PPARα agonists, and fasting suggests that ADFP is likely more important for LD coating during fasting.

DISCUSSION

We have previously demonstrated that the two LD-associated proteins, S3-12 and perilipin, which share high sequence homology to ADFP, are regulated by PPARγ in adipose tissue (12). Here, we provide substantial evidence to establish ADFP as a novel direct target gene for the PPARα/RXR heterodimer in liver. Increased transcription

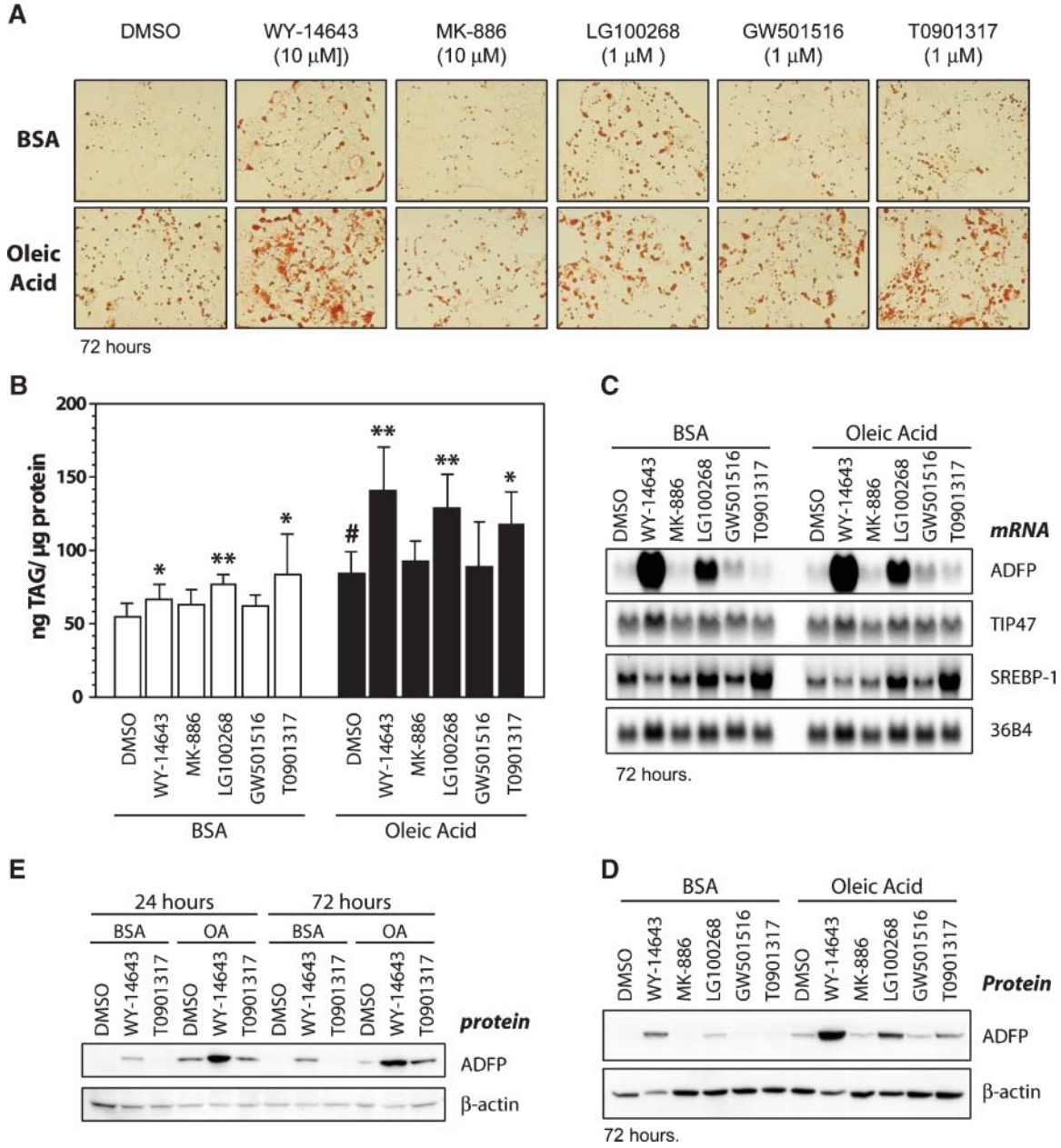

Fig. 7. Stimulation of ADFP mRNA transcription in the presence of OA greatly enhances cellular ADFP protein content in rat 7800-C1 cells. Rat 7800-C1 cells were stimulated with WY-14643 (10 μM), MK-886 (10 μM), LG100268 (1 μM), GW501516 (1 μM), or T0901317 (1 μM) in the presence of either BSA or BSA-bound OA (100 μM) for 72 h. A: Oil Red O-colored lipids in stimulated cells. B: Cellular TAG content. Each bar represents the mean ± SD of two independent experiments. In both experiments, 12 wells were harvested in triplicate (four wells per sample; n = 6). Statistical differences from controls (BSA or OA) were evaluated using a two-tailed Student's *t*-test (* $P < 0.05$, ** $P < 0.01$). TAG was higher in OA-treated compared with BSA-treated cells ($^{\#} P < 0.001$). C: ADFP, TIP47, and SREBP-1 mRNA levels. D: ADFP and β-actin protein content. E: ADFP and β-actin protein content in 7800-C1 cells stimulated with WY-14643 or T0901317 in the presence of BSA or BSA-bound OA for 24 and 72 h. Experiments in C–E were performed twice. Each lane contains total RNA (20 μg) or protein (50 μg) pooled from six wells.

of ADFP after activation of PPARα with a synthetic PPARα agonist occurs rapidly in rat hepatoma 7800-C1 cells, with a transactivating potential in agreement with the potency of PPARα activation (35). Transcription of ADFP is furthermore increased by WY-14643 treatment in C57Bl/6J and Sv/129 mice. ADFP mRNA level is not altered in PPARα$^{-/-}$ mice upon PPARα agonist treatment, which demonstrates that the induction of ADFP mRNA is dependent on the expression of a functional PPARα protein. PPARα-mediated transcriptional regulation of ADFP in liver was recently also demonstrated by others (50), which supports our findings.

Activation of PPARα stimulates transcription of the ADFP gene through a DR-1 element that is conserved among the human and mouse ADFP promoters. This DR-1 element has previously been found to bind PPARβ/δ (25) and more

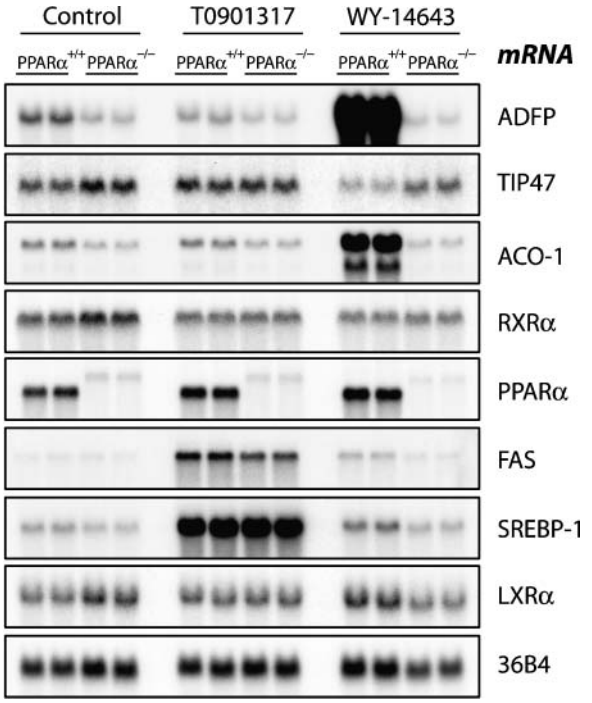

Fig. 8. ADFP mRNA expression is not induced by stimulation of the lipogenic pathway. Male PPARα$^{+/+}$ and PPARα$^{-/-}$ mice were gavaged with vehicle (CMC), T0901317 (2 × 10 mg/kg), or WY-14643 (2 × 50 mg/kg) 24 and 8 h before being euthanized. Livers were rapidly taken out and subjected to RNA isolation and Northern analysis. Each lane contains total RNA (20 μg) pooled from six individuals applied in duplicate on the gel.

recently also PPARα (51) by other investigators. Long-chain FAs, which are believed to be functional ligands for PPARα (35), have been found to increase ADFP mRNA levels in adipocyte precursor cells cultured in vitro (23). Our analyses suggest that FAs increase ADFP mRNA levels by activating the transcription of ADFP through the conserved DR-1 element, possibly by functioning as physiological ligands for the PPARα/RXR heterodimer. Similarly, it has been proposed that VLDL/LDL activates the transcription of ADFP in macrophages through PPARβ/δ/RXR hererodimerization (25). Thus, although there are likely other important transcription sites in the ADFP promoter, the DR-1 element seems to be fundamentally important in the lipid-mediated regulation of ADFP transcription.

The expression of ADFP can be stimulated by PPARβ/δ (25, 26) or by PPARα ligands (51; this report), but there are conflicting reports on whether ADFP is regulated by PPARγ ligands. Increased ADFP mRNA levels have been reported after stimulation with PPARγ ligands in colorectal cancer cell lines (52), human blood monocytes (34), and human trophoblasts (53) but not in human macrophages (27) or adipose cells (12). A recently characterized hepatic cell line overexpressing PPARγ2 accumulates LDs with a corresponding increase in ADFP protein (54). Although aFABP/aP2 mRNA, a well-characterized PPARγ target gene (29), is induced by PPARγ2 overexpression, the ADFP mRNA remains unchanged (54). The human adipophilin promoter is not activated by PPARγ2 cotransfection in COS-1 cells (results not shown), but others have reported that it might respond to PPARγ1 cotransfection and activation in CV-1 and JEG-3 cells (53). Therefore, transcriptional regulation of ADFP by PPARγ might depend on the PPARγ isoform-expressed or -specific coactivators/corepressors present in the cell. Another, and perhaps more likely, possibility is that transcriptional regulation of ADFP by PPARγ agonists is secondarily initiated in some cell types but not in others (e.g., as a result of altered FA uptake after PPARγ activation).

The ADFP mRNA and ADFP protein levels were highly induced in mice after 24 h of fasting. PPARα has been ascribed an important role in the adaptive response to fasting and is known to initiate the transcription of genes that encode key proteins involved in liver β-oxidation and peroxisomal proliferation (32, 33). Although expression of ADFP is induced by fasting, this effect is apparently not dependent on PPARα, because ADFP mRNA and protein levels were induced similarly during fasting in PPARα$^{+/+}$ and PPARα$^{-/-}$ mice. In contrast, induction of ACO-1 mRNA by fasting was dependent on the presence of PPARα. ME-1 mRNA remained unchanged after fasting but was induced after refeeding in a pattern very similar to RXRα mRNA expression. Hence, there are marked differences in the requirements for PPARα and the response to fasting/refeeding among characterized PPARα target genes. It should be emphasized that ME-1 was recently demonstrated to be alternatively regulated by RXR homodimers through the DR-1 element previously characterized as a PPRE (55). Even though ME-1 mRNA is enhanced after treatment with a potent PPARα agonist (46, 47; this report), the gene might not be activated by PPARα in the presence of more physiological PPARα ligands, which questions the classification of ME-1 as a PPARα target gene. Our results from the fasting/refeeding experiment clearly demonstrate that ADFP is not solely regulated by PPARα in liver. Another compensatory stimulating transcription mechanism exists that ensures the induction of ADFP mRNA during fasting in the absence of a functional PPARα protein. Further studies will be required to identify these transcription factors.

An interesting finding from our experiments performed in vivo and in vitro is the close correlation between accumulation of the ADFP protein and the formation of LDs. Increased expression of ADFP mRNA by the synthetic PPARα agonist resulted in only a moderate increase in ADFP protein content. In contrast, increased ADFP mRNA under situations in which LDs were formed (i.e., FA supplementation or fasting) resulted in a much greater cellular content of ADFP. Such a difference in ADFP mRNA and protein levels has also been found in mature adipocytes, in which perilipin is thought to displace ADFP from the LD surface (7, 19). It is known that forced expression of ADFP might increase FA uptake (21) and enhance the formation of LDs in cell culture experiments (7, 9, 22). In agreement with this, increased mRNA expression and intracellular ADFP protein content have been observed in different cell types in various diseases

(24, 56–58) and in a rodent model of obesity (12). These observations were all obtained in cells or tissues that had been exposed to high levels of FAs with enhanced formation of intracellular LDs. All of these observations jointly suggest that the cellular availability of FAs and the subsequent formation of LDs are required for cellular accumulation of the ADFP protein. A more recent publication suggests that ADFP is actively degraded by proteasomes when not bound to LDs (59). This might explain the low content of ADFP protein in cells lacking intracellular LDs compared with the high ADFP content observed in cells containing LDs.

In marked contrast to the induction of ADFP by fasting or the PPARα agonist, we could not observe any transcriptional regulation of ADFP with an LXR agonist. Similarly, the LXR agonist barely changed the TAG or ADFP protein content in 7800-C1 cells. Activation of LXRs stimulates lipogenesis and the synthesis of TAG in liver and increases the secretion of VLDL particles (49). The transcriptional regulation of ADFP in liver cells is apparently stimulated by increased cellular FA uptake but not by increased cellular FA synthesis. This suggests that ADFP likely does not have a role in the binding of lipid bodies synthesized through activated lipogenesis. The expression and presence of ADFP in the liver are likely much more important during fasting.

TIP47 is another PAT protein known to be expressed at significant levels in liver (12). We failed to detect any relevant changes in TIP47 mRNA levels in any of our experiments. TIP47 also seems not to be transcriptionally regulated by PPARγ in adipose tissue (12). Although we cannot exclude the possibility that the TIP47 protein is targeting to LDs when they are formed, it seems reasonable that ADFP is the major PAT protein that coats the LDs in these experiments. Therefore, we propose that TIP47 is a less specialized and more ubiquitously expressed PAT protein whose function is the packaging of small LDs under normal physiological conditions. In contrast, ADFP seems to be a more specialized and physiologically regulated PAT protein in liver. The main function of ADFP in liver is to ensure the efficient uptake and trapping of FAs that are released from peripheral tissues by coating the TAG-containing LDs formed in that organ upon fasting.

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