PPARα activation increases triglyceride mass and adipose differentiation-related protein in hepatocytes

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Abstract **Adipose differentiation-related protein (ADRP) is a lipid droplet-associated protein that is expressed in various tissues. In mice treated with the peroxisome proliferator-activated receptor α (PPARα) agonist Wy14,643 (Wy), hepatic mRNA and protein levels of ADRP as well as hepatic triglyceride content increased. Also in primary mouse hepatocytes, Wy increased ADRP expression and intracellular triglyceride mass. The triglyceride mass increased in spite of unchanged triglyceride biosynthesis and increased palmitic acid oxidation. However, Wy incubation decreased the secretion of newly synthesized triglycerides, whereas apolipoprotein B secretion increased. Thus, decreased availability of triglycerides for VLDL assembly could help to explain the cellular accumulation of triglycerides after Wy treatment. We hypothesized that this effect could be mediated by increased ADRP expression. Similar to PPARα activation, adenovirus-mediated ADRP overexpression in mouse hepatocytes enhanced cellular triglyceride mass and decreased the secretion of newly synthesized triglycerides. In ADRP-overexpressing cells, Wy incubation resulted in a further decrease in triglyceride secretion. This effect of Wy was not attributable to decreased cellular triglycerides after increased fatty acid oxidation because the triglyceride mass in Wy-treated ADRP-overexpressing cells was unchanged. In summary, PPARα activation prevents the availability of triglycerides for VLDL assembly and increases hepatic triglyceride content in part by increasing the expression of ADRP.**—Edvardsson, U., A. Ljungberg, D. Lindén, L. William-Olsson, H. Peilot-Sjögren, A. Ahnmark, and J. Oscarsson. **PPARα activation increases triglyceride mass and adipose differentiation-related protein in hepatocytes.** ***J. Lipid Res.*** **2006.** 47: **329–340.**

Supplementary key words Wy14,643 • primary hepatocytes • triglyceride synthesis • fatty acid oxidation • triglyceride secretion • apolipoprotein B-100 • apolipoprotein B-48 • peroxisome proliferator-activated receptor α

Peroxisome proliferator-activated receptor α (PPARα) is a ligand-activated transcription factor that plays a key role in the regulation of genes involved in carbohydrate, lipid, and lipoprotein metabolism (for review, see 1). PPARα is highly expressed in tissues with high mitochondrial and peroxisomal β-oxidation activities, such as liver, heart, kidney, and skeletal muscle (2–5). In humans, treatment with PPARα agonists (i.e., fibrates) results in decreased plasma levels of triglycerides and increased plasma HDL cholesterol levels (6, 7). The triglyceride-lowering effect of fibrates is partly explained by increased lipoprotein lipase expression (8) and downregulation of hepatic apolipoprotein C-III expression (9, 10), which results in increased turnover of VLDL. In addition, fibrates have been shown to decrease the plasma concentration of atherogenic small dense LDL particles (11, 12), indicating decreased hepatic triglyceride secretion, because small dense LDLs are products of triglyceride-rich VLDL ($VLDL_1$) particles (13). Indeed, fibrates have been shown to decrease VLDL triglyceride secretion in both humans and rats (14, 15). In primary rat hepatocytes, fibrates reduced triglyceride secretion and decreased the size of the secreted apolipoprotein B (apoB)-containing lipoproteins (16). The reduced triglyceride secretion may be explained by decreased triglyceride biosynthesis in rat hepatocytes (16, 17). However, in vivo in rats, incorporation of palmitate into liver triglycerides was unchanged, whereas triglyceride secretion decreased (15), indicating that other effects of PPARα activation than decreased triglyceride biosynthesis gave rise to the decreased triglyceride secretion. One suggested possibility is that PPARα activation increases diacylglycerol acyltransferase (DGAT) activity in the cytoplasm while inhibiting DGAT activity in the microsomal compartment, thereby diverting triglycerides away from the secretory pathway without influencing total cellular triglyceride synthesis (18).

Adipose differentiation-related protein (ADRP) is a lipid storage droplet-associated protein belonging to the PAT (Perilipin, ADRP, and TIP 47) family (19). ADRP was first

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Abbreviations: ADRP, adipose differentiation-related protein; apoB, apolipoprotein B; DGAT, diacylglycerol acyltransferase; MTP, microsomal triglyceride transfer protein; PPARα, peroxisome proliferator-activated receptor α; PPRE, peroxisome proliferator-activated receptor response element; Wy, Wy14,643; 36B4, acidic ribosomal phosphoprotein P0.

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identified as an early marker of adipocyte differentiation (20). However, studies have shown that ADRP is expressed in a variety of tissues and cultured cells (21, 22). It has been suggested to be a marker of lipid accumulation, as the cellular protein level of ADRP is related to the total mass of neutral lipids within the cell (21, 23). A few studies have explored the function of ADRP. Overexpression of ADRP in fibroblasts resulted in lipid accumulation and lipid droplet formation without induction of adipogenic genes (24). Also in macrophages, ADRP overexpression gave rise to lipid accumulation without changed expression of the genes involved in lipid efflux (25). Furthermore, transfection of COS-7 cells with ADRP was shown to promote the uptake of long-chain fatty acids (26). Together, these results indicate that ADRP increases intracellular lipids without changing the expression of genes involved in lipid metabolism, but the mechanisms of this effect are still unclear.

ADRP expression has been reported to be regulated by long-chain fatty acids at the transcriptional level (27). In macrophages and in colorectal cancer cells, ADRP expression was induced by agonists of PPAR subtypes α, γ, and δ (28–30). Peroxisome proliferator-activated receptor response elements (PPREs) were recently identified both in the murine and human ADRP promoters (31, 32), showing that PPAR agonists regulate ADRP expression by increasing transcription of the gene. However, the regulation of ADRP by PPARα in liver and hepatocytes has not been investigated.

The aims of this study were to investigate the effects of the specific PPARα agonist Wy14,643 (Wy) (33) on the expression of ADRP in mouse liver in vivo and in primary mouse hepatocytes in vitro and to determine the importance of changed ADRP expression for the effects of PPARα activation on triglyceride secretion and intracellular triglyceride accumulation.

MATERIALS AND METHODS

Animals and treatment

C57BL/6 mice were from Taconic Europe (Ry, Denmark). Homozygous PPARα null mice and corresponding wild-type control mice, on a pure Sv/129 genetic background, were used for in vitro experiments (kindly provided by Dr. F. J. Gonzalez, National Institutes of Health, Bethesda, MD) (34). PPARα null mice and littermate controls, backcrossed for two generations with C57BL/6, were used for in vivo experiments. The animals were housed individually and maintained under standardized conditions of temperature (21–22°C) and humidity (40–60%), with light from 6:00 AM to 6:00 PM for at least 1 week before the experiments. The mice were given either standard laboratory chow containing (energy %) 12% fat, 62% carbohydrates, and 26% protein, with a total energy content of 12.6 kJ/g (R3; Lactamin AB, Kimstad, Sweden), or a high-fat diet containing 48% fat (mainly saturated), 15% protein, and 37% carbohydrates, with a total energy content of 21.4 kJ/g (Lactamin). The mice were fed laboratory chow or high-fat diet for 3 weeks and treated with Wy (30 μmol/kg/day; Chemsyn Science Laboratories, Lenaxa, KS) in 0.5% (w/v) methyl cellulose by gavage once daily for the last 2 weeks. Age-matched control mice received only vehicle. Food intake was estimated by weighing the food twice weekly. The mice were anesthetized with isoflurane (Forene; Abbot Scandinavia AB), and the livers were removed, immediately frozen in liquid nitrogen, and stored at − 70°C. The study protocol was approved by the Ethics Committee of Göteborg University. All experiments were conducted in accordance with accepted standards of humane animal care.

Liver triglycerides

Frozen livers were homogenized in isopropanol (1 ml/50 mg tissue) and incubated at 4°C for 1 h. The samples were centrifuged at 4°C for 5 min at 2,500 rpm, and triglyceride concentrations in the supernatants were measured with an enzymatic colorimetric assay (Roche, Mannheim, Germany).

Production of recombinant adenoviruses

A pBluescript SK(+) vector containing full-length ADRP was kindly provided by Björn Magnusson (Wallenberg Laboratory for Cardiovascular Research, Sahlgrenska University Hospital, Göteborg, Sweden). The ADRP construct was then transferred to a pENTR vector (Invitrogen, Carlsbad, CA) and recombined into pAd/CMV/V5-DEST (Invitrogen) according to the manufacturer's manual. The packaging cell line Ad-293 (Stratagene, La Jolla, CA) was grown in Dulbecco's modified Eagle's medium with 10% FBS supplemented with 100,000 IU/l penicillin and 100 mg/l streptomycin. Cells were seeded in 25 cm^2 culture flasks, cultured to 90–95% confluence, and transfected with adenoviral constructs for ADRP or the control zsGreen (35) digested with Pac-1 using Lipofectamine 2000 in Opti-MEM according to the manufacturer's manual. After 4 h of incubation, DMEM containing 20% FBS was added, resulting in a concentration of 10% FBS. Cells were cultured for 10–14 days until cytopathic effects were ~80%. Viruses were harvested by repeated freeze/thaw cycles in 10 mM Tris-HCl, pH 8.0. After large-scale amplification using Cell Factories (Nunc, Rochester, NY), recombinant adenoviruses were purified by two rounds of CsCl density gradient ultracentrifugation. The purified virus stocks were desalted over 10DG columns (Bio-Rad, Hercules, CA) and eluted in sterile PBS. Glycerol (65%) was added (1:5 dilution with virus suspension) before the virus stocks were divided into aliquots and stored at −80°C until use. Infectious viral titers were determined using the Adeno-X Rapid Titer Kit (Clontech, Palo Alto, CA). All purified virus stocks were screened for possible wild-type virus contamination according to Zhang, Koch, and Roth (36) before use.

Primary hepatocyte cultures

Mouse hepatocytes were obtained by nonrecirculating collagenase perfusion through the portal vein of the mice (10–16 weeks of age) as described (16, 37, 38). In brief, the cells were seeded at 100,000 cells/cm^2 in dishes (Falcon, Plymouth, UK) coated with laminin-rich matrigel (BD Biosciences, Bedford, MA). The cells were cultured during the first 16–18 h in Williams' E medium with Glutamax (Invitrogen) supplemented as described (37). The cells were then treated for up to 3 days with 1 or 10 μM Wy (Chemsyn Science Laboratories) dissolved in DMSO [final concentration, 0.15% (v/v)] in medium supplemented as described above plus 1 nM dexamethasone (Sigma, St. Louis, MO) and 3 nM insulin (Actrapid; Novo Nordisk A/S). In experiments with adenoviral overexpression of ADRP or the control zsGreen, cells were infected with virus (500 infectious units/cell) in 0.75 ml of culture medium (10 cm^2 dish) starting 4 h after seeding. Two hours later, medium was added to a final volume of 2 ml and infection was continued overnight. After 17 h of infection with

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virus, the medium was replaced with virus-free medium. Analyses were performed 3 days after infection.

Quantitative real-time PCR analysis

Total RNA of cultured primary hepatocytes and mouse liver was isolated with TriReagent™ (Sigma) according to the manufacturer's protocol, and the concentration of RNA was determined spectrophotometrically at 260 nm. To remove contaminating DNA, total RNA was treated with DNA free (Ambion, Austin, TX) before being retrotranscribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Quantitative real-time PCR analysis was performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems) using SYBR Green detection chemistry. All samples were analyzed in triplicate. To exclude that the amplification-associated fluorescence was associated with residual genomic DNA and/or from the formation of primer dimers, controls without reverse transcriptase or DNA template were analyzed. RT-PCR products were also analyzed by electrophoresis in ethidium bromide-stained agarose gels to check that a single amplicon of the expected size was obtained. The expression data were normalized to the endogenous control acidic ribosomal phosphoprotein P0 (36B4). The expression of 36B4 was not influenced by the various treatments in this study. The relative expression levels were calculated according to the formula $2^{-\Delta Ct}$, where ΔCt is the difference in threshold cycle (Ct) values between the target and the 36B4 endogenous control. Specific primers for each gene (**Table 1**) were designed using Primer Express software (Applied Biosystems).

Protein preparation and Western blot

Matrigel was removed from cultured primary hepatocytes by incubation on ice for 60 min in PBS containing 5 mM EDTA followed by washings in PBS. Total protein extracts from frozen livers and cultured hepatocytes were prepared as described previously (39), and protein concentrations were determined with the RC/DC Protein Assay Kit II (Bio-Rad). Proteins were separated on 10–20% Tris-glycine gels (Invitrogen) and transferred to Hybond-P polyvinylidene difluoride transfer membrane (Amersham Biosciences, Bucks, UK). Equal loading was confirmed by staining the membranes with 0.2% Ponceau S (Serva, Heidelberg, Germany). Immunoblotting was performed using a guinea pig polyclonal anti-ADRP antibody at 1:2,000 (Research Diagnostics, Inc., Flanders, NJ) and a horseradish peroxidase-conjugated anti-guinea pig antibody at 1:20,000 (Dako, Glostrup, Denmark), followed by detection using the enhanced chemiluminescence plus detection system (Amersham Biosciences). Band intensity was quantified with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Palmitic acid oxidation

Williams' E medium with Glutamax, supplemented as described above, containing [9,10(n)-^{3}H]palmitic acid was prepared as described by Leung and Ho (40). To each culture dish (10 cm^2), 1 ml of medium containing 110 μM unlabeled palmitic acid and 8.3 μCi of [9,10(n)-^{3}H]palmitic acid (specific activity, 54 Ci/mmol) was added. The fatty acid oxidation was shown to be linear between 30 and 120 min of incubation at 37°C (data not shown). Fatty acid oxidation was thereafter determined after 60 or 90 min of incubation with labeled palmitic acid. Labeled water-soluble products were isolated and analyzed as described previously (35) but with one additional precipitation step. Background radioactivity was determined by precipitation of fatty acids in medium that had not been in contact with cells. The fatty acid oxidation was related to the DNA content in each culture dish, which was determined according to Labarca and Paigen (41).

Triglyceride biosynthesis and accumulation of triglycerides in cell medium

Triglyceride biosynthesis in cultured hepatocytes was estimated by measurement of incorporated [9,10(n)-^{3}H]palmitic acid (concentration as described above) in cellular triglycerides after 60–90 min of incubation at 37°C. Accumulation of newly synthesized triglycerides in the medium was determined after 2–6 h of incubation with [9,10(n)-^{3}H]palmitic acid at 37°C. Cells and medium were then collected and lipid extraction was performed according to Bligh and Dyer (42). Lipids were separated by thin-layer chromatography (silica gel 60 on plastic sheets; Merck, Darmstadt, Germany) with chloroform-acetic acid (96:4). The bands corresponding to triglycerides were recovered and extracted from the silica gel with 1 ml of cyclohexane followed by the addition of 10 ml of scintillation solution (Ready Safe™; Beckman Coulter, Fullerton, CA) before the radioactivity was measured. Triglyceride synthesis and the accumulation of newly synthesized triglycerides in the cell culture medium were related to the DNA content in each culture dish as described above.

Estimation of apoB secretion

The secretion of apoB-48 and apoB-100 from primary mouse hepatocyte cultures was estimated by labeling the cells with a [^{35}S]methionine-cysteine mix (Amersham Biosciences) for 2 h followed by a 4 h chase in culture medium supplemented with 10 mM methionine, as described (38, 43, 44). Labeled apoB-48 and apoB-100 were isolated by immunoprecipitation with 10 μl of polyclonal rabbit anti-human apoB antibodies (DakoCytomation, Glostrup, Denmark), followed by 5% polyacrylamide gel electrophoresis containing SDS. The bands corresponding to apoB-48 and apoB-100 were quantified using a FLA-3000 phosphorimager (Fujifilm). The densities were related to the total amount of DNA in each culture dish, as described above.

Triglyceride mass in hepatocytes

Intracellular triglyceride content in hepatocytes was determined by HPLC separation of neutral lipids as described (45). Briefly, after extraction with Folch reagent (46) and evaporation,

TABLE 1. Primers used for quantitative real-time PCR

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
ADRP	TGGCAGCAGCAGTAGTGGAT	CAGGTTGGCCACTCTCATCA
CPT-I	TGAGTGGCGTCCTCTTTGG	CAGCGAGTAGCGCATAGTCATG
LCAD	GCGAAATACTGGGCATCTGAA	TCCGTGGAGTTGCACACATT
ACO	CAGCAGGAGAAATGGATGCA	GGGCGTAGGTGCCAATTATCT
36B4	GAGGAATCAGATGAGGATATGGGA	AAGCAGGCTGACTTGGTTGC

ACO, acyl-coenzyme A oxidase; ADRP, adipose differentiation-related protein; CPT-I, carnitine palmitoyltransferase I; LCAD, long-chain acyl-coenzyme A dehydrogenase; 36B4, acidic ribosomal phosphoprotein P0.

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lipids were dissolved in hexane-isopropanol-acetic acid (98.7:1.2:0.1) and separated by HPLC in an isocratic system with 40% hexane (0.6 ml/min). Lipids were detected using a light-scattering detector (PL-ELS 1000; Polymer Laboratories, Shropshire, UK), and the amount of triglycerides was quantified using the standard HPLC Mix 42 (Larodan, Malmö, Sweden). The amount of triglycerides was related to the total amount of DNA in each culture dish as described above.

Statistics

Values are expressed as means ± SEM. Comparisons between groups were made by Kruskal-Wallis test and Mann-Whitney *U*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of Wy on ADRP expression and liver triglycerides in vivo

C57BL/6 mice were fed ordinary chow or high-fat diet for 3 weeks and treated with Wy (30 µmol/kg/day) by gavage during the last 2 weeks. Body weight gain during the treatment did not differ between the groups. However, the food consumption decreased, whereas the energy intake increased in the groups fed the high-fat diet (**Table 2**). Irrespective of diet, Wy treatment resulted in increased hepatic ADRP mRNA expression (**Fig. 1A**). ADRP protein expression increased by 2-fold with Wy treatment in mice on the chow diet, whereas Wy had no significant effect on ADRP protein expression in mice fed the high-fat diet (Fig. 1B). The high-fat diet per se increased ADRP protein expression, whereas mRNA expression was unaffected. Wy treatment increased hepatic triglyceride concentration by 33% in chow-fed mice, and to a lesser degree (15%) in mice fed the high-fat diet (Fig. 1C). When taking into account the increased liver weight as a result of Wy treatment, the total triglyceride content increased significantly in the chow-fed group by 42%, and by 64% in the high-fat diet group (data not shown). The experiment in which the mice were on the ordinary chow diet was repeated. Also in this experiment, Wy significantly increased ADRP mRNA (500%) and protein (90%) expression as well as hepatic triglyceride concentration (54%) (data not shown).

TABLE 2. Food consumption and body weight gain

Treatment and Diet	Food Consumption	Energy Intake	Body Weight Gain
	g/day	*kJ/day*	*g*
Vehicle chow	3.64 ± 0.09	45.9 ± 1.1	1.07 ± 0.36
Wy chow	3.74 ± 0.11	47.1 ± 1.4	1.67 ± 0.46
Vehicle high-fat diet	2.66 ± 0.06[a]	56.9 ± 1.3[a]	1.33 ± 0.17
Wy high-fat diet	2.72 ± 0.06[a]	58.2 ± 1.3[a]	0.16 ± 0.35

Wy, Wy14,643. C57BL/6 mice were fed the chow or high-fat diet for 3 weeks and were treated with vehicle or Wy (30 µmol/kg/day) for the last 2 weeks. Values are means ± SEM (n = 7).

[a] $P < 0.05$, vehicle chow versus vehicle high-fat diet or Wy chow versus Wy high-fat diet (Kruskal-Wallis test followed by Mann-Whitney *U* test).

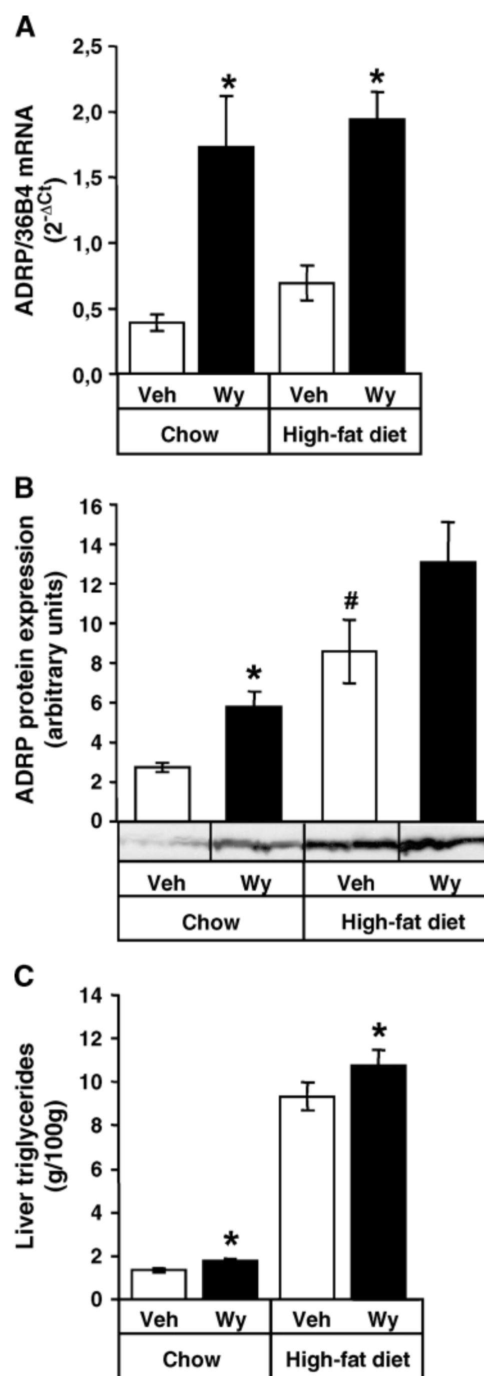

Fig. 1. Effects of Wy14,643 (Wy) treatment on adipose differentiation-related protein (ADRP) mRNA expression (A), protein expression (B), and hepatic triglyceride concentration (C) in vivo. C57BL/6 mice were fed the chow or high-fat diet for 3 weeks and treated with Wy (30 µmol/kg/day) or vehicle (Veh) for the last 2 weeks. ADRP mRNA and protein levels were estimated by quantitative real-time PCR and Western blotting, respectively. Liver triglyceride concentration was estimated as described in Materials and Methods. The Western blot in B shows two representative individuals per group. 36B4, acidic ribosomal phosphoprotein P0. Values are means ± SEM based on six (A), four (B), or seven (C) observations. *$P < 0.05$, vehicle chow versus Wy chow or vehicle high-fat diet versus Wy high-fat diet; #$P < 0.05$, vehicle chow versus vehicle high-fat diet (Kruskal-Wallis test followed by Mann-Whitney *U*-test).

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Effects of Wy on ADRP expression in PPARα null mice

To investigate whether the effect of Wy was mediated via PPARα activation, littermate wild-type and PPARα null mice were fed a high-fat diet for 3 weeks and treated the last 2 weeks with Wy (as described above). Food consumption and body weight gain during the treatment did not differ between the groups (**Table 3**). The effect of Wy on ADRP mRNA and protein expression was dependent on PPARα, as shown in **Fig. 2**. ADRP mRNA expression was lower, whereas ADRP protein levels were higher in the PPARα null mice than in littermate controls. The hepatic triglyceride concentration was not significantly influenced by Wy treatment or PPARα deficiency in this experiment.

Effects of Wy on ADRP expression in primary mouse hepatocytes

To determine whether the stimulatory effect on ADRP expression was a direct effect on hepatocytes, we incubated primary mouse hepatocytes from C57BL/6 mice with 10 μM Wy for 3 days. Wy increased the ADRP mRNA expression (**Fig. 3A**) and protein expression (Fig. 3B) in parallel with increased cellular triglyceride mass (Fig. 3C). Experiments were also performed on hepatocytes derived from PPARα null mice and wild-type mice on a pure Sv/129 genetic background. Incubation with 10 μM Wy for 3 days increased ADRP mRNA expression 200% in wild-type cells but had no effect in cells from PPARα null mice (data not shown).

Effects of Wy on palmitic acid oxidation

The increased triglyceride mass in hepatocytes after Wy treatment was unexpected, as PPARα activation is known to increase the oxidation of fatty acids. Therefore, we characterized the effect of Wy on metabolic events that influence the amount of triglycerides in hepatocytes. First, we measured the oxidation of palmitic acid in mouse hepatocytes after 3 days of exposure to 1 or 10 μM Wy. As expected, the fatty acid oxidation was enhanced by Wy, resulting in a 4-fold increase when cells were incubated with 10 μM Wy (**Table 4**). The enhanced fatty acid oxidation was paralleled by increased mRNA levels of carnitine palmitoyltransferase I and long-chain acyl-coenzyme A dehydrogenase (Table 4), enzymes involved in the mitochondrial β-oxidation of palmitic acid. We also measured the mRNA expression of acyl-coenzyme A oxidase, which participates in the peroxisomal fatty acid oxidation and is known to be PPAR-responsive (47). Wy (10 μM) increased the expression of acyl-coenzyme A oxidase by 14-fold, as shown in Table 4. Thus, these cells responded well to Wy incubation in terms of fatty acid oxidation and gene expression.

TABLE 3. Food consumption and body weight gain

Treatment and Genotype	Food Consumption	Body Weight Gain
	g/day	*g*
Vehicle wild type	2.35 ± 0.04	0.66 ± 0.40
Wy wild type	2.27 ± 0.07	−0.47 ± 0.42
Vehicle PPARα null	2.44 ± 0.12	0.81 ± 0.36
Wy PPARα null	2.45 ± 0.12	1.23 ± 0.40

PPARα, peroxisome proliferator-activated receptor α. Littermate wild-type and PPARα null mice were fed a high-fat diet for 3 weeks and treated with vehicle or Wy (30 μmol/kg/day) for the last 2 weeks. Values are means ± SEM (n = 7). No significant differences were found between the groups (Kruskal-Wallis test).

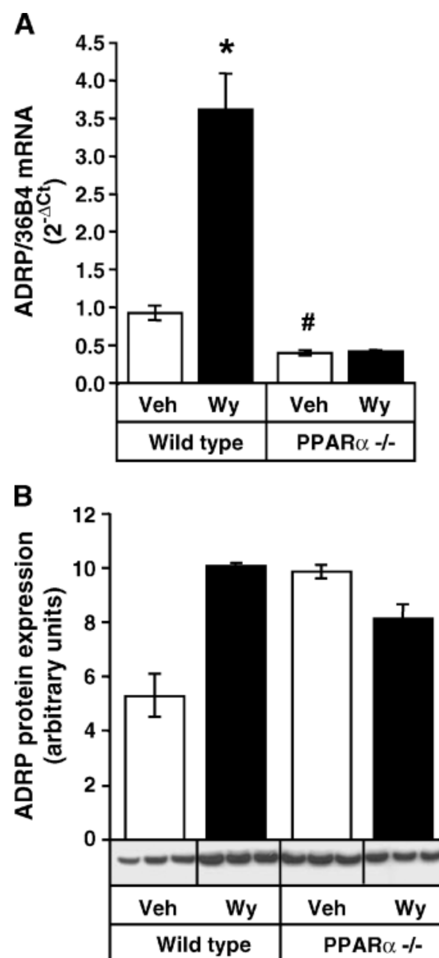

Fig. 2. Effects of Wy treatment on ADRP mRNA expression (A) and protein expression (B) in peroxisome proliferator-activated receptor α (PPARα) null mice in vivo. PPARα null mice and wild-type littermates were fed a high-fat diet for 3 weeks and treated with Wy (30 μmol/kg/day) or vehicle (Veh) for the last 2 weeks. ADRP mRNA and protein levels were estimated by quantitative real-time PCR and Western blotting, respectively. Values are means ± SEM based on seven (A) or three (B) observations. *$P < 0.05$, vehicle wild type versus Wy wild type; $^{\#}P < 0.05$, vehicle wild type versus vehicle PPARα null (Kruskal-Wallis test followed by Mann-Whitney *U*-test). The statistical analysis of protein expression (B) showed a significant difference between the groups, $P < 0.05$ (Kruskal-Wallis test; but the Mann-Whitney *U*-test showed no significant differences between individual groups).

Effects of Wy on triglyceride biosynthesis and the accumulation of triglycerides in the medium

To further characterize the effects of Wy on lipid metabolism in primary mouse hepatocytes, we estimated triglyceride biosynthesis using [^{3}H]palmitic acid as a tracer. Three days of exposure to Wy did not affect the

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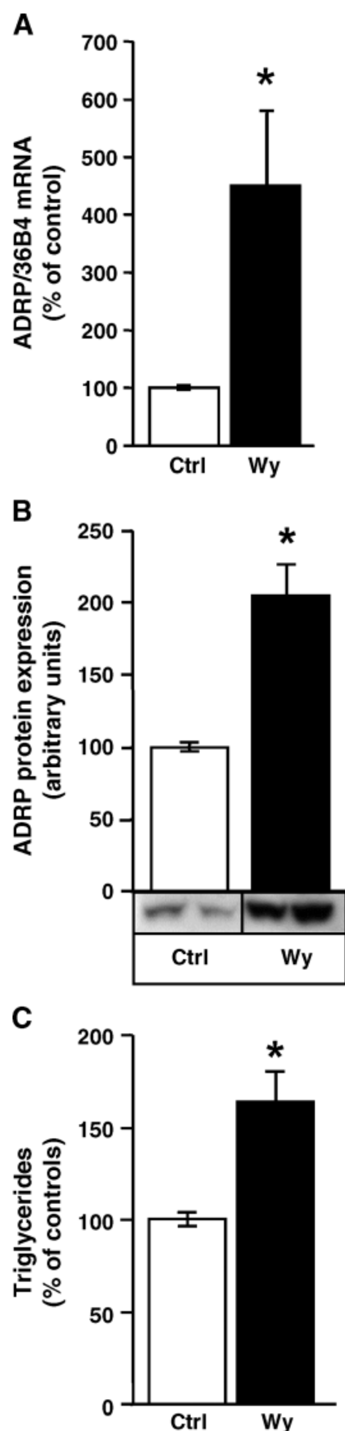

Fig. 3. Effects of Wy on ADRP mRNA expression (A), protein expression (B), and intracellular triglyceride mass (C) in primary mouse hepatocytes. Hepatocytes were isolated from C57BL/6 mice by liver perfusion and incubated with 10 μM Wy for 72 h. ADRP mRNA and protein expressions were quantified by quantitative real-time PCR and Western blotting, respectively. Triglyceride mass was determined by HPLC separation of neutral lipids as described in Materials and Methods. Data are based on measurements of $2^{-\Delta Ct}$ (A) and μg triglyceride/μg DNA (C) and presented as percentages of control (Ctrl). Values are means ± SEM based on five (A) and three (B, C) independent liver perfusions with one to two (A, B) or three (C) culture dishes in each group. *$P < 0.05$, Mann-Whitney *U*-test.

TABLE 4. Effect of Wy on fatty acid oxidation and gene expression

Fatty Acid or Gene	Control	Wy (1 μM)	Wy (10 μM)
Palmitic acid oxidation (n = 4)	100.0 ± 4.6	319.6 ± 38.3[a]	412.3 ± 78.8[a]
CPT-I expression (n = 4)	100.0 ± 5.3	167.9 ± 9.7[a]	270.7 ± 20.8[a]
LCAD expression (n = 5)	100.0 ± 3.0	227.5 ± 27.1[a]	375.1 ± 72.2[a]
ACO expression (n = 3)	100.0 ± 6.2	763.5 ± 115.6[a]	1431.8 ± 212.1[a]

Mouse hepatocytes were isolated and cultured with Wy for 72 h before a 90 min incubation in the presence of [9,10(*n*)-^{3}H]palmitic acid. mRNA expression of CPT-I, LCAD, and ACO was estimated by quantitative real-time PCR, and the expression data were normalized to 36B4. Values are means ± SEM (% of control) of three to five independent liver perfusions (as indicated) with one or two culture dishes per group.

[a] $P < 0.05$, control versus Wy (Kruskal-Wallis test, followed by Mann-Whitney *U* test).

triglyceride biosynthesis, estimated as palmitic acid incorporation into cellular triglycerides during 90 min (data not shown). Because Wy increased the intracellular triglyceride content despite an increased fatty acid oxidation and unchanged triglyceride biosynthesis, we also investigated whether PPARα activation influenced the partitioning of newly synthesized triglycerides between the cellular and extracellular compartments. **Figure 4A** shows data from a representative experiment, which illustrates the distribution of newly synthesized triglycerides in cells and in the medium after 2–6 h of incubation with [^{3}H]palmitic acid. In control cells, 30% of the newly synthesized triglycerides were recovered in the medium after 6 h of incubation, compared with 12% in the Wy-incubated cells. As shown in Fig. 4B, Wy decreased the accumulation of newly synthesized triglycerides in the medium by 50%, whereas no change in intracellular [^{3}H]triglycerides was detected after 6 h of incubation. Therefore, we conclude that PPARα activation results in decreased availability of newly synthesized triglycerides for VLDL assembly, although the cellular triglyceride mass increases.

Effects of Wy on the secretion of apoB-containing lipoprotein particles

Wy incubation of primary rat hepatocytes has been shown to result in increased apoB-100 secretion, whereas apoB-48 secretion was unaffected (16, 48). Because it is not known whether PPARα activation also influences apoB secretion from mouse hepatocytes, the effect of Wy on the secretion of apoB-100 and apoB-48 from primary mouse hepatocytes was investigated. As shown in **Fig. 5**, Wy incubation increased apoB-100 and apoB-48 secretion by 2.5-fold and 2-fold, respectively. Because triglyceride secretion was reduced, it can be concluded that Wy incubation of mouse hepatocytes gives to the secretion of an increased number of triglyceride-poor apoB-containing lipoprotein particles.

Effects of ADRP overexpression in mouse hepatocytes

Because ADRP overexpression in fibroblasts and in macrophages has been shown to stimulate lipid accumulation (24, 25), we hypothesized that the increase in ADRP ex-

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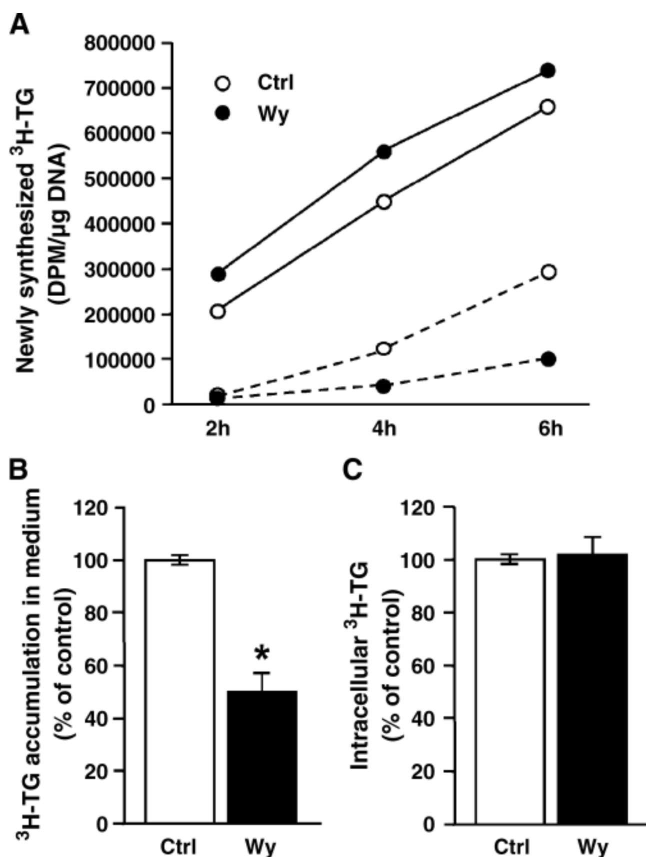

Fig. 4. Effect of Wy on the distribution of newly synthesized triglycerides (TG) between the cells and culture medium. Mouse hepatocytes were isolated from C57BL/6 mice by liver perfusion and treated with Wy for 72 h. Triglycerides were labeled using [^{3}H]palmitic acid as described in Materials and Methods. A: Data from one representative experiment show the distribution of newly synthesized triglycerides between the cell culture medium (dashed lines) and the cells (solid lines) after 2, 4, and 6 h of incubation with [^{3}H]palmitic acid. B, C: Recovery of newly synthesized triglycerides in the cell culture medium (B) and intracellularly (C) after 6 h of incubation with [^{3}H]palmitic acid. Data are based on measurements of dpm/μg DNA and presented as percentages of control (Ctrl). Values are means ± SEM based on three independent liver perfusions with two culture dishes in each group. *P 0.05, Mann-Whitney U-test.

pression after Wy treatment might be responsible for the increased cellular triglyceride content and decreased triglyceride secretion. Adenovirus-mediated overexpression of ADRP in primary hepatocytes resulted in a 2-fold increase in ADRP protein expression, as shown in **Fig. 6A**. To estimate the effect of ADRP overexpression on triglyceride biosynthesis, cells were incubated with [^{3}H]palmitic acid for 60 min. ADRP overexpression resulted in a slight increase in triglyceride biosynthesis (Fig. 6B). However, ADRP overexpression reduced the secretion of newly synthesized triglycerides by 50% (Fig. 6C), an effect that was paralleled by increased intracellular accumulation of newly synthesized triglycerides (Fig. 6D). Thus, these findings support the hypothesis that the increased ADRP expression by Wy may contribute to increased cellular triglyceride mass and decreased triglyceride secretion.

Effects of ADRP overexpression in the presence of Wy

To further explore the importance of ADRP for the effects of Wy, ADRP-overexpressing mouse hepatocytes were incubated with Wy. As shown in **Fig. 7A**, Wy did not further increase the ADRP protein expression in ADRP-overexpressing cells. Nevertheless, Wy decreased the accumulation of triglycerides in the medium (Fig. 7B). ADRP overexpression resulted in a slightly decreased palmitic acid oxidation (−10%) (Fig. 7C). However, also in ADRP-overexpressing cells, Wy increased the oxidation of palmitic acid (Fig. 7C). This result indicated that Wy might decrease the secretion of newly synthesized triglycerides by increasing the oxidation of fatty acids in ADRP-overexpressing cells. Therefore, we also measured the intracellular triglyceride mass (Fig. 7D). In spite of increased fatty acid oxidation, Wy incubation tended to further increase the triglyceride mass in ADRP-overexpressing cells ($P = 0.09$). This finding showed that the increased fatty acid oxidation after Wy incubation did not result in a lack of cellular triglycerides for VLDL secretion. Thus, increased ADRP expression in combination with other effects of PPARα activation are responsible for the decreased availability of the cytosolic triglycerides for VLDL assembly.

DISCUSSION

In this study, we extend previous findings of a regulation of ADRP by PPARα agonists (29, 30, 49) by showing that ADRP is regulated by a PPARα agonist also in the liver and in hepatocytes. The regulation of ADRP was at the level of mRNA, with a similar change in mRNA and protein levels both in vitro and in vivo. Interestingly, this regulation was shown to be accompanied by an increased cellular accumulation of triglycerides in vivo and in vitro. Because no change in triglyceride synthesis was observed, and as expected, the fatty acid oxidation was markedly enhanced by PPARα activation, we concluded that the decreased availability of triglycerides for VLDL assembly contributed to the increased intracellular content of triglycerides, an effect that could be mediated by increased ADRP expression. Therefore, we investigated the effect of ADRP overexpression and found decreased secretion of newly synthesized triglycerides from ADRP-overexpressing cells, although triglyceride synthesis increased. Thus, increased ADRP expression could contribute to the decreased secretion of newly synthesized triglycerides after Wy incubation. However, in cells overexpressing ADRP, incubation with Wy further decreased triglyceride secretion, indicating that increased ADRP expression is not the sole mechanism responsible for the decreased hepatic triglyceride secretion.

Few studies have addressed the effect of PPARα activation on the triglyceride content of hepatocytes. Ten days of fenofibrate treatment of rats resulted in a 50% increase in liver triglycerides (18). Moreover, it has been shown that incubation of primary rat hepatocytes with bezafibrate for 48 h resulted in increased cellular triglyceride mass (50). Because bezafibrate has been shown to activate the murine PPARα and PPARγ promoters with similar potency (33),

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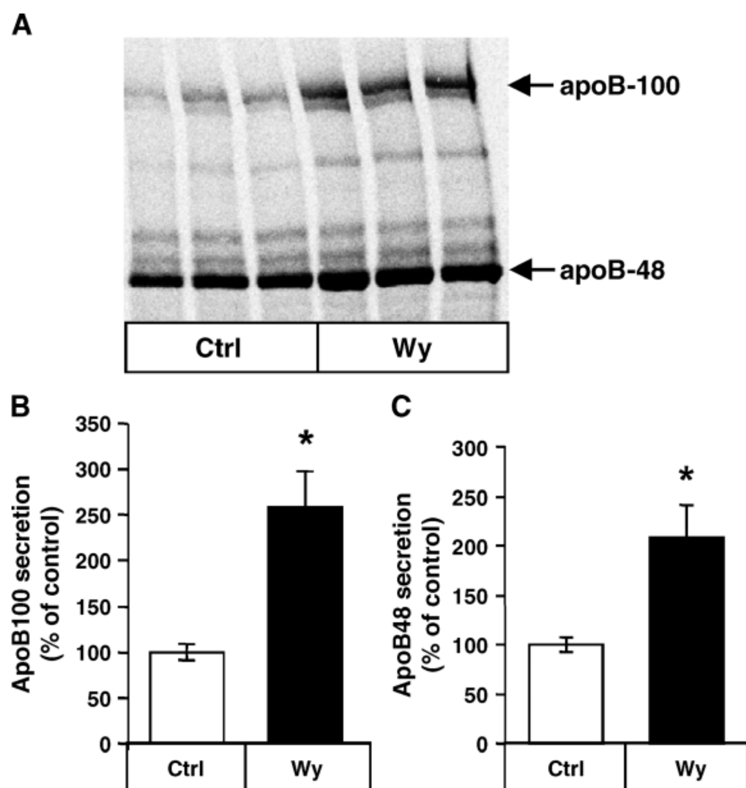

Fig. 5. Effect of Wy on apolipoprotein B-100 (apoB-100) and apoB-48 secretion. Mouse hepatocytes were isolated from C57BL/6 mice by liver perfusion and treated with Wy for 72 h. ApoB-100 and apoB-48 secretion was determined by labeling the cells with [^{35}S]methionine-cysteine mix for 2 h followed by a 4 h chase with an excess of cold methionine and immunoprecipitation of the culture medium as described in Materials and Methods. A: Autoradiogram from one representative experiment demonstrating the effect of Wy on radiolabeled apoB-48 and apoB-100 in the medium from three culture dishes in each group. B, C: Data are based on band densities/μg DNA, and apoB-100 secretion (B) and apoB-48 secretion (C) are presented as percentages of controls (Ctrl). Values are means ± SEM based on four independent liver perfusions with three culture dishes in each group. * $P < 0.05$, Mann-Whitney *U*-test.

the relative importance of PPARα and PPARγ activation for the increased triglyceride mass cannot be determined. In contrast to these findings, there are studies in lipoatrophic mice and mice fed a methionine- and choline-deficient diet demonstrating that treatment with fibrates alleviates hepatic steatosis (51, 52). However, these animals have a severe liver steatosis that may explain the different effect of Wy treatment. In addition, the mice were given a markedly higher dose of Wy than the dose used in our study (400 vs. 30 μmol/kg/day). This high dose of Wy resulted in a marked decrease in body weight gain in one of the studies (52), probably because of decreased food intake. Thus, the decreased body weight gain may explain the decreased triglyceride content of the liver. The lower dose of Wy used in the present study results in decreased plasma triglycerides and apoB levels without influencing food intake or body weight gain (48). Thus, the increased liver content of triglycerides occurs when a therapeutic dose of a PPARα agonist is given. The hepatic expression of ADRP might also increase in human subjects upon PPARα activation, because a PPRE has been demonstrated in the human ADRP (adipophilin) promoter (32). To the best of our knowledge, Wy has not been used in published clinical trials. Therefore, it is difficult to relate the present findings to the effects of fibrates used for the treatment of patients. However, the published maximal plasma concentrations for the commonly used fibrates are in the same range (fenofibrate, ∼40 μmol/l; gemfibrozil, ∼80 μmol/l) as the concentrations of Wy used in the in vitro experiments (53). The clinical importance of increased ADRP expression for the liver concentration of triglycerides in human subjects after PPARα activation awaits further studies.

From our results, it can be concluded that the hepatic triglyceride concentration increased less by Wy treatment when the animals were on a high-fat diet. The reason for this is probably that the high-fat diet induced ADRP protein expression. Interestingly, increased mRNA levels did not parallel the increased ADRP protein expression induced by a high-fat diet. It has been shown that *trans*-10, *cis*-12-conjugated linoleic acid induces ADRP protein to a much greater extent than ADRP mRNA in adipocytes (54). *Trans*-10, *cis*-12-conjugated linoleic acid was suggested to increase the translation of ADRP via increased mTOR/p70S6K/S6 signaling. In addition, oleic acid has been shown to stabilize the ADRP protein in Chinese hamster ovary cells by inhibition of proteasomal degradation (55). Thus, PPARα activation and dietary fat seem to regulate ADRP expression via different mechanisms.

Increased ADRP expression also results in increased cellular accumulation of triglycerides in cells that do not secrete lipoproteins (24, 25). Therefore, the decreased secretion of triglyceride-rich lipoproteins is not a prerequisite for the intracellular accumulation of triglycerides as a result of overexpression of ADRP. We showed that increased ADRP expression decreases fatty acid oxidation and increases net incorporation of palmitic acid into triglycerides. Thus, ADRP seems to compartmentalize fatty acids toward glycerolipid synthesis and away from oxidation, which could contribute to the increased accumulation of triglycerides in the cells. Therefore, the finding that Wy did not affect triglyceride synthesis in isolated hepatocytes might be the result of other effects of PPARα activation, such as increased fatty acid oxidation, that may counteract the effect of increased ADRP expression.

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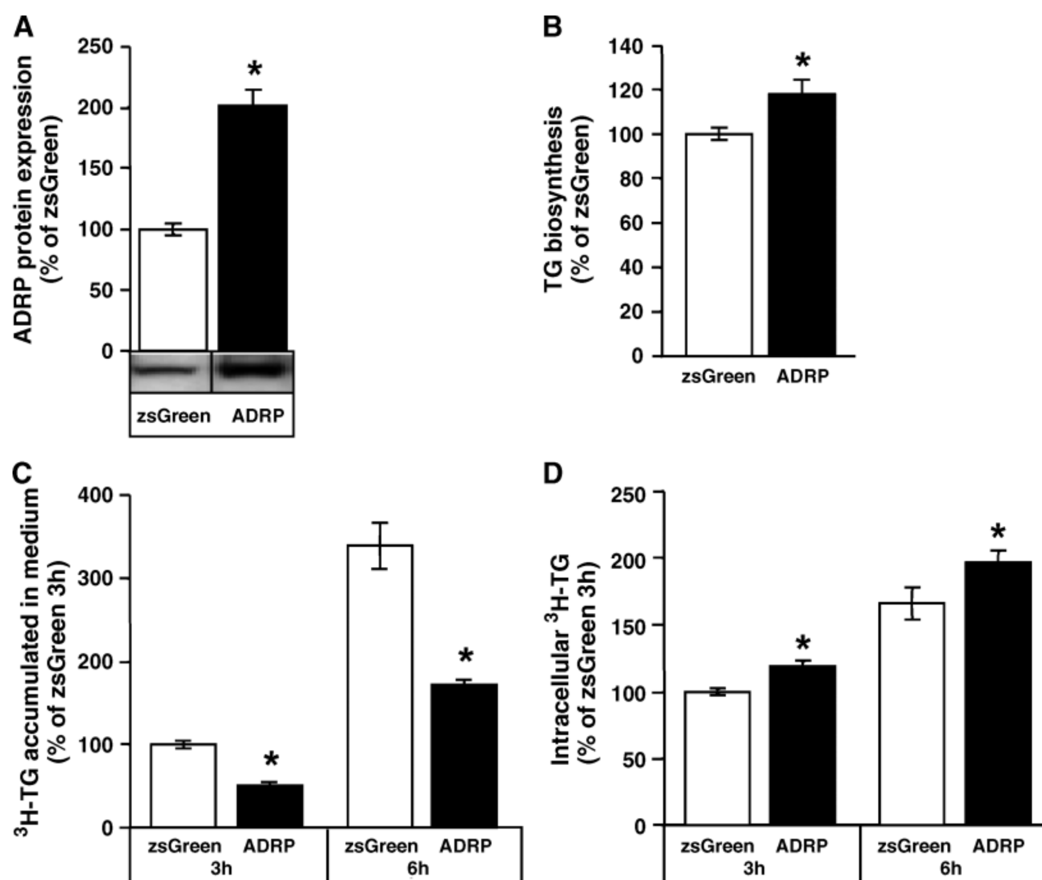

Fig. 6. Effect of ADRP overexpression on ADRP protein expression (A), triglyceride (TG) biosynthesis (B), and distribution of newly synthesized triglycerides to the cell culture medium (C) and intracellularly (D). Mouse hepatocytes were isolated from C57BL/6 mice by liver perfusion and transduced with adenoviruses expressing zsGreen or ADRP 4 h after seeding. Analyses were performed after 3 days of culture in control medium. A: ADRP protein expression was estimated by Western blotting. B: Triglyceride biosynthesis was determined by incubation with [^{3}H]palmitic acid for 60 min. C, D: Distribution of newly synthesized triglycerides in cell culture medium (C) and intracellularly (D) after 3 and 6 h of incubation with [^{3}H]palmitic acid. Data are based on measurements of dpm/μg DNA (B–D) and presented as percentages of zsGreen. Values are means ± SEM based on three independent liver perfusions with two culture dishes in each group. *$P < 0.05$, zsGreen versus ADRP at each time point [Mann-Whitney *U*-test (A, B) and Kruskal-Wallis test followed by Mann-Whitney *U*-test (C, D)].

ADRP overexpression has also been shown to increase fatty acid uptake in COS-7 cells (26). Interestingly, PPARα activation also results in changed expression of other genes that may take part in the increased uptake of fatty acids, such as acyl-CoA synthase, fatty acid transport protein (56), and liver fatty acid binding protein (57, 58). Thus, ADRP may increase cellular triglyceride accumulation in hepatocytes by increasing fatty acid uptake (26), diverting fatty acids to triglyceride formation, and preventing the use of triglycerides for VLDL assembly.

In a previous study using primary rat hepatocytes, we observed that Wy decreased both triglyceride synthesis and secretion (16). Thus, PPARα agonists seem to have different effects on triglyceride synthesis in cultured mouse and rat hepatocytes, although secretion of newly synthesized triglycerides decreases as a result of PPARα activation in both species. In this study, we showed that decreased triglyceride synthesis is not a prerequisite for the decreased availability of triglycerides for VLDL assembly.

PPARα activation increased the expression of microsomal triglyceride transfer protein (MTP) in both mouse and rat hepatocytes (48). MTP catalyzes the transfer of neutral lipids to apoB in the endoplasmic reticulum (59), and MTP expression has been shown to determine the rate of apoB secretion (60–62). In agreement with increased MTP expression, PPARα activation increased the secretion of both apoB-100 and apoB-48 from mouse hepatocytes. These data are partially in agreement with earlier results from rat hepatocytes. ApoB-100 secretion increased, whereas apoB-48 secretion was unaffected by Wy incubation of rat hepatocytes (16, 48). In cultured rat hepatocytes, the effect of Wy on apoB-100 secretion was not explained by the changed editing of apoB mRNA (16). Moreover, it is unlikely that the increased apoB-100 secretion from mouse hepatocytes is the result of decreased editing of apoB mRNA (63), because both apoB-48 and apoB-100 secretion increased to a similar degree. Thus, the reason for the different effects of Wy on apoB-48 secretion in mouse and rat hepatocytes is unclear.

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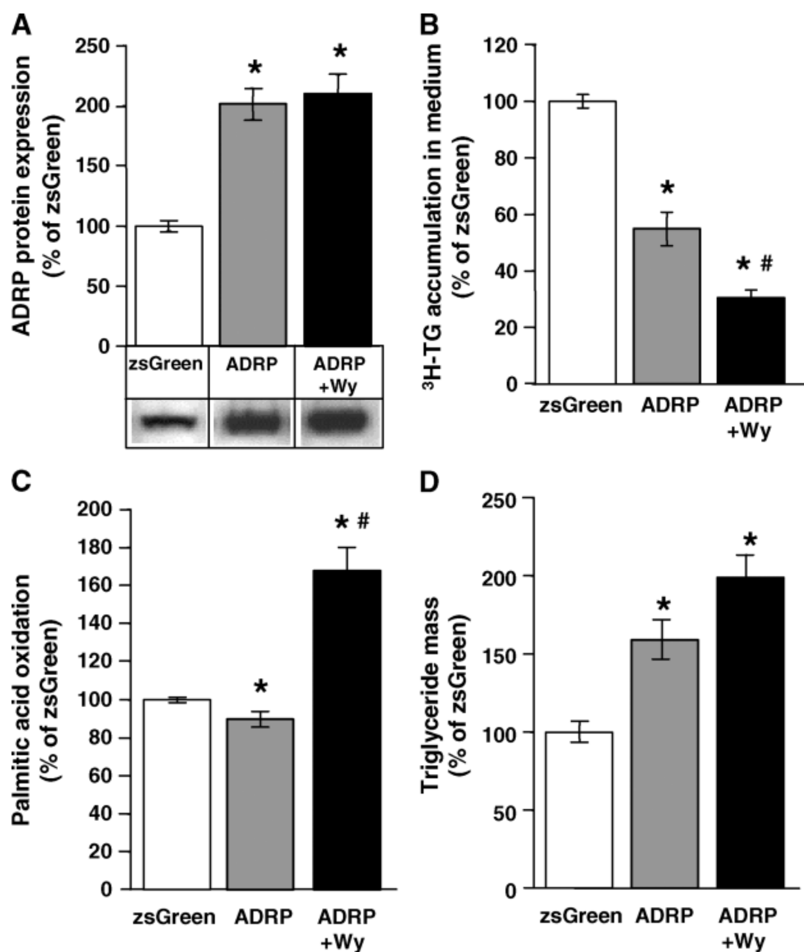

Fig. 7. Effect of Wy in cells overexpressing ADRP on ADRP protein expression (A), secretion of newly synthesized triglycerides (TG; B), palmitic acid oxidation (C), and intracellular triglyceride mass (D). Hepatocytes from C57BL/6 mice were isolated by liver perfusion and transduced with adenoviruses expressing zsGreen or ADRP 4 h after seeding. After 17 h of infection, medium was replaced with medium with or without 10 μM Wy. Analyses were performed 3 days after infection. A: ADRP protein expression was determined by Western blot. B: Accumulation of newly synthesized triglycerides in the cell culture medium was determined after 6 h of incubation with [^{3}H]palmitic acid. C: Fatty oxidation was determined after 1 h of incubation with [^{3}H]palmitic acid. D: Total triglyceride mass in hepatocytes was determined by HPLC separation of neutral lipids. Data are based on measurements of dpm/μg DNA (B, C) and μg triglyceride/μg DNA (D) and presented as percentages of zsGreen. Values are means ± SEM based on one to two (A), two (B, C), or three (D) culture dishes of three (A–C) or two (D) independent liver perfusions. $^*P < 0.05$, zsGreen versus ADRP and zsGreen versus ADRP + Wy; $^\#P < 0.05$, ADRP versus ADRP + Wy (Kruskal-Wallis test followed by Mann-Whitney *U*-test).

It has been shown that the fatty acids taken up by liver cells are not immediately available for VLDL assembly. A large part of the fatty acids are esterified in the cytosol, and cytosolic triglycerides need to be hydrolyzed into diacylglycerol and fatty acids to be available for the triglyceride synthesis for VLDL assembly (64, 65). Because Wy increases MTP activity (48) and cytosolic triglyceride levels, the availability of cytosolic triglycerides for the MTP-mediated lipidation of apoB in the VLDL assembly process must be prevented by PPARα activation. Moreover, PPARα activation must result in other changes than increased ADRP expression that prevents the flux of substrates for triglyceride synthesis to endoplasmic reticulum for VLDL assembly, because treatment with Wy had an effect also in ADRP-overexpressing cells. Fenofibrate treatment of rats increased DGAT activity in the cytoplasm and decreased DGAT activity in the endoplasmic reticulum (18). These changes would, like increased ADRP expression, result in the selective accumulation of triglycerides in the cytoplasm and decrease the availability of triglycerides for the MTP-dependent lipidation of apoB. It is less likely that the reduced activity of triglyceride hydrolase takes part in the decreased triglyceride secretion, because clofibrate treatment of mice did not influence the lipolytic activity of microsomes (66).

In summary, PPARα activation does not primarily decrease triglyceride secretion via enhanced fatty acid oxidation, because the intracellular triglyceride content of hepatocytes is not limiting for the availability of triglycerides. Rather, PPARα activation prevents the use of cytosolic triglycerides for VLDL assembly, in part by increasing the expression of ADRP.

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