Chapter 3

Induction of hepatic ABC transporter expression is part of the PPARα-mediated fasting response in the mouse

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ABSTRACT

Background & Aims: Fatty acids are natural ligands of the peroxisome proliferator-activated receptor alpha (PPARα). Synthetic ligands of this nuclear receptor, i.e., fibrates, induce the hepatic expression of the multidrug resistance 2 gene (Mdr2), encoding the canalicular phospholipid translocator, and affect hepatobiliary lipid transport. We tested whether fasting-associated fatty acid release from adipose tissues alters hepatic transporter expression and bile formation in a PPARα-dependent manner. Methods: A 24-hour fasting/48-hour refeeding schedule was used in wild-type and Ppara(/−/) mice. Expression of genes involved in the control of bile formation was determined and related to secretion rates of biliary components. Results: Expression of Ppara, Fxr and Lxra, genes encoding nuclear receptors that control hepatic bile salt and sterol metabolism was induced on fasting in wild-type mice only. The expression of Mdr2 was 5-fold increased in fasted wild-type mice and increased only marginally in Ppara(/−/) mice, and it normalized on refeeding. Mdr2 protein levels and maximal biliary phospholipid secretion rates were clearly increased in fasted wild-type mice. Hepatic expression of the liver X receptor target genes Abca1, Abcg5 and Abcg8, implicated in hepatobiliary cholesterol transport, was induced in fasted wild-type mice only. However, the maximal biliary cholesterol secretion rate was reduced by approximately 50%. Conclusions: Induction of Mdr2 expression and function is part of the PPARα-mediated fasting response in mice. Fasting also induces expression of putative hepatobiliary cholesterol transport genes Abca1, Abcg5 and Abcg8, but, nonetheless, maximal biliary cholesterol excretion is decreased after fasting.
INTRODUCTION

Fasting induces Mdr2 expression

Fasting triggers a complex array of adaptive metabolic responses. The switch to reliance on fatty acids and ketones, rather than on glucose, for energy production requires augmentation of the capacity for mitochondrial fatty acid oxidation in tissues with high oxidative energy demands, such as the liver. The nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα; NR1C1) plays a pivotal role in the control of this metabolic response. Fatty acids that are liberated from adipose tissue during fasting are, in part, taken up by the liver, where they may serve as ligands for PPARα. Activated PPARα heterodimerizes with the 9-cis-retinoic acid receptor (RXR; NR2B1) and alters transcription of its target genes, including those controlling hepatic β-oxidation. The generation of Ppara(-/-) mice has established that PPARα is critical in the coordinate transcriptional activation of the fatty acid oxidation machinery in the liver. It is becoming clear that PPARα is also involved in the control of other metabolic pathways, including those of glucose, amino acid and bile salt metabolism. Furthermore, through cross-talk with other transcription factors, for instance the liver X receptor alpha (LXRα; NR1H3), PPARα may also influence sterol metabolism and transport.

It has been reported that synthetic PPARα ligands, i.e., fibrates, induce hepatic expression of the multidrug resistance 2 gene Mdr2 (or Abcb4). Mdr2 P-glycoprotein (Pgp) is a member of the Abcb subfamily of adenosine triphosphate-binding cassette (ABC) transport proteins. Mdr2 Pgp is exclusively present at the canalicular membrane and controls phospholipid secretion into bile. Recent studies from our laboratory, employing Ppara-deficient mice, showed that the induction of murine Mdr2 mRNA and Mdr2 Pgp protein by fibrates is strictly PPARα dependent. Fibrates have been shown to increase biliary cholesterol secretion in humans and in rodents. Cholesterol secretion into bile is coupled to that of phospholipids and requires active secretion of bile salts. Bile salt secretion into bile is mediated by the bile salt export pump (Bsep; Abcb11). Bsep expression is controlled by the farnesoid X receptor (FXR; NR1H4). Transporters involved in the actual cholesterol secretion process have not yet been identified. A number of ABC transporters, i.e., Abca1 and Abcg5/Abcg8, have recently been suggested to play a role, but definite proof is not yet available. Hepatic expression of rodent Abca1, as well as of Abcg5 and Abcg8, is induced by synthetic agonists of LXR and by cholesterol feeding.

We speculate that PPARα-controlled expression of the ABC transporter genes involved in hepatobiliary lipid transport may constitute a physiologically relevant process. We have tested this hypothesis by comparing hepatic expression of transporters with parameters of bile formation in wild-type and Ppara(-/-) mice subjected to a fasting/refeeding schedule.

MATERIALS AND METHODS

Animals
Mice homozygous for disruption of the Ppara gene (Ppara(-/-)) and wild-type (Ppara(+/+)) mice on a SV129 background were used. Male mice of 25-30 g were housed in a light- and temperature-controlled facility. Food and water were available ad libitum. Wild-type and Ppara(-/-) mice were divided into three groups. Of each genotype, mice were maintained on standard laboratory chow (RMH-B; Hope Farms BV, Woerden, The Netherlands), were fasted for 24 h, or were fasted for 24 h and then refed a high-carbohydrate/low-fat diet (containing 38.5% wt saccharose, 38.5% wt cornstarch, 16% wt casein, 5% wt vitamins
and minerals and less than 1% wt of fat; Hope Farms BV) for 48 h prior to sacrifice. For a second experiment, mice homozygous for disruption of the *Mdr2* gene (*Mdr2* (-/-)) and wild-type (*Mdr2* (+/+)) on an FVB background were used. They were obtained from the breeding colony at the Animal Facility of the Academic Medical Center, Amsterdam. Of each genotype four male mice were maintained on standard laboratory chow (RMH-B), four were fasted for 24 h and four were fasted for 48 h. All experiments were approved by the Ethical Committee on animal testing of the University of Groningen.

**Experimental procedures**

After the fasting/refeeding schedule, mice were anesthetized with Hypnorm (1ml/kg) and Diazepam (10 mg/kg) (Janssen Pharmaceutica, Beerse, Belgium). Of each group of *Ppara* (-/-) and wild-type mice, six animals were subjected to bile duct cannulation for collection of bile. During the 30 min bile collection period, animals were placed in an humidified incubator to ensure 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. From all mice, blood was obtained by cardiac puncture and collected in EDTA-containing tubes. Plasma was obtained by centrifugation at 9000 rpm for 10 minutes (Eppendorf centrifuge, Hamburg, Germany) and stored at -80°C until 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 and for biochemical analyses. From four separate animals of each experimental group, the livers were excised without bile collection, weighed, cut into small pieces, snap-frozen in liquid nitrogen, and stored at -80°C until use for isolation of RNA and for biochemical analyses.

In fed and fasted wild-type mice only, maximal biliary lipid secretion rates were determined during infusion of a hydrophilic bile salt at supraphysiological rates. After three basal 10 min bile collections, tauroursodeoxycholate (TUDC; 45 mM in phosphate buffered saline (PBS), pH 7.4) was continuously infused via a jugular vein in stepwise increasing rates: 150, 300, 450 nmol/min during 30 min periods followed by 600 nmol/min during a 60 min period. Bile was collected throughout the experiment at 10 min intervals.

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

Total RNA was isolated from frozen mouse liver using TRIzol Reagent (Gibco BRL, Grand Island, NY) according to the manufacturer’s instructions. RNA was checked on an agarose gel for integrity, and RNA concentration was measured using the Ribogreen RNA quantitation kit (Molecular Probes, Leiden, The Netherlands). Single-stranded complementary DNA (cDNA) was synthesized from 4.5 µg RNA, 30 U of Moloney murine leukemia virus reverse transcriptase, 6 µl of 5-fold concentrated buffer, 12 U of RNase inhibitor, 0.406 µg of random primer and 3 µl of dNTP mix (10 mM) (all from Roche, Mannheim, Germany) in a total volume of 30 µl. Reverse transcription was performed for 10 minutes 25°C and for 1 hour 45°C and the samples were subsequently heated for 5 minutes at 95°C to terminate the reverse transcription reaction. Real-time quantitative PCR was performed on cDNA samples as described by Heid et al. to detect mRNA levels. Primer and probe sequences for *β-actin*, *Fxr*, 3-hydroxy-3-methylglutaryl-coenzyme A synthase (*Hmgs*), *Mdr1a*, *Mdr1b*, *Ppara*, *Pparγ* and small heterodimer partner (*Shp*) are listed in Table 1. Primer and probe sequences for *Abca1, Abcg5, Abcg8, Bsep*, cholesterol 7α-hydroxylase (*Cyp7a1*), sterol 27-hydroxylase (*Cyp27*), *Lxrα, Mdr2, Na+-taurocholate
Fasting induces Mdr2 expression

co-transporting polypeptide (Ntcp), organic anion-transporting polypeptide (Oatp1), sterol regulatory-element binding protein (Srebp1a), Srebp1c and Srebp2 have recently been described by Plösch et al.\textsuperscript{30}. Primers and detection probes for the gene of interest, labeled with a fluorescent reporter dye (6-carboxy-fluorescein) and a fluorescent quenching dye (6-carboxy-tetramethyl-rhodamine), were added. Fluorescence was measured in each PCR tube by an ABI Prism 7700 Sequence Detector v. 1.6 software (Perkin-Elmer Corp., Foster City, CA). For every PCR reaction, $\beta$-actin was used as the internal control. The cycle number at the threshold (CT), after which the intensity of reporter fluorescent emission increases, was used to quantitate the PCR product.

### Liver plasma membrane isolation

Liver plasma membranes were isolated by density gradient ultracentrifugation as described previously\textsuperscript{31}. 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.\textsuperscript{32} using bovine serum albumin as the standard. Relative enrichments of alkaline phosphatase\textsuperscript{33} as 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.

### Western blotting

Approximately 10 µg of protein of plasma membrane fraction of each group, normalized for enrichment in alkaline phosphatase, was separated using 4-15% Tris-HCL ready gradient gels (Bio-Rad laboratories, Hercules, CA) and transferred to nitrocellulose (Amersham, Buckinghamshire, UK), using a tankblotting system (Bio-Rad laboratories). The anti-Ntcp

<p>| Table 1. Oligonucleotide primers used for the analysis of gene expression by real-time PCR |
|---------------------------------|-----------------|-----------------|-----------------|</p>
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<th>reverse</th>
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<td>$Fxr$ (Nr1h4)</td>
<td>U09416</td>
<td>CCAATG</td>
</tr>
<tr>
<td>$Hmg$</td>
<td>U12790</td>
<td>GCCGACACTC</td>
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<td>$Mdr1a$ (Abcb1a)</td>
<td>M33581</td>
<td>TGGTGTGGTAAGGGAAG</td>
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<td></td>
<td>TGGTCATTGTGGTCG</td>
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<tr>
<td>$Mdr1b$ (Abcb1b)</td>
<td>M14757</td>
<td>GAGCGGCACTCAT</td>
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<td>TTGGCTGAT</td>
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<td></td>
<td></td>
<td>CTAG</td>
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<td>$Shp$ (Nr0b2)</td>
<td>L76567</td>
<td>CTTCGTCAGTTGTCG</td>
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Other oligonucleotide primers used, can be found in reference \textsuperscript{30}.
and anti-Oatp1 immunoglobuline G (IgG) K4 and K10, respectively, were kindly provided by Dr. B. Stieger and Prof. P. Meier-Abt (University Hospital, Zürich, Switzerland). Antibodies for detection of Na⁺/K⁺-ATPase were a kind gift from Dr. W. Peters (Nijmegen, The Netherlands). Mouse monoclonal antibody P3II-26, raised against the human MDR3, but also detecting mouse Mdr2 Pgp was kindly provided by Dr. R. Scheper (Free University Hospital, Amsterdam, Netherlands). The polyclonal antibody raised against Bsep (k12) has been described before. The blots were incubated with the first antibody diluted in Tris-buffered saline containing 5% dried milk powder and 0.1% polyoxyethylene sorbitan monolaurate (Tween 20; Sigma, St. Louis, MO), washed in Tris-buffered saline / 0.1% Tween 20 and incubated with horseradish peroxidase-labeled donkey anti-rabbit IgG, sheep anti-mouse IgG or rabbit anti-goat IgG (dilution 1:1000; Amersham, Little Chalfont, UK). Detection was done using the ECL Western blotting kit (Amersham).

Analyses
Bile salt concentrations in plasma and bile were determined by an enzymatic fluorimetric assay. Plasma cholesterol and triglycerides concentrations were measured enzymatically using commercially available kits (Roche). Levels of biliary cholesterol, phospholipids and glutathione (GSH) were measured as described by Kuipers et al. Free fatty acids and glucose concentrations in plasma were determined using commercially available kits (respectively WAKO Chemicals GmbH, Neuss, Germany and Sigma). Aspartate transaminase (ASAT) and alanine transaminase (ALAT) activities, total bilirubin and β-hydroxybutyrate 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.

Statistical analyses
All results are presented as means ± SD. Differences between the different 2 genotypes treated groups were determined by 1-way analysis of variance (ANOVA), with post hoc comparison by Newman-Keuls t-test. Differences between fasted and fed wild-type mice during the infusion studies were tested for significance by the Mann-Whitney U 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).

RESULTS
Effects of fasting/refeeding on metabolic parameters in wild-type and Pparα(−/−) mice
Twenty-four hours of fasting led to reductions in body weight (~12%) and liver weight (~20%) in both wild-type and Pparα(−/−) mice, which normalised on refeeding. Plasma markers of hepatic function were not affected by fasting/refeeding. Likewise, no changes in plasma bile salt concentrations were found. As expected, fasting was associated with reduced plasma glucose levels in both strains and increased plasma free fatty acid (FFA) levels (data not shown). Plasma β-hydroxybutyrate levels were strongly elevated in fasted wild-type mice (Figure 1A). The absence of this ketogenic response upon fasting in Pparα(−/−) mice is caused by an inability to induce the expression of genes involved in β-oxidation and ketogenesis. This is illustrated by the induction of mitochondrial HMG-CoA synthase (Hmgs) in fasted wild-type mice only (Figure 1B). Fasting was associated with a more than 5-fold increase in hepatic triglyceride content in wild-type mice (Figure 1C). Hepatic
Fasting induces Mdr2 expression

Figure 1. Plasma concentrations of β-hydroxybutyrate (A), steady-state mRNA levels of Hmg-CoA synthase (Hmgs) in liver (B), and hepatic triglyceride contents (C) of fed, fasted and refed wild-type and Pparα (-/-) mice. Total mRNA was isolated from livers of wild-type (white bars) and Pparα (-/-) (black bars) mice, and 4.5 µg RNA was transcribed into cDNA and subjected to real-time PCR analysis as described in Material and Methods. Hepatic triglyceride concentrations were measured in liver homogenates and expressed per milligram of liver. aSignificant difference between wild-type and Pparα (-/-) mice on the same treatment. bSignificant difference between fasted-refed and fed mice of the same genotype.

Figure 2. Steady-state mRNA levels of Pparα (A), Pparγ (B), Srebp1c (C), Lxrα (D) Fxr (E) and Shp (F) in livers of fed, fasted and refed wild-type and Pparα (-/-) mice. Total 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 Material and Methods. The presence of a Pparα transcript in Pparα (-/-) mice, also described by Muoio et al.40, is explained by the presence of residual, non-functional mRNA. The strategy used for targeted disruption of the Pparα gene results in a non-functional transcript in Pparα (-/-) mice, which is recognized by our primer set described in Table 1. aSignificant difference between wild-type and Pparα (-/-) mice on the same treatment. bSignificant difference between fasted-refed and fed mice of the same genotype.

Triglyceride concentrations in fed Pparα (-/-) mice tended to be higher than those in fed wild-type mice, and fasting induced a more pronounced fat accumulation in absolute terms which persisted after refeeding in the Pparα (-/-) mice.

Fasting/refeeding differentially affects hepatic expression of nuclear receptors in wild-type and Pparα (-/-) mice
In view of the potential interference of PPARα with other nuclear receptors and transcription factors involved in control of hepatic transporter expression10-12, we first evaluated the
consequences of fasting/refeeding and PPARα deficiency on mRNA levels of these transcription factors themselves. As shown previously, the expression of Pparα was induced 2.5-fold by fasting in wild-type mice (Figure 2A). It can be expected that PPARα protein was similarly induced under fasting conditions, because PPARα protein level strongly correlates with the expression of its mRNA. Hepatic mRNA levels of Pparγ were clearly higher in fed Pparα (-/-) mice than in the wild-type mice (Figure 2B). The expression of Srebp1a and Srebp2 did not change, but fasting caused a strong decrease in Srebp1c expression (Figure 2C), as previously reported in wild-type mice. The mRNA levels of Lxrα (Figure 2D) and Fxr (Figure 2E) were elevated in livers of fasted wild-type mice; this induction was completely absent in Pparα (-/-) mice. Despite fasting-associated changes in Fxr expression in wild-type mice, no significant effects on Shp expression, a well-established FXR target gene, were found (Figure 2F). It should be noted that changes in mRNA levels of the transcription factors studied do not necessarily directly correlate with their corresponding protein levels and activities.

**Fasting/refeeding-associated changes in the expression of hepatic transporters and bile formation in wild-type and Pparα (-/-) mice**

Hepatic Mdr2 mRNA levels were clearly induced (5-fold) by fasting in wild-type mice (Figure 3A). Likewise, the amount of Mdr2 protein (Figure 4) in hepatic membranes was induced by fasting in wild-type mice and decreased again upon refeeding. The induction of Mdr2 mRNA levels by fasting was only marginal in Pparα (-/-) mice (Figure 3A) and Mdr2 protein levels remained unaffected (Figure 4). Fasting significantly induced the expression of Mdr1a in wild-type mice, and this effect persisted after two days of refeeding (Figure 3B). No effects on Mdr1b expression were found in wild-type and Pparα (-/-) mice (Figure 3C). In contrast, mRNA levels of Bsep were transiently reduced by fasting in both

![Figure 3](image-url)

**Figure 3.** Steady-state mRNA levels of Mdr2 (A), Bsep (B), Mdr1a (C), Mdr1b (D), Ntcp (E) and Oatp1 (F) during fasting or refeeding in livers of wild-type and Pparα (-/-) mice. Total mRNA was isolated from livers of wild-type (white bars) and Pparα (-/-) (black bars) mice, transcribed into cDNA and subjected to realtime PCR analysis as described in Material and Methods. aSignificant difference between wild-type and Pparα (-/-) mice on the same treatment. bSignificant difference between fasted-refed and fed mice of the same genotype.
Fasting induces Mdr2 expression

groups (Figure 3D). This, however, was not associated with reduced Bsep protein levels (data not shown). Divergent effects of fasting were observed for the bile salt uptake carriers Ntcp and Oatp1; Ntcp gene expression was induced by fasting in wild-type mice (Figure 3E), whereas Oatp1 expression was reduced in fasted Pparaα(-/-) mice (Figure 3F). Neither of these effects, however, resulted in marked changes in the amounts of the respective proteins. Three other ABC transporter genes putatively involved in cholesterol transport, i.e., Abca1, Abcg5 and Abcg8, were also regulated by fasting/refeeding. Abca1 expression was slightly induced by fasting in wild-type mice only (Figure 5A). Basal levels of Abcg5 and Abcg8 expression were markedly lower in Pparaα(-/-) mice than in wild-type controls (Figure 5B, C). Expression of both genes was induced at the mRNA level by fasting in the wild-type mice and remained induced after refeeding. No changes in Abcg5/8 expression upon fasting/refeeding was observed in Pparaα(-/-) mice.

Fasting/refeeding did not significantly affect basal bile flow, as determined during a 30 min collection of bile (Table 2). Biliary bile salt secretion was enhanced in fasted wild-type mice, but not in Pparaα(-/-) mice. The difference between both models may be related to PPARα-dependent effects of fasting on the expression of Cyp7a1. This gene encodes cholesterol 7α-hydroxylase, which catalyzes the first step of the so-called acidic pathway

Figure 4. Western blot analysis of Mdr2 Pgp and Na+/K+-ATPase (β-subunit) protein levels in total liver membrane fractions of fed, fasted and refeed wild-type and Pparaα(-/-) mice. Several preparations (n=3, each from 2 pooled livers) were tested in the same way, and similar results were obtained. Apparent molecular weights are indicated at the right side of the blots.

Figure 5. Steady-state mRNA levels of Abca1 (A), Abcg5 (B) and Abcg8 (C) during fasting or refeeding in livers of wild-type and Pparaα(-/-) mice. Total mRNA was isolated from livers of wild-type (white bars) and Pparaα(-/-) (black bars) mice, transcribed into cDNA, and subjected to real-time PCR analysis as described in Material and Methods. *Significant difference between wild-type and Pparaα(-/-) mice on the same treatment. **Significant difference between fasted-refed and fed mice of the same genotype.

59
of bile salt synthesis\textsuperscript{40}. \textit{Cyp7a1} was induced in fasted wild-type mice only (Figure 6A). The expression of \textit{Cyp27}, encoding sterol 27-hydroxylase, which catalyzes the initial step of the neutral pathway of bile salt synthesis\textsuperscript{40}, was slightly induced in both strains upon fasting (Figure 6B). Most importantly, basal biliary phospholipid secretion was increased in fasted and refed wild-type mice (Table 2). This effect persisted when phospholipid output was corrected for differences in bile salt output but was relatively small. Biliary cholesterol secretion tended to be increased in fasted compared to fed wild-type mice. However, when corrected for differences in bile salt output, it appeared that fasting did not affect basal biliary cholesterol output in these animals.

To investigate whether the induction of \textit{Mdr2}, \textit{Abca1} and \textit{Abcg5/8} expression by fasting would lead to a hypersecretion of biliary phospholipids and cholesterol under conditions of high hepatobiliary bile salt fluxes, gallbladder-cannulated fed and fasted wild-type mice were intravenously infused with tauroursodeoxycholate (TUDC) at supraphysiological rates. Figure 7 shows that the bile flow (Figure 7A) was stimulated to a similar extent by TUDC in fed and fasted wild-type mice. Biliary bile salt secretion (Figure 7B) was slightly but significantly increased in the fasted compared to the fed mice. To evaluate the relationship between the biliary bile salt and lipid secretion, we plotted the phospholipid and cholesterol secretion against the bile salt secretion\textsuperscript{18}. The biliary phospholipid secretion was consistently approximately 40-50\% higher in fasted mice over the whole range of bile salt output rates. In contrast, biliary secretion of cholesterol was markedly reduced in fasted mice at high bile salt secretion rates (Figure 7D). Hepatic free cholesterol content, on the other hand, was not affected by fasting, i.e., 40.2 \( \pm \) 1.7 and 44.6 \( \pm \) 5.5 nmol/mg protein in livers from fed and fasted mice, respectively.

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<th>Genotype Treatment</th>
<th>wild-type fed</th>
<th>\textit{Ppara} \textsuperscript{(-/-)} fed</th>
<th>wild-type fasted</th>
<th>\textit{Ppara} \textsuperscript{(-/-)} fasted</th>
<th>wild-type refed</th>
<th>\textit{Ppara} \textsuperscript{(-/-)} refed</th>
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<td>5.2 ( \pm ) 0.9</td>
<td>6.6 ( \pm ) 1.5</td>
<td>5.1 ( \pm ) 0.9</td>
<td>4.5 ( \pm ) 0.7</td>
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<td>Biliary output\textsuperscript{2}</td>
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<td>Bile salts (BS)</td>
<td>453 ( \pm ) 184</td>
<td>412 ( \pm ) 175</td>
<td>765 ( \pm ) 256\textsuperscript{ab}</td>
<td>475 ( \pm ) 230</td>
<td>437 ( \pm ) 36</td>
<td>356 ( \pm ) 149</td>
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<td>Phospholipids (PL)</td>
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<td>31.4 ( \pm ) 10.6</td>
<td>76.6 ( \pm ) 20.7\textsuperscript{ab}</td>
<td>28.2 ( \pm ) 7.2</td>
<td>53.8 ( \pm ) 7.3\textsuperscript{ab}</td>
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<td>7.2 ( \pm ) 2.2</td>
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<td>Gluthathione</td>
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<td>21.0 ( \pm ) 3.7</td>
<td>11.1 ( \pm ) 3.8\textsuperscript{b}</td>
<td>10.2 ( \pm ) 2.4\textsuperscript{b}</td>
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<td>0.079 ( \pm ) 0.009</td>
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\textsuperscript{1}\( \mu \)l/min/100 g body wt
\textsuperscript{2}nmol/min/100 g body wt

Values are expressed as means \( \pm \) SD (\( n = 5-10 \) per group). \textsuperscript{a}Significant difference between wild-type and \textit{Ppara} \textsuperscript{(-/-)} mice on the same treatment. \textsuperscript{b}Significant difference between fasted/refed and fed mice of the same genotype.
Fasting induces Mdr2 expression

Figure 6. Steady-state mRNA levels of Cyp7a1(A) and Cyp27(B) during fasting or refeeding in livers of wild-type and Ppar α (-/-) mice. Total mRNA was isolated from livers of wild-type (white bars) and Ppar α (-/-) (black bars) mice, transcribed into cDNA, and subjected to real-time PCR analysis as described in Material and Methods. *Significant difference between wild-type and Ppar α (-/-) mice on the same treatment. \(^{a}\)Significant difference between fasted-refed and fed mice of the same genotype.

Figure 7. Bile flow and biliary bile salt, phospholipid and cholesterol secretion in fed and fasted wild-type mice during intravenous administration of increasing amounts of TUDC. The gallbladders of mice were cannulated, and, at 30 min after starting bile collection, TUDC was infused via a jugular vein in step-wise increasing dosages, as indicated by the numbers (nmol/min) in the figure. Open circles represent fed mice, and filled circles represent fasted mice. Data are presented as mean ± SD from four mice of each group. (A) bile flow expressed in µl/min/100 g BW during TUDC infusion in time; (B) bile salt secretion expressed in nmol/min/100 g BW during TUDC infusion in time; (C) phospholipid secretion versus bile salt secretion, both expressed in nmol/min/100 g BW during TUDC infusion; (D) cholesterol secretion versus bile salt secretion, both expressed in nmol/min/100 g BW during TUDC infusion. * Significant difference between fasted and fed wild-type mice.
Mdr2 deficiency does not aggravate fasting-associated fat accumulation in the mouse liver

Biliary phospholipid secretion constitutes a quantitatively important route for fatty acid turnover in the liver. Mdr2 (-/-) mice are unable to secrete phospholipids into bile and, therefore, lack the possibility to remove excess fatty acids from the liver via output of phospholipid-associated fatty acids into bile. To evaluate whether the induction of Mdr2 Pgp by fasting is of metabolic relevance, particularly with respect to hepatic triglyceride accumulation, Mdr2 (-/-) and wild-type mice were subjected to prolonged fasting. It was found that 24 and 48 hours of fasting was associated with similarly decreased plasma glucose levels and similarly increased plasma free fatty acid concentrations in wild-type and Mdr2 (-/-) mice (data not shown). Likewise, plasma β-hydroxybutyrate levels increased to a similar extent in both genotypes. Twenty-four and 48 hours of fasting was associated with an approximately 5-fold increase in hepatic triglyceride content in both wild-type and Mdr2 (-/-) mice. The triglyceride content per milligram of liver tissue was higher in wild-type than in Mdr2 (-/-) mice, but the absolute amount of hepatic triglycerides was similar in both genotypes due to the larger liver size in Mdr2 (-/-) mice (Figure 8). No changes in hepatic phospholipid contents were observed in either genotype during prolonged fasting (data not shown).

![Figure 8. Hepatic triglyceride contents of fed, 24 h fasted and 48 h fasted wild-type and Mdr2 (-/-) mice.](image)

DISCUSSION

This study demonstrates that the induction of Mdr2 expression is part of the PPARα-controlled fasting response in the murine liver. Twenty-four hours of starvation transiently increased Mdr2 mRNA levels, Mdr2 Pgp protein levels, and Mdr2 Pgp function, as reflected by an increased phospholipid output into bile. These effects of fasting were found in wild-type mice, but not in Pparα-deficient mice. Fasting also induced the hepatic expression of Abca1, Abcg5, and Abcg8 in a PPARα-dependent manner. This may reflect a consequence of fasting-associated induction of Lxrα expression, suggesting cross-talk between PPARα and LXRα. However, neither direct effects of PPARα activation on Abca1, Abcg5 or Abcg8 expression nor contribution of alternative regulating mechanisms can be excluded at this
Fasting induces Mdr2 expression point. Such a cross-talk also follows from the elevated mRNA levels of \( \text{Fx}r \) in fasted wild-type, but not in \( \text{Ppar}a \) \((-/-)\) mice. An interaction between PPAR\( \alpha \) and FXR has, to the best of our knowledge, not been reported yet. However, the increased \( \text{Fx}r \) mRNA expression was not associated with an increase in expression of \( \text{Bsep} \) and \( \text{Shp} \), two established FXR-target genes. These apparently contradicting results may be explained by an impaired enterohepatic cycling of the natural FXR ligands, i.e., the bile salts\(^{42} \), which are stored in the gallbladder during fasting. The cascade of fasting-associated adaptations in the liver mediated by PPAR\( \alpha \) is schematically shown in Figure 9.

As expected\(^1 \), plasma \( \beta \)-hydroxybutyrate levels and \( \text{Hmgs} \) mRNA levels were strongly increased in wild-type mice, reflecting the anticipated increase in fatty acid oxidation and

\[ \text{Figure 9. Proposed role of PPAR}\alpha \text{ in regulation of hepatic ABC transporters during fasting.} \]

During fasting, plasma insulin concentrations decrease, and triglycerides (TG) stored in adipose tissue are mobilized to yield glycerol and free fatty acids (FA). Part of these fatty acids are taken up by the liver to enter the \( \beta \)-oxidation pathway, allowing formation of ketone bodies. FAs taken up by the liver in excess of requirements for direct oxidation are re-esterified into TG, which gives rise to fasting-associated development of a fatty liver. FAs stimulate expression of genes involved in their uptake, \( \beta \)-oxidation, and ketogenesis via activation of the ligand-activated transcription factor PPAR\( \alpha \), which appears to be a critical event in the control of the metabolic fasting response. Enhanced expression of the \( \text{Ppar}a \) gene itself provides a feed-forward loop to accelerate this response. This current study shows that activation of PPAR\( \alpha \) by fasting also leads to a PPAR\( \alpha \)-dependent induction of \( \text{Mdr}2 \) gene expression, protein levels and function, leading to increased phospholipid output into bile. Furthermore, fasting induces the expression of \( \text{Abca}1 \) and \( \text{Abcg}5/\text{g8} \), which could be a direct consequence of PPAR\( \alpha \) activation or an indirect consequence of PPAR\( \alpha \)-dependent induction of \( \text{Lxr}\alpha \), a nuclear receptor involved in control of \( \text{Abca}1 \) and \( \text{Abcg}5/\text{Abcg}8 \) expression. Besides LXR, fasting also induces the mRNA expression of the bile salt-activated nuclear receptor \( \text{Fx}r \) in a PPAR\( \alpha \)-dependent manner. Increased expression of \( \text{Abcg}5/\text{Abcg}8 \), encoding ABC transporters that have recently been implicated in cholesterol transport, did not affect basal biliary cholesterol excretion but appeared to be associated with decreased rather than increased maximal hepatobiliary cholesterol excretion. This suggests that other factors control the rate of cholesterol excretion under these conditions.
ketogenesis that was absent in \textit{Ppara}(-/-) mice. A 24 hour fasting period led to a markedly increased triglyceride content of the liver, caused by storage of fatty acids released from adipose tissues in excess of the requirements for direct oxidation. This fasting-induced increase in hepatic triglycerides was more severe in the absence of PPAR\(\alpha\) and did not return to baseline values upon refeeding. This is probably due to the inability of \textit{Ppara}(-/-) mice to induce \(\beta\)-oxidation. The markedly higher expression of \textit{Ppar\(\gamma\)} in livers of \textit{Ppara}(-/-) mice, which was even further induced upon fasting, apparently could not compensate for PPAR\(\alpha\) deficiency. Induction of \textit{Ppar\(\gamma\)} expression in livers of \textit{Ppara}(-/-) mice may be related to the accumulation of fat per se since high hepatic \textit{Ppar\(\gamma\)} expression has been reported in murine models of hepatic steatosis\(^43\). In addition to the PPAR\(\alpha\)-mediated induction of the \(\beta\)-oxidation machinery, fasting is also associated with impaired hepatic \textit{de novo} lipogenesis\(^38\). The latter is related to a strongly reduced expression of \textit{Srebp1c}, confirmed in our study (Figure 2C), which has been attributed to low levels of circulating insulin\(^44\).

It has been shown\(^10\) that PPAR\(\alpha\) and \(\gamma\) activation induces expression of \textit{Lxr} in macrophages, which, in turn, induces expression of \textit{Abca1} and cholesterol efflux by these cells. Cross-talk between fatty acid and cholesterol metabolism mediated by LXR\(\alpha\) has also been demonstrated in hepatocytes. Liver expression of \textit{Lxra} has been reported to be responsive to dietary fatty acids in mice\(^12\). The induction of hepatic \textit{Abca1}, \textit{Abcg5} and \textit{Abcg8} in wild-type mice may be due to a similar cascade in which activation of PPAR\(\alpha\) by influx of fatty acids represents the primary event. The induction of \textit{Cyp7a1} in wild-type mice, a gene known to be positively controlled by LXR\(^24\), supports this suggestion. However, based on the current studies, a direct effect of PPAR\(\alpha\) or alternative alterations of other signalling mechanisms on the expression of \textit{Abca1} and \textit{Abcg5/Abcg8} cannot be ruled out.

The induction of \textit{Mdr2} expression on fasting in wild-type mice is in accordance with earlier studies by us\(^15\) and by others investigators\(^13,45\) in which PPAR\(\alpha\) was activated pharmacologically through treatment with PPAR\(\alpha\) agonists. In the current study, we show that also under physiological conditions with metabolic stress leading to PPAR\(\alpha\) activation, \textit{Mdr2} expression is induced in a PPAR\(\alpha\)-dependent manner. The slight induction of \textit{Mdr2} expression seen in fasted \textit{Ppara}(-/-) mice may be related to the fasting-associated reduction of plasma insulin levels. We have recently shown that the induction of insulin-dependent diabetes in rats leads to a strong induction of \textit{Mdr2} expression that is reversible upon insulin treatment\(^46\). Likewise, the expression of another member of the B subfamily of ABC transporters, i.e., \textit{Mdr1a}, was approximately 3-fold higher in fasted than in fed wild-type mice and remained induced upon refeeding. Unlike the situation observed for \textit{Mdr2}, the induction of \textit{Mdr1a} expression after a fast was much less pronounced than that observed after pharmacological PPAR\(\alpha\) activation (~10-fold): the physiological relevance of PPAR\(\alpha\)-induced \textit{Mdr1a} expression is unknown.

It should be stressed that in the fed state, neither \textit{Mdr2} nor \textit{Mdr1a} expression differed between wild-type and \textit{Ppara}(-/-) mice, indicating that PPAR\(\alpha\) is not involved in the control of basal expression of these genes. Likewise, the expression of genes encoding the major bile salt transporting proteins and the key enzymes in bile salt biosynthesis were similar in both strains in the fed situation. As a consequence, there were no differences in basal bile formation as determined during 30 min of bile collection immediately after gallbladder cannulation. Biliary phospholipid secretion was induced in fasted wild-type mice, an effect that persisted when phospholipid output was corrected for differences in bile salt output, and reflected the increased \textit{Mdr2} Pgp levels\(^47\). The increased capacity for biliary
phospholipid secretion became particularly clear when wild-type mice received an intravenous infusion of TUDC at supraphysiological rates: fasted wild-type mice persistently secreted more phospholipid per bile salt than fed wild-type mice did, demonstrating that the induction of \textit{Mdr2} expression indeed has functional consequences.

The secretion of cholesterol into bile is tightly coupled to that of phospholipids under normal conditions. Recently, it has been suggested that \textit{Abca1} and/or \textit{Abcg5}/\textit{Abcg8} may be involved herein\textsuperscript{23,24}. Although these LXR-controlled genes were induced to a variable extent in fasted wild-type mice, fasting was associated with an impaired rather than with an increased maximal cholesterol secretion rate. Basal biliary cholesterol secretion, when corrected for differences in bile salt secretion, was not affected. Although it should be stressed that we were not able to assess protein levels of these ABC transporters, these results can be interpreted to indicate that factors unrelated to their expression must be rate-controlling in the cholesterol secretion process under fasting conditions. A reason for the uncoupling of cholesterol from phospholipid secretion during fasting may be a reduced supply of bile-destined HDL cholesterol. We found reduced amounts of the scavenger receptor class B type I (SR-BI), the HDL receptor\textsuperscript{48}, by Western analysis on plasma membrane fractions from livers of fasted wild-type mice when compared to those of fed controls (unpublished observation, Kok \textit{et al.} 2002). It has recently been shown that SR-BI-deficient mice secrete less cholesterol into bile than the corresponding wild-type mice\textsuperscript{49}, whereas transient overexpression of SR-BI is associated with cholesterol hypersecretion\textsuperscript{50}. In addition, impaired hepatic cholesterogenesis during fasting\textsuperscript{51,52}, may contribute to a diminished supply of bile-destined cholesterol.

The results of this study clearly demonstrate that stimulation of biliary phospholipid secretion via induction of \textit{Mdr2} expression is part of the physiological PPAR\textit{\alpha}-controlled fasting response. The obvious next question refers to the physiological significance of this response. Our initial hypothesis was that accelerated removal of phospholipid-associated fatty acids might attenuate the development of fasting-induced fat accumulation, which represents a potentially harmful condition\textsuperscript{53}. This hypothesis was based on the fact that the amount of phospholipid-associated fatty acids secreted into bile (~25 \(\mu\)mol/day/mouse) is considerable when compared to the amount of triglyceride-associated fatty acids in the liver (~75 \(\mu\)mol) and that a 50\% increase in biliary fatty acid removal might thus be beneficial in this respect. However, it was found that prolonged fasting resulted in a very similar increase in hepatic triglyceride content in wild-type and in \textit{Mdr2} Pgp-deficient mice that are unable to secrete phospholipids into bile. Although there are evidently adaptations in hepatic phospholipid metabolism in \textit{Mdr2} \((-/-)\) mice that may hamper interpretation of these results, it seems plausible to conclude that an increased output of phospholipid-associated fatty acids into bile is not of physiological importance in prevention or attenuation of fasting-induced hepatic fat accumulation. An alternative function of increased biliary phospholipid concentrations could be in protection of the cells lining the biliary tree and the gallbladder wall from the potentially toxic actions of high concentrations of bile salts that build up during fasting.

In conclusion, this work demonstrates that the induction of \textit{Mdr2} gene expression and \textit{Mdr2} Pgp function in hepatobiliary phospholipid secretion is part of the PPAR\textit{\alpha}-controlled fasting response in the liver of the mouse. The physiological relevance remains to be established. The PPAR\textit{\alpha}-dependent increase in hepatic expression of \textit{Abca1}, \textit{Abcg5} and \textit{Abcg8} upon fasting was not associated with increased biliary cholesterol excretion. In contrast, maximal cholesterol excretion appeared to be impaired in fasted mice. This
indicates that hepatobiliary cholesterol excretion is, in quantitative terms, not controlled by these ABC transporters under the physiological conditions employed.

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Fasting induces Mdr2 expression

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Chapter 3

Fasting induces Mdr2 expression

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