Peroxisome proliferator-activated receptor α (PPAR α)-mediated regulation of multidrug resistance 2 (Mdr2) expression and function in mice

Tineke KOK^{*1}, Vincent W. BLOKS^{*}, Henk WOLTERS^{*}, Rick HAVINGA^{*}, Peter L. M. JANSEN^{*}, Bart STAELS^{†‡} and Folkert KUIPERS^{*}

^{*}Groningen University Institute for Drug Exploration, Center for Liver, Digestive and Metabolic Diseases, Laboratory of Pediatrics, University Hospital Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands, [†]UMR 545 INSERM Département d'Athérosclérose, Institut Pasteur de Lille, Lille, France, and [‡]Faculté de Pharmacie, Univ. Lille II, Lille, France

Peroxisome proliferator-activated receptor α (PPAR α) is a nuclear receptor that controls expression of genes involved in lipid metabolism and is activated by fatty acids and hypolipidaemic fibrates. Fibrates induce the hepatic expression of murine multidrug resistance 2 (*Mdr2*), encoding the canalicular phospholipid translocator. The physiological role of PPAR α in regulation of *Mdr2* and other genes involved in bile formation is unknown. We found no differences in hepatic expression of the ATP binding cassette transporter genes *Mdr2*, *Bsep* (bile salt export pump), *Mdr1a/1b*, *Abca1* and *Abcg5/Abcg8* (implicated in cholesterol transport), the bile salt-uptake systems *Ntcp* (Na⁺-taurocholate co-transporting polypeptide gene) and *Oatp1* (organic anion-transporting polypeptide 1 gene) or in bile formation between wild-type and *Ppar α ^(-/-)* mice. Upon treatment of wild-type mice with ciprofibrate (0.05 %, w/w, in diet for 2 weeks), the expression of *Mdr2* (+3-fold), *Mdr1a* (+6-fold) and *Mdr1b* (+11-fold) mRNAs was clearly induced, while that of *Oatp1* (–5-fold) was reduced. *Mdr2* protein levels were increased, whereas *Bsep*, *Ntcp*

and *Oatp1* were drastically decreased. Exposure of cultured wild-type mouse hepatocytes to PPAR α agonists specifically induced *Mdr2* mRNA levels and did not affect expression of *Mdr1a/1b*. Altered transporter expression in fibrate-treated wild-type mice was associated with a $\approx$ 400 % increase in bile flow: secretion of phospholipids and cholesterol was increased only during high-bile-salt infusions. No fibrate effects were observed in *Ppar α ^(-/-)* mice. In conclusion, our results show that basal bile formation is not affected by PPAR α deficiency in mice. The induction of *Mdr2* mRNA and *Mdr2* protein levels by fibrates is mediated by PPAR α , while the induction of *Mdr1a/1b* *in vivo* probably reflects a secondary phenomenon related to chronic PPAR α activation.

Key words: ATP binding cassette (ABC) transporter, bile, bile salt, peroxisome proliferator-activated receptor α (PPAR α)-null mouse, phospholipid.

INTRODUCTION

Bile formation critically depends on active secretion of osmotically active compounds from the liver into the bile canalicular lumen. Several transporter proteins at the hepatocyte canalicular membrane are involved in secretion of biliary constituents in an active, ATP-dependent manner. Bile salts and glutathione provide the major driving force for bile formation [1]. The bile salt export pump (*Bsep* or *Abcb11*) has been identified as the major canalicular bile salt-transporting protein [2], while glutathione is transported by *Mrp2* (or *Abcc2*) [3]. Bile salt secretion drives the secretion of phospholipids and cholesterol by as yet unknown mechanisms [4], but only when multidrug resistance 2 (*Mdr2*) P-glycoprotein (*Pgp*) is present. *Mdr2* *Pgp*, encoded by *Mdr2* (or *Abcb4*), is absolutely essential for phospholipid secretion into bile [5]. Transporters involved in the actual cholesterol secretion process have not been identified: a number of ATP binding cassette (ABC) transporters, i.e. *Abca1* [6] and *Abcg5/g8* [7], have recently been shown to play a role. The B subfamily of ABC transporters contains at least two additional members that are localized to the canalicular domain of hepatocytes in rodents, i.e. *Mdr1a* (or *Abcb1a*) and *Mdr1b* (or *Abcb1b*). The physiological function of *Mdr1a/1b* lies presumably in hepatobiliary transport

of bulky amphiphilic compounds, such as steroids, hydrophobic peptides and cationic drugs [8]. High biliary bile salt secretion rates, required for bile formation and effective dietary lipid absorption, are maintained by effective bile salt reabsorption from the intestine. Bile salts are taken up from portal blood, prior to resecretion into bile, by the Na⁺-taurocholate co-transporting polypeptide (*Ntcp*; *Slc10a1*) and Na⁺-independent organic anion-transporting polypeptides, including *Oatp1* (or *Slc21a1*) [9].

Insight into the molecular regulation of these transporters is still limited. Bile salts have been shown to control expression of *Bsep* [10] and *Ntcp* [11] as well as *Mrp2* [12] via interactions with the recently identified nuclear 'bile salt receptor' FXR (farnesoid X-activated receptor; NR1H4), while hepatic *Abca1*, *Abcg5* and *Abcg8* expression is controlled by the liver X-receptor (or NR1H2/3) [13,14]. Expression of *Mdr1b* is specifically induced in situations associated with metabolic stress (inflammation, partial hepatectomy [15,16]) and appears to involve nuclear factor- κ B signalling [17]. Expression of *Mdr2* appears to be controlled in a complex fashion, as it is induced in rodents by bile salt feeding [18], treatment with cholesterol synthesis inhibitors [19,20], insulin deficiency [21] and treatment with hypolipidaemic fibrates [22].

Abbreviations used: ABC, ATP binding cassette; ALAT, alanine transaminase; ASAT, aspartate transaminase; *Bsep*, bile salt export pump; Hmgs, 3-hydroxy-3-methylglutaryl-CoA synthase; *Mdr*, multidrug resistance; *Ntcp*, Na⁺-taurocholate co-transporting polypeptide; *Oatp*, organic anion-transporting polypeptide; *Pgp*, P-glycoprotein; PPAR α , peroxisome proliferator-activated receptor α ; TUDC, tauroursodeoxycholate.

¹ To whom correspondence should be addressed (e-mail T.Kok@med.rug.nl).

Fibrates exert their metabolic actions via activation of peroxisome proliferator-activated receptor α (PPAR α ; NR1C1) [23,24]. Natural ligands of PPAR α are (un)saturated fatty acids and their derivatives. Activated PPAR α heterodimerizes with the 9-*cis*-retinoic acid receptor (NR2B1) and alters transcription of target genes after binding to specific peroxisome proliferator-response elements. PPAR α is highly expressed in the liver and controls the expression of genes involved in the β -oxidation of fatty acids and those encoding apolipoproteins A-I, A-II and C-III [23,24]. Fibrates are widely used in the treatment of hyperlipidaemia: the drugs lower plasma triglycerides and increase high-density lipoprotein cholesterol [23,25]. Fibrates may adversely affect bile composition, as they have been shown to reduce expression of rate-limiting enzymes in bile salt synthesis [26–28]. An increased incidence of cholesterol gallstones in patients undergoing long-term therapy with clofibrate has been reported [29,30].

In rodents, but not in humans, fibrates induce hyperplasia and hypertrophy of hepatocytes as a consequence of rodent-specific effects on peroxisome proliferation [31]. Therefore, earlier data [22,32] describing fibrate effects on hepatic transporter expression should be interpreted with caution, as they may reflect aspecific consequences of hepatic hyperplasia. The aim of the present study was to evaluate the role of PPAR α in control of bile formation in mice, with emphasis on its potential role in regulating the expression of Mdr2 and other hepatic transporter proteins. Therefore, we have studied the effect of fibrates on the expression of transporter proteins *in vitro* in cultured mouse hepatocytes and *in vivo* in wild-type and *Ppar α ^(-/-)* SV129 mice.

MATERIALS AND METHODS

Animals

Mice homozygous for disruption of the PPAR α gene [*Ppar α ^(-/-)*] and wild-type mice [*Ppar α ^(+/+)*] of the same SV129 background [33] were used. Male mice of 25–30 g were housed in a light- and temperature-controlled facility and received either standard laboratory chow (Souris AO4; UAR, Lille, France) or the same diet containing 0.05 % (w/w) ciprofibrate for 2 weeks. Food and water were available *ad libitum*. All experiments were approved by the ethical committee on animal testing of the University of Groningen, Groningen, The Netherlands, and the Pasteur Institute, Lille, France.

Experimental procedures

After 2 weeks on experimental diets, wild-type and *Ppar α ^(-/-)* mice ($n = 10$ per group) were anaesthetized with Hypnorm (1 ml/kg) and Diazepam (10 mg/kg). Of each group, six animals were subjected to gall-bladder catheterization to allow collection of bile [34]. During the 30 min bile-collection period, animals were placed in a humidified incubator to ensure the maintenance of body temperature. Bile flow was determined gravimetrically, assuming a density of 1 g/ml for bile. Bile was stored at -20°C until analysis. Blood was obtained by cardiac puncture and the samples were collected in EDTA-containing tubes. Plasma was obtained by centrifugation in an Eppendorf centrifuge at 15000 g for 10 min (Eppendorf, Hamburg, Germany) and stored at -80°C until further analysis. The livers were excised, weighed, cut into small pieces, snap-frozen in liquid nitrogen and stored at -80°C until use for isolation of membranes for Western blot analysis or for biochemical analyses. From the other four animals of each group, the livers were also excised, weighed, cut into small pieces, snap-frozen in liquid nitrogen and stored at -80°C until use for isolation of RNA. For microscopic evaluation,

small pieces of liver were collected in paraformaldehyde or in liquid isopentane and stored at -80°C until further use.

In a second experiment, after three basal 10 min bile collections, tauroursodeoxycholate (TUDC; in 45 mM PBS, pH 7.4) was continuously infused via the jugular vein with the rate increased in a stepwise manner: 150, 300 and 450 nmol/min for 30 min periods, followed by 600 nmol/min for 60 min. Bile was collected throughout the experiment at 10 min intervals.

Mouse hepatocyte isolation and culture

Primary mouse hepatocytes were isolated from male wild-type and *Ppar α ^(-/-)* mice as described previously [35]. The viability of isolated wild-type and *Ppar α ^(-/-)* hepatocytes was similar, i.e. $\approx 80\%$. Hepatocytes were suspended in William's E medium (Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 10 % fetal calf serum, 20 m-units/ml insulin, 50 nM dexamethasone, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 50 $\mu\text{g}/\text{ml}$ gentamycin and seeded on to 35 mm six-well plastic culture dishes (Costar Corp., Cambridge, MA, U.S.A.), pre-coated with collagen (Serva, Feinbiochemica, Heidelberg, Germany) at a density of 1.0×10^6 cells/well in 2 ml of William's E medium. After a 5 h attachment period, the medium was refreshed. After overnight culture, cells were incubated in serum-free William's E medium supplemented with 1.7 % albumin (fat-free), insulin, penicillin/streptomycin and gentamycin. After 4 h, ciprofibrate (Sigma, St. Louis, MO, U.S.A.) or WY14,643 (Alexis, San Diego, CA, U.S.A.) dissolved in DMSO was added at the indicated concentrations and cells were exposed for the subsequent 24 h. The cells were maintained in a humidified incubator at 37°C and 5 % CO_2 .

Steady-state mRNA levels determined by real-time quantitative PCR

Total RNA was isolated from frozen mouse liver using TRIzol Reagent (Gibco BRL) according to the manufacturer's instructions. Total RNA was isolated from cultured hepatocytes using the SV Total RNA isolation system (Promega, Madison, WI, U.S.A.). RNA was checked on an agarose gel for integrity, and RNA concentration was measured spectrophotometrically. Single-stranded cDNA was synthesized from 4.5 μg of RNA, 30 units of Moloney murine leukaemia virus reverse transcriptase, 6 μl of 5-fold concentrated buffer, 12 units of RNase inhibitor, 0.406 μg of random primer and 3 μl of 10 mM dNTP mix (all from Roche, Mannheim, Germany) in a total volume of 30 μl . Reverse transcription was performed for 10 min at 25°C and for 1 h at 45°C and the samples were subsequently heated for 5 min at 95°C to terminate the reverse transcription reaction. Real-time quantitative PCR was performed on cDNA samples as described by Heid et al. [36] to detect mRNA levels.

Primer and probe sequences for *Abca1*, *Abcg5*, *Abcg8*, *Bsep* (*Abcb11*), *Lxra* (*Nr1h3*), *Mdr2* (*Abcb4*), *Ntcp* (*Slc10a1*) and *Oatp1* (*Slc21a1*) have been described by Plösch et al. [37]. Primer and probe sequences for β -actin, *Exr* (*Nr1h4*), mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (*Hmgs*), *Mdr1a* (*Abcb1a*) and *Mdr1b* (*Abcb1b*) have recently been described by Kok et al. [38]. The following primer sequences for *Mrp2* (*Abcc2*) were used: sense primer, 5'-GGA TGG TGA CTG TGG GCT GAT-3'; antisense primer, 5'-GGC TGT TCT CCC TTC TCA TGG-3'; and probe 5'-AGC TGC ATC GTC AGG AAT TTC CTC CAC A-3' (accession number, NM_013806). Primers and detection probes for the gene of interest, labelled with a fluorescent reporter dye (6-carboxy-fluorescein) and a fluorescent quenching dye (6-carboxy-tetramethyl-rhodamine) were

added. Fluorescence was measured with ABI Prism 7700 Sequence Detector v. 1.6 software (Perkin-Elmer, Foster City, CA, U.S.A.). For every PCR reaction, β -actin was used as the internal control. The cycle number at the threshold, where-after the intensity of reporter fluorescent emission increases, was used to quantify the PCR product.

Liver plasma membrane isolation

Liver plasma membranes were isolated by density-gradient ultracentrifugation as described in [39]. The membrane aliquots were frozen and stored until use at -80°C in 10 mM Tris/HCl (pH 7.4) and 250 mM sucrose, supplemented with complete protease inhibitor cocktail (Roche). Protein concentrations were determined according to Lowry et al. [40] using BSA as a standard. Relative enrichments of Na^+/K^+ -ATPase as a marker enzyme for the basolateral fraction and alkaline phosphatase as a marker enzyme for the canalicular fraction, i.e. the specific activity of the enzyme in the isolated plasma membrane preparation divided by the activity in the homogenate, were used to determine the degree of purification of the isolated membranes in the different experimental groups. Na^+/K^+ -ATPase [41] and alkaline phosphatase [42] activities were measured.

Western blotting

Protein of the plasma membrane fraction of each group ($\approx 10\ \mu\text{g}$), normalized for enrichment in Na^+/K^+ -ATPase or alkaline phosphatase, was separated using 4–15% Tris/HCl ready gradient gels (Bio-Rad, Hercules, CA, U.S.A.) and transferred to nitrocellulose (Amersham Biosciences, Little Chalfont, Bucks., U.K.), using a tank-blotting system (Bio-Rad). The respective anti-Ntcp and anti-Oatp1 IgG K4 and K10 antibodies were kindly provided by Dr Bruno Stieger and Professor Peter Meier-Abt (University Hospital, Zürich, Switzerland). Antibodies for detection of Na^+/K^+ -ATPase were a kind gift from Dr Wilbert Peters (University Medical Centre, Nijmegen, The Netherlands). Mouse monoclonal antibody P3II-26, raised against the human MDR3, but also detecting mouse Mdr2 Pgp, was kindly provided by Dr J. Scheper (Free University Hospital, Amsterdam, The Netherlands). The polyclonal antibody raised against Bsep (K12) has been described before [16]. The blots were incubated with the first antibody diluted in Tris-buffered saline containing 5% dried milk powder and 0.1% Tween 20 (Sigma), washed in Tris-buffered saline/0.1% Tween 20, incubated with horseradish peroxidase-labelled donkey anti-rabbit IgG, sheep anti-mouse IgG or rabbit anti-goat IgG (dilution, 1:1000; Amersham Biosciences). Detection was done with the ECL Western blotting kit (Amersham Biosciences).

Confocal scanning laser microscopy

Localization of Bsep was studied by confocal scanning laser microscopy on $4\ \mu\text{m}$ frozen sections, as described previously [16].

Analyses

Bile salts concentrations in plasma and bile were determined by an enzymic fluorimetric assay [43]. Levels of biliary cholesterol, phospholipids and GSH were measured as described by Kuipers et al. [44]. Aspartate transaminase (ASAT) and alanine transaminase (ALAT) activities and total bilirubin concentrations in plasma were determined by routine clinical chemistry. Total protein content of tissue homogenates was measured using the method described by Lowry et al. [40]. Biliary bile salt composition was determined by capillary GC as described in [34]

after extraction of the bile salts from bile by use of Sep-Pack C_{18} cartridges (Waters Associates, Milford, MA, U.S.A.).

Statistical analyses

All results are presented as means $\pm$ S.D. Differences between the groups were determined by one-way ANOVA, with *post-hoc* comparison by Newman-Keuls *t* test. The level of significance for all statistical analyses was set at $P < 0.05$. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL, U.S.A.).

RESULTS

Animal characteristics

Body and liver weights of untreated wild-type and *Ppar α* ^(-/-) mice were similar but, as expected, liver weights were increased significantly upon ciprofibrate treatment in wild-type mice (Table 1). No increase in liver weight occurred in fibrate-treated *Ppar α* ^(-/-) mice. ASAT and ALAT levels in plasma were slightly increased upon ciprofibrate treatment only in wild-type mice. Plasma bilirubin and bile salt concentrations did not differ between the groups.

Effects of PPAR α deficiency and/or ciprofibrate on hepatic transporter mRNA levels *in vivo* and *in vitro*

Real-time quantitative PCR was used to evaluate the expression of transporter genes as influenced by PPAR α deficiency and ciprofibrate *in vivo* in mice (Figure 1). Mitochondrial Hmgs, involved in ketogenesis, was used as a PPAR α -responsive gene for comparative purposes [45,46]. *Hmgs* mRNA levels increased 4-fold upon ciprofibrate treatment in wild-type mice but did not change in *Ppar α* ^(-/-) mice. PPAR α deficiency itself did not affect expression of any of the hepatic transporters studied. *Mdr2* was significantly (3-fold) up-regulated in wild-type mice treated with ciprofibrate but not in *Ppar α* ^(-/-) mice. Likewise, *Mdr1a* (6-fold) and *Mdr1b* (11-fold) were up-regulated in the wild-type mice treated with ciprofibrate, but not in *Ppar α* ^(-/-) mice. *Mrp2* mRNA levels were not significantly affected by PPAR α deficiency and/or ciprofibrate. *Bsep* and *Ntcp* mRNA levels were slightly, though non-significantly, decreased in ciprofibrate-treated wild-type mice and somewhat induced in ciprofibrate-treated *Ppar α* ^(-/-) mice. *Oatp1* expression, on the other hand, was drastically decreased in treated wild-type mice but increased in the treated *Ppar α* ^(-/-) mice. Expression of three other ABC genes, *Abca1*, *Abcg5* and *Abcg8*, encoding transporters recently

Table 1 Body and liver weights and plasma liver function parameters in untreated and ciprofibrate-treated wild-type and *Ppar α* ^(-/-) mice

Values are expressed as means $\pm$ S.D. ($n = 10$ in all groups).

Strain	Wild-type	<i>Pparα</i> ^(-/-)	Wild-type	<i>Pparα</i> ^(-/-)
Ciprofibrate	—	—	+	+
Body weight (g)	28.8 $\pm$ 1.6	29.1 $\pm$ 2.2	28.4 $\pm$ 2.6	28.6 $\pm$ 3.7
Liver weight (g)	1.3 $\pm$ 0.1	1.3 $\pm$ 0.2	2.6 $\pm$ 0.5†	1.3 $\pm$ 0.2*
ASAT (units/l)	113 $\pm$ 29	148 $\pm$ 89	260 $\pm$ 72†	168 $\pm$ 96
ALAT (units/l)	49 $\pm$ 32	44 $\pm$ 20	142 $\pm$ 117†	40 $\pm$ 18*
Bilirubin (μM)	7 $\pm$ 2	7 $\pm$ 3	8 $\pm$ 4	7 $\pm$ 2
Bile salts (μM)	15 $\pm$ 11	10 $\pm$ 2	21 $\pm$ 16	13 $\pm$ 3

* Significant difference between wild-type and *Ppar α* ^(-/-) mice on the same treatment.

† Significant difference between ciprofibrate-treated and untreated mice with the same genotype.

Figure 1 Steady-state mRNA levels of *Hmgs*, *Mdr2*, *Mdr1a*, *Mdr1b*, *Bsep*, *Mrp2*, *Ntcp* and *Oatp1* in livers of wild-type and *Pparα*^(-/-) mice after ciprofibrate treatment

Mice were fed on either a control diet or a diet supplemented with 0.05% ciprofibrate for 2 weeks. mRNA was isolated from livers of wild-type (white bars) and *Pparα*^(-/-) (black bars) mice, transcribed into cDNA and subjected to real-time PCR analysis as described in the Materials and methods section. Data represent means $\pm$ S.D. from four mice of each group. ^aSignificant difference between wild-type and *Pparα*^(-/-) mice on the same treatment. ^bSignificant difference between ciprofibrate-treated and untreated mice with the same genotype.

Figure 2 Steady-state mRNA levels of *Abca1*, *Abcg5* and *Abcg8* in livers of wild-type and *Pparα*^(-/-) mice after ciprofibrate treatment

Mice were fed on either a control diet or a diet supplemented with 0.05% ciprofibrate for 2 weeks. mRNA was isolated from livers of wild-type (white bars) and *Pparα*^(-/-) (black bars) mice, transcribed into cDNA and subjected to real-time PCR analysis as described in the Materials and methods section. Data represent means $\pm$ S.D. from four mice of each group.

suggested to play a role in cholesterol secretion, did not change significantly, although *Abcg5/g8* expression tended to be increased in ciprofibrate-treated wild-type mice (Figure 2). In view of the potential interference of PPAR α with other nuclear receptors involved in control of hepatic transporter expression, we also evaluated the mRNA levels of a number of these receptors. However, neither ciprofibrate treatment nor PPAR α deficiency affected the mRNA levels of *Lxrα*, *Fxr* and *Shp* in these experiments (results not shown).

To discriminate between direct PPAR α -mediated effects of ciprofibrate on hepatocellular transporter expression and indirect effects potentially related to drug-induced metabolic adaptations in the *in vivo* situation, *in vitro* studies were conducted. Primary mouse hepatocytes from wild-type and *Pparα*^(-/-) mice were incubated with ciprofibrate, WY14,643 or the vehicle (DMSO) for 24 h and analysed for expression of transporters as shown in Figure 3. Ciprofibrate and WY14,643 treatment caused a marked increase (4-fold) in levels of *Hmgs* mRNA and a 2.5-fold increase in *Mdr2* mRNA levels in hepatocytes of wild-type mice. Neither of these effects were observed in hepatocytes of *Pparα*^(-/-) mice, in which the basal expression of *Hmgs* and *Mdr2* was lower than in wild-type hepatocytes. No effects of ciprofibrate or WY14,643 treatment on mRNA levels of *Mdr1a/1b*, *Bsep*, *Ntcp*,

Oatp1, *Abca1*, *Abcg5/g8* or nuclear receptors like *Lxrα* were observed (results not shown).

Effects of PPAR α deficiency and/or ciprofibrate on hepatic transporter protein levels *in vivo*

Protein contents of hepatic transporter proteins were analysed by Western blotting on liver plasma membrane fractions (Figure 4). Ciprofibrate treatment resulted in a $\approx$ 2.5-fold increase of *Mdr2* Pgp levels of wild-type mice but not in *Pparα*^(-/-) mice. Protein levels of *Bsep* and of the uptake transporters *Ntcp* and *Oatp1* were dramatically decreased in the treated wild-type mice. This fibrate effect was not seen in *Pparα*^(-/-) mice. Na⁺/K⁺-ATPase was clearly present, although in somewhat reduced amounts, in liver membrane fractions of ciprofibrate-treated wild-type mice, showing that the virtual absence of *Bsep*, *Ntcp* and *Oatp1* in membrane fractions of these mice is not due to an isolation artifact.

Localization of *Bsep* in livers of ciprofibrate-treated mice

Immunofluorescence microscopy on frozen liver sections revealed that, in livers of untreated wild-type mice, *Bsep* was exclusively

Figure 3 Steady-state mRNA levels of *Hmgs* and *Mdr2* in primary hepatocytes of wild-type and *Pparα*^{-/-} mice after ciprofibrate and WY14,643 treatment

Mouse hepatocytes were incubated for 24 h in the presence or absence of 300 μ M ciprofibrate or 100 μ M WY14,643. mRNA was isolated from cells of wild-type (white bars) and *Pparα*^{-/-} (black bars) mice, transcribed into cDNA and subjected to real-time PCR analysis as described in the Materials and methods section. Data represent means $\pm$ S.D. and the experiments were done in triplicate and repeated three times in cells obtained from three individual mice/genotype. ^aSignificant difference between hepatocytes of wild-type and *Pparα*^{-/-} mice. ^bSignificant difference between ciprofibrate and WY14,643-treated hepatocytes compared with untreated hepatocytes.

present at the canalicular domain of hepatocytes and uniformly present across the liver lobule (Figure 5A). In the livers of wild-type mice treated with ciprofibrate, Bsep was still expressed canalicularly, but the signal was clearly reduced (Figure 5C), confirming the results obtained with Western analysis. In the untreated and treated *Pparα*^{-/-} mice, the localization of Bsep was identical with that in the untreated wild-type mice (Figures 5B and 5D). Because of the absence of a suitable antibody, we were not able to perform immunofluorescence microscopic studies on Mdr2.

Effects of PPAR α deficiency and/or ciprofibrate on bile flow and bile composition

There were no apparent differences in bile formation between chow-fed wild-type and *Pparα*^{-/-} mice, showing that PPAR α deficiency itself does not affect bile formation in mice (Table 2). The concentrations of bile salts, phospholipids, cholesterol and glutathione were significantly reduced in the wild-type mice treated with ciprofibrate. Also the concentration of acylcarnitines was reduced in this group (results not shown). Interestingly, bile flow in wild-type mice treated with ciprofibrate was markedly increased compared with the other groups. In spite of induced Mdr2 Pgp expression, biliary phospholipid and cholesterol output were significantly decreased in these animals. Analysis of biliary bile salt composition by GLC did not reveal marked differences between the groups (Table 3). In all four groups, cholate constituted the major fraction of biliary bile salts. In ciprofibrate-treated wild-type mice, the relative contents of lithocholate and chenodeoxycholate were slightly but significantly increased.

Lipid secretion during infusion of TUDC

To gain insight in the physiological consequences of Mdr2 induction and Bsep down-regulation, we administered increasing amounts of TUDC to ciprofibrate-treated and untreated wild-type and *Pparα*^{-/-} mice. TUDC is a non-toxic hydrophilic bile salt, which does not cause cholestasis upon infusion in mice and effectively induces the secretion of phospholipids and cholesterol into bile. As shown in Figure 6(A), bile flow remained markedly higher in ciprofibrate-treated wild-type mice than in the untreated

Figure 4 Western blot analysis of Mdr2, Bsep, Ntcp, Oatp1 and Na⁺/K⁺-ATPase (β -subunit) protein levels in liver plasma membrane fractions of untreated and ciprofibrate-treated wild-type (WT) and *Pparα*^{-/-} mice

All liver samples ($n = 3$, each from two pooled livers) were tested in the same way, and similar results were obtained. The appearance of Ntcp protein bands as doublets has been described previously and is probably due to partial deglycosylation of the protein [1]. Transport proteins are indicated to the left of each blot and apparent molecular masses to the right.

wild-type and the *Pparα*^{-/-} mice during the course of the experiment. Biliary bile salt output rates increased to a similar extent in all groups to levels clearly exceeding endogenous output rates (Figure 6B). This is surprising, in view of the fact that Bsep levels were dramatically decreased in wild-type mice treated with ciprofibrate. Phospholipid secretion (Figure 6C) decreased during

Figure 5 Confocal scanning laser microscopy of Bsep protein expression on frozen liver sections of untreated and ciprofibrate-treated wild-type and *Pparα*^{-/-} mice

Frozen liver sections were stained with primary antibodies directed against Bsep using K12. (A) Wild-type mice; (B) *Pparα*^{-/-} mice; (C) ciprofibrate-treated wild-type mice; (D) ciprofibrate-treated *Pparα*^{-/-} mice. Arrows depict the canalicular membrane. Scale bar, 40 μ m.

Table 2 Concentrations of organic solutes in bile and biliary output rates in untreated and ciprofibrate-treated wild-type and *Pparα*^(-/-) mice

Values are expressed as means ± S.D. (*n* = 7–10 per group). Units for bile flow and biliary output are given per 100 g of body weight.

	Strain ... Ciprofibrate ...	Wild type —	<i>Pparα</i> ^(-/-) —	Wild type +	<i>Pparα</i> ^(-/-) +
Bile					
Bile salts (mM)		31.7 ± 11.4	37.4 ± 16.6	6.4 ± 1.9†	35.8 ± 15.9*
Phospholipids (mM)		3.7 ± 0.7	3.8 ± 0.6	0.4 ± 0.3†	3.6 ± 1.2*
Cholesterol (mM)		0.5 ± 0.1	0.5 ± 0.2	0.1 ± 0.05†	0.6 ± 0.2*
Glutathione (mM)		5.7 ± 0.6	5.4 ± 0.7	1.4 ± 0.3†	5.4 ± 0.4*
Bile flow (μl · min ⁻¹ · 100 g ⁻¹)		6.6 ± 1.1	6.7 ± 1.6	30.3 ± 5.0†	7.2 ± 1.1*
Biliary output					
Bile salts (nmol · min ⁻¹ · 100 g ⁻¹)		206 ± 53	239 ± 82	188 ± 66	254 ± 100
Phospholipids (nmol · min ⁻¹ · 100 g ⁻¹)		24.8 ± 6.2	25.4 ± 7.3	10.5 ± 7.2†	25.7 ± 8.9*
Cholesterol (nmol · min ⁻¹ · 100 g ⁻¹)		3.6 ± 0.7	3.7 ± 1.5	2.2 ± 1.5†	4.1 ± 1.5*
Glutathione (nmol · min ⁻¹ · 100 g ⁻¹)		42.1 ± 3.8	40.8 ± 6.5	39.1 ± 12.1	40.3 ± 3.5

* Significant difference between wild-type and *Pparα*^(-/-) mice on the same treatment.

† Significant difference between ciprofibrate-treated and untreated mice with the same genotype.

Table 3 Biliary bile salt composition (as a percentage of the total) in untreated and ciprofibrate-treated wild-type and *Pparα*^(-/-) mice

Values are expressed as means ± S.D. (*n* = 6 in all groups).

	Biliary bile salt composition (% of total)			
	Wild-type —	<i>Pparα</i> ^(-/-) —	Wild-type +	<i>Pparα</i> ^(-/-) +
Lithocholate	4.9 ± 1.0	4.9 ± 1.7	11.6 ± 5.0†	4.5 ± 0.9*
Deoxycholate	4.2 ± 1.0	3.5 ± 0.7	1.7 ± 0.9†	4.0 ± 1.8
α-Muricholate	5.1 ± 1.4	7.0 ± 1.0	7.1 ± 2.1	6.9 ± 0.9
β-Muricholate	8.6 ± 3.9	10.1 ± 2.6	4.1 ± 1.9†	10.7 ± 1.6*
ω-Muricholate	6.0 ± 0.9	5.4 ± 2.2	5.6 ± 1.1	4.6 ± 0.5
Chenodeoxycholate	1.7 ± 0.4	1.8 ± 0.5	4.0 ± 0.5†	1.5 ± 0.5*
Cholate	66.5 ± 5.1	63.9 ± 5.0	61.5 ± 5.5	63.5 ± 4.5

* Significant difference between wild-type and *Pparα*^(-/-) mice on the same treatment.

† Significant difference between ciprofibrate-treated and untreated mice with the same genotype.

bile depletion (0–30 min) and the output of phospholipids in wild-type mice treated with ciprofibrate was lower than in the two other groups despite induction of *Mdr2* expression (see also Table 3). During infusion of TUDC at low rates, the phospholipid secretion was similar in the untreated and fibrate-treated wild-type mice. At 80 min, when biliary bile salt concentration reached a level of ≈ 30 mM, the secretion of phospholipids by the ciprofibrate-treated wild-type mice started to increase much more rapidly than in the untreated wild-type mice, to reach a 2-fold higher rate at the end of the experiment. The untreated and ciprofibrate-treated *Pparα*^(-/-) mice secreted slightly fewer phospholipids during the final period of the infusion. Since the secretion of cholesterol is coupled to that of phospholipids [4] a secretion pattern similar to that of phospholipids was observed for cholesterol in all groups (Figure 6D).

DISCUSSION

This study demonstrates that the absence of PPARα, a nuclear receptor critically involved in control of hepatic lipid [24] and bile salt metabolism [26–28], does not affect the expression of important transport systems in the liver or bile formation in SV129 mice under normal chow-fed conditions. Stimulation of

PPARα activity with synthetic agonists, however, resulted in the specific induction of *Mdr2* expression and function that was independent from the rodent-specific PPARα-mediated induction of liver enlargement. In contrast, increased expression of other members of the B subfamily of ATP-dependent ABC transporters upon ciprofibrate treatment *in vivo*, i.e. of *Mdr1a* and *Mdr1b*, appeared to be related to the peroxisome-proliferating capacities or to the metabolic actions of the drug rather than to direct control of PPARα on expression of these genes.

As expected [47], liver weights were significantly increased in ciprofibrate-treated wild-type mice but not in *Pparα*^(-/-) mice. Liver enlargement in fibrate-treated rodents is due to both cellular hypertrophy and hyperplasia: the hepatocytes display proliferation of peroxisomes and, to a lesser extent, of the smooth endoplasmic reticulum [31]. ASAT and ALAT activities in plasma were slightly increased in treated wild-type mice, while plasma bile salt and bilirubin levels remained unaffected. Elevated transaminases may represent a direct effect of the fibrate on the liver, independent of hepatotoxicity [48].

Mdr2 mRNA and *Mdr2* Pgp levels were clearly induced in ciprofibrate-treated wild-type mice and this induction was not seen in the *Pparα*^(-/-) mice. The induction of *Mdr2* expression is in accordance with earlier studies, in which mice were treated with different fibrates, including ciprofibrate [22], or other peroxisome proliferators [32]. From the latter studies, however, it cannot be concluded whether this fibrate effect is actually PPARα-dependent or whether it is an aspecific consequence of liver enlargement. The absence of *Mdr2* induction in ciprofibrate-treated *Pparα*^(-/-) mice demonstrates PPARα dependency of this process. Furthermore, our study shows that ciprofibrate and WY14,643 also rapidly induce *Mdr2* mRNA levels in cultured hepatocytes of wild-type mice, but not those of *Pparα*^(-/-) mice. Thus our combined results firmly establish that the induction of *Mdr2* mRNA and *Mdr2* Pgp upon ciprofibrate treatment is directly PPARα-mediated. Two other *Mdr* genes, i.e. *Mdr1a* and *Mdr1b*, were strongly induced at the mRNA level *in vivo*. Chianale et al. [22] did not observe induction of *Mdr1a/Mdr1b* expression by fibrates in mice, but these authors used a Northern blotting procedure which did not allow detection of *Mdr1a* and *Mdr1b* mRNA in normal mouse liver. Also in rats treated with clofibrate [49], no induction of *Mdr1a/1b* expression was observed. This deviation from our data could be related to the use of different species and/or treatment strategies. In contrast with the *in vivo* situation, *in vitro* studies did not show induction of *Mdr1a* and

Figure 6 Bile flow and biliary bile salt, phospholipid and cholesterol secretion in untreated and ciprofibrate-treated wild-type and *Ppar α ^{-/-}* mice during intravenous administration of increasing amounts of TUDC

Mice were cannulated in the gall-bladder, and 30 min after starting bile collection TUDC was infused via the jugular vein in stepwise increasing dosages, as indicated by the numbers (nmol/min) in the boxes. $\circ$, Wild-type mice; $\bullet$, untreated and ciprofibrate-treated *Ppar α ^{-/-}* mice; $\triangle$, ciprofibrate-treated wild-type mice. Data represent means $\pm$ S.D. from four mice of each genotype. Data for the treated and untreated *Ppar α ^{-/-}* mice were combined for reasons of clarity: both groups showed very similar results. (A) Bile flow; (B) bile salt secretion; (C) phospholipid secretion; (D) cholesterol secretion. *Significant difference between ciprofibrate-treated and untreated mice with the same genotype. BW, body weight.

Mdr1b. We propose therefore that *Mdr1a/1b* induction by fibrates *in vivo* represents an indirect effect that may be related to liver hyperplasia or other metabolic changes. Rat *Mdr1b* is up-regulated under various conditions associated with 'metabolic stress', such as partial hepatectomy [15] and lipopolysaccharide treatment [16]. The expression of *Mdr1a* is largely unchanged under these experimental conditions, but is clearly induced by fibrates. The underlying mechanism remains elusive at the moment.

Surprisingly, protein levels of the canalicular bile salt transporter (Bsep) and of the major bile salt-uptake systems Ntcp and Oatp1 were drastically decreased in wild-type mice treated with ciprofibrate, but not in *Ppar α ^{-/-}* mice. At mRNA level *Bsep* and *Ntcp* expression tended to be decreased in treated wild-type mice, while *Oatp1* expression was dramatically decreased in these animals. Ciprofibrate and WY14,643 did not induce any change in *Bsep*, *Ntcp* and *Oatp1* mRNA levels in cultured hepatocytes. Immunofluorescence studies confirmed that Bsep was down-regulated in ciprofibrate-treated wild-type mice and revealed that Bsep was still mainly localized canalicularly under these conditions. Despite the fact that Bsep, Ntcp and Oatp1 protein levels were all down-regulated, plasma bile salt concentrations and biliary bile salt secretion rates were hardly affected, even during the course of supraphysiological TUDC infusions. Recent studies by Meerman et al. [50] have shown that in *fch/fch* mice, in which Ntcp and Oatp1 are also strongly down-regulated, the majority of intravenously administered radiolabelled taurocholate was present in liver and bile within 30 min of injection, possibly indicative for taurocholate uptake by the liver via alternative mechanisms. Unaffected biliary bile salt secretion in the face of strongly reduced Bsep levels probably illustrates the overcapacity of this excretory system. We speculate that an excessive amount

of Bsep is present in mouse liver under 'normal conditions' and that the decreased amounts of Bsep in fibrate-treated wild-type mice are still able to fully accommodate bile salt transport. Alternatively, it has recently been shown [51] that Bsep-deficient mice are able to secrete limited amounts of primary bile salts into bile. Therefore, alternative mechanisms for biliary bile salt secretion should be considered.

Despite the marked alterations in transporter expression upon ciprofibrate treatment of wild-type mice, hepatobiliary bile salt flux remained unaffected but bile flow was strongly increased. This latter effect was not seen in ciprofibrate-treated *Ppar α ^{-/-}* mice. The cause of the increased bile flow is currently unknown, but may be related to excretion into bile of PPAR α -dependent ciprofibrate metabolites or other metabolites/solutes generated by PPAR α activation. The biliary concentrations of bile salts, phospholipids, cholesterol and glutathione were markedly reduced in treated wild-type mice. For bile salts and glutathione, this reduction was probably secondary to the increased bile flow. However, when corrected for bile flow, phospholipid and cholesterol output rates remained significantly reduced in fibrate-treated wild-type mice compared with untreated controls. This despite the fact that Mdr2 Pgp levels were clearly increased. The determinants of biliary phospholipid (and cholesterol secretion) are (i) the actual biliary bile salt concentration, which is partly determined by the magnitude of the bile acid-independent flow [52], (ii) the biliary bile salt composition, (iii) the presence of non-bile-salt organic anions in bile [4] and (iv) the Mdr2 Pgp activity [5]. The biliary bile salt concentration was strongly reduced by ciprofibrate in wild-type mice, due to an unexplained increase of bile acid-independent flow. A high bile acid-independent flow influences the 'exposure time' of the bile canalicular membrane to the intracanalicular bile salts and thereby reduces the efficacy

of these bile salts to induce the secretion of lipids. Biliary bile salt composition was similar in all groups of mice and, therefore, cannot be responsible for impaired phospholipid and cholesterol secretion in ciprofibrate-treated wild-type mice. The lowered phospholipid secretion under basal conditions can theoretically be due to an 'uncoupling' effect of ciprofibrate metabolites in bile [53]. We infused increasing amounts of TUDC to see if *Mdr2* Pgp was indeed functionally up-regulated in ciprofibrate-treated wild-type mice. Figure 5 shows that, at a certain time point, the fibrate-treated wild-type mice started to secrete much more phospholipids and cholesterol into bile than the untreated wild-type and *Ppar α ^{-/-}* mice did. At the point of onset of phospholipid hypersecretion, the biliary bile salt concentration was about 30 mM. It therefore seems appropriate to suggest that a certain threshold bile salt concentration is needed for 'optimal' secretion of phospholipids by *Mdr2* Pgp. The secretion of cholesterol is coupled to that of phospholipids [4], and for cholesterol a similar pattern of secretion was observed during these experiments. With these infusion studies, we have unequivocally demonstrated a functional induction of *Mdr2* Pgp by ciprofibrate in mice. A role of *Abcg5/g8* in control of cholesterol secretion is emerging on the basis of studies in mice overexpressing both half-transporters [7]. In view of the modest changes seen in their expression, *Abcg5/g8* are unlikely to be responsible for the observed changes in biliary cholesterol secretion.

Recent studies indicate a multifactorial regulation of *Mdr2* expression in rodents. An increase in *Mdr2* mRNA and *Mdr2* protein levels has been detected in rats treated with statins [19,20]. Statins cause a transient state of cellular cholesterol deprivation, resulting in enhanced transcription of sterol-regulated genes by sterol-regulatory-element-binding proteins (SREBPs), which possibly play a role in regulation of *Mdr2* gene expression [54]. Bile salts may also be involved in the regulation of *Mdr2* expression. Bile diversion in rats lowers *Mdr2* mRNA levels [55], while bile salt feeding has the opposite effect [18]. Bile salts presumably do not play a role in our study, because the bile salt flux did not differ between the groups. Finally, recent work from our laboratory has shown that insulin deficiency is associated with strongly induced *Mdr2* expression in rats [21]. Based on our observation that PPAR α deficiency itself did not alter *Mdr2* expression, we propose that PPAR α -mediated control of *Mdr2* is of physiological relevance only under conditions of metabolic stress associated with PPAR α activation, e.g. during fasting [38] and other conditions associated with enhanced free fatty acid flux towards the liver such as diabetes [21].

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