Nuclear receptors in control of cholesterol transport
van der Veen, Jelske Nynke

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Reduced cholesterol absorption upon PPARδ activation coincides with decreased intestinal expression of NPC1L1

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ABSTRACT

Objectives- Peroxisome proliferator-activated receptors (PPARs) control transcription of genes involved in lipid metabolism. Activation of PPARδ may have anti-atherogenic effects through elevation of plasma HDL, theoretically promoting reverse cholesterol transport from peripheral tissues towards the liver for removal via bile and feces. Methods and results- Effects of PPARδ activation by GW610742 were evaluated in wild-type and Abca1-deficient (Abca1−/−) mice that lack HDL. Treatment with GW610742 resulted in a ~50% increase of plasma HDL-cholesterol in wild-type mice, whereas plasma cholesterol levels remained extremely low in Abca1−/− mice. Yet, biliary cholesterol secretion rates were similar in untreated wild-type and Abca1−/− mice and unaltered upon treatment. Unexpectedly, PPARδ activation led to enhanced fecal neutral sterol loss in both groups without any changes in intestinal Abca1, Abcg5, Abcg8 and HMG-CoA reductase expression. Moreover, GW610742 treatment resulted in a 43% reduction of fractional cholesterol absorption in wild-type mice, coinciding with a significantly reduced expression of the cholesterol absorption protein Niemann-Pick C1 Like 1 (Npc1l1) in the intestine. Conclusions- PPARδ activation is associated with elevated plasma HDL and reduced intestinal cholesterol absorption efficiency that may be related to decreased intestinal Npc1l1 expression. Thus, PPARδ is a promising target for drugs aimed to treat or prevent atherosclerosis.
INTRODUCTION

Plasma levels of high-density lipoprotein (HDL) cholesterol are inversely related to development of atherosclerosis. This protective effect has been attributed to a role of HDL in reverse cholesterol transport (RCT), defined as the flux of excess cholesterol from peripheral cells to nascent HDL particles followed by transport to the liver. The liver is able to secrete cholesterol into bile, either as free cholesterol or after conversion into bile salts, for removal via the feces. Stimulation of HDL-mediated cholesterol efflux is considered an attractive approach to diminish the development of atherosclerosis.

The ATP-binding cassette transporter A1 (ABCA1) is considered to be essential in RCT. ABCA1 is ubiquitously expressed and probably involved in formation of pre-β-HDL particles and the efflux of cholesterol from peripheral tissues towards HDL. HDL is considered a major source for bile-destined cholesterol. However, we have recently demonstrated that, despite the absence of HDL, hepatobiliary cholesterol flux and fecal sterol excretion are not affected in Abca1-deficient (Abca1−/−) mice. The ABCG5/ABCG8 heterodimer was recently shown to be of crucial importance for hepatobiliary cholesterol secretion and for transport of cholesterol from enterocytes back into the intestinal lumen, thereby promoting net cholesterol removal from the body.

Several genes involved in the control of cholesterol metabolism are transcriptionally regulated by nuclear receptors. Peroxisome proliferator-activated receptors (PPARs) comprise a subgroup of the nuclear receptor superfamily, designated PPARα (NR1C1), PPARδ (NR1C2) and PPARγ (NR1C3), which all serve functions in lipid homeostasis and energy metabolism. PPARδ is ubiquitously expressed and activated by long-chain fatty acids and prostacyclins. Recent work suggests that activation of PPARδ may induce RCT and hence have anti-atherogenic effects. Whether or not PPARδ activation, like PPARα activation, is associated with altered bile formation and fecal sterol loss is not known.

This study shows that PPARδ activation in mice raised plasma HDL concentrations and accelerated fecal cholesterol removal from the body without changing hepatobiliary sterol excretion. Moreover, intestinal cholesterol absorption efficiency was reduced upon PPARδ activation, which coincided with down-regulation of intestinal gene expression of the very recently identified cholesterol absorption protein Niemann-Pick C1 Like 1 (Npc1l1).

EXPERIMENTAL PROCEDURES

Animals
Female Abca1−/− mice with a DBA/1 background and age-matched DBA/1 wild-type mice were purchased from IFFA Credo (Saint-Germain-sur-l’Arbresle, France). Separate groups of wild-type DBA/1 mice were obtained from Harlan (Horst, The Netherlands). All experimental procedures were in accordance with local guidelines for use of experimental animals.

PPARδ agonist
GW610742, previously referred to as GW0742 (GlaxoSmithKline Pharmaceuticals, Stevenage, UK) (figure 1) is a high affinity ligand for PPARδ. Specificity of GW610742, as evaluated by ligand binding studies, revealed EC50 values for GW610742 of 28 nM for
murine PPARα versus 8900 nM and >10000 nM for murine PPARα and PPARγ, respectively. For human PPARα, PPARα and PPARγ the EC50 values are 1, 1200 and 4100 nM, respectively14. L. Patel, personal communication). The specificity of GW1516 has previously been described10,14.

![Figure 1. Chemical structure of the PPARα-specific agonist GW601742.](image)

**Experimental Methods**
Abca1+/- and DBA/1 wild-type mice (n = 6 per group) were fed GW610742 mixed through chow at a level of 0.017% w/w for 8 days. With an average daily food intake of 3 gram, this provided an approximate intake of 20 mg/kg/day leading to an average plasma concentration of 1 μM (L. Patel, personal communication). Control mice received standard chow without GW610742. From day 7 to 8, feces was collected from individual mice. Mice were then anesthetized by intraperitoneal injection of ketamine (1 ml/kg) and diazepam (10 mg/kg). Bile was collected for 30 minutes after cannulating the gallbladder and a blood sample was taken by cardiac puncture. Livers and small intestines were excised. Parts of both liver and intestine were snap-frozen in liquid nitrogen and stored at –80 °C for RNA isolation and biochemical analysis. Samples for microscopic evaluation were frozen in isopentane and stored at –80 °C or fixed in paraformaldehyde.

**Analytical Methods**
Livers were homogenized and hepatic and biliary lipids were extracted15. Hepatic, biliary and plasma concentrations of cholesterol, triglycerides and phospholipids were determined as previously described6. Fecal neutral sterols and fatty acids were analyzed by gas chromatography. Bile salts in feces and in bile were measured enzymatically. Pooled plasma samples were used for lipoprotein separation by fast protein liquid chromatography (FPLC).

**RNA Isolation and Measurement of mRNA Levels by Realtime PCR (Taqman)**
RNA isolation, cDNA synthesis and real-time quantitative PCR were performed as described by Plösch et al6. Primer and probe sequences for Abca1, Abcg5, Abcg8, Acat2, Hmgr, Lxrα, Mdr2 and Sr-b1 as well as for β-actin11 have been published. Furthermore, the following primers/probes were used: For Pparα, sense 5'-AGA TGG TGG TGG CAG AGC TAT GAC C-3'; antisense 5'-TCT CCT CCT GTG GCT GTT CC-3'; and probe 5'-CCC ACT TGG CGT GGC GCC T-3' (accession number, NM_013141). For Pdk4, sense 5'-GCA TTT CTA CTC GGA TGC TCA TG-3'; antisense 5'-CCA ATG TGG CTT GGG TTT CC-3'; and probe 5'-CAG CAC ATC CTC ATA TTC AGT GAC TCA AAG AC-3' (accession number, NM_013743). For Npc1l1, sense 5'-GAG AGC CAA AGA TGC TAC TAT CCT CA-3'; antisense 5'-CCC GGG AAG TTG GTC ATG-3'; and probe 5'-ACT CCA GCA AAC ACC GCA CTG CC-3'
Isolation of Peritoneal Macrophages

DBA/1 wild-type mice were treated with the GW610742-containing diet for 8 days. Thioglycollate-elicited peritoneal macrophages of treated and untreated DBA/1 wild-type mice were harvested as described by Herijgers et al. The cells were washed and RNA isolation, cDNA synthesis and real-time PCR were performed as described.

Plasma Dual Isotope Ratio Method

Cholesterol absorption was measured using the plasma dual isotope ratio method. DBA/1 wild-type mice (n = 5 per group) received a diet with or without 0.017% w/w GW610742. After six days, mice received an intravenous injection of 1.1 µCi [3H]-cholesterol dissolved in intralipid and an oral dose of 1.0 µCi [14C]-cholesterol dissolved in medium-chain triglyceride oil. At 24, 48, and 72 hours after administration, blood samples were taken by retro-orbital puncture and feces was collected. At day 10, mice were anaesthetized and bile was collected for 30 minutes. [14C] and [3H] activity in plasma, bile and feces was measured by liquid scintillation counting. Blood samples obtained 72 hours after administration were used for the calculation of cholesterol absorption.

In vitro activation of PPARs in Caco-2 cells

Cell culture reagents were obtained from Eurobio (Les Ulis, France) and microporous PET membrane inserts (23.1 mm, 3 µm pore size) from Becton Dickinson (Le Pont de Claix, France). Caco-2 cells were routinely grown in plastic flasks (TPP, ATGC, Marne la Vallée, France) under a humidified atmosphere containing 10 % CO2, at 37°C, in Dulbecco’s modified essential medium containing 25 mM glucose and glutamax, supplemented with penicillin-streptomycin (100 IU/ml and 100 µg/ml, respectively), 1 % nonessential amino-acids, and 20 % heat-inactivated Fetal Calf Serum (FCS). To establish the intestinal barrier model for the assay, Caco-2 cells (between passages 40-45) were plated at a density of 0.25 × 106 cells per insert and grown in the complete medium. Confluence was routinely reached 8 days after seeding. Cells were then cultured in asymmetric conditions, with medium containing FCS in the lower compartment and serum-free medium in the upper compartment. Media were changed every day. Three weeks after, cells were activated with ligands for PPARα (Wy14643 at 50 µM), PPARγ (rosiglitazone at 100 nM) or PPARβ/δ (GW1516 at 100 nM) for 24 hours in the upper compartment. After incubation, cell layers were briefly rinsed twice with ice-cold phosphate-buffered saline (PBS, 10 mM phosphate buffer, pH 7.5 ; 2.7 mM KCl ; 150 mM NaCl) and total cellular RNA was extracted using RNA-Plus (Q-BIOgene, Illkirch, France). For quantitative PCR, total RNA were reverse transcribed using random hexameric primers and Superscript reverse transcriptase (Life Technologies, France). cDNA were quantified by real-time PCR on a MX 4000 (Stratagene) using specific primers for NPC1L1 (sense 5'-GGG GCA TCA GTT ACA ATG CT-3'; antisense 5'-AAA CAC CGC ACT TCC CAT AG-3'). PCR amplification was performed in a volume of 25 µl containing 100 nmol/L of each primer, 4 mmol/L MgCl2, the Brilliant Quantitative PCR Core Reagent Kit mix as recommended by the manufacturer.
(Stratagene) and SYBR Green 0.33 X (Sigma-Aldrich, Saint Quentin Fallavier, France). The conditions were 95°C for 10 minutes, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C. NPC1L1 mRNA levels were subsequently normalized to 28S mRNA (sense 5'-AAT CTC GGG TGG AGG TCC GT-3'; antisense 5'-CTT ACC AAA AGT GGC CCA CTA-3'). The activated condition was then normalized to the control condition set at 100%. Each point was performed in triplicate.

**Immunohistochemistry**

Histology of livers and small intestines was examined after hematoxilin/eosin staining on paraformaldehyde-fixed sections. Neutral lipids were stained by oil-red-O on frozen sections and peroxisome proliferation was determined by catalase staining. Intestinal cell proliferation was examined after Ki67 staining on paraformaldehyde-fixed sections, using a Ki-67 polyclonal antibody (Novo Castra, Newcastle, UK) (1:500).

**Statistics**

Statistical analyses were performed using SPSS Version 10.0 for Windows (SPSS Inc., Chicago, IL). Treated and untreated groups were compared by Student’s t-test. A p-value < 0.05 was considered statistically significant.

**TABLE 1.** Animal characteristics and plasma and hepatic lipid concentrations in untreated and GW610742-treated DBA/1 wild-type and Abca1<sup>-/-</sup> mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild-type</th>
<th>Abca&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Wild-type</th>
<th>Abca&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>GW610742</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>20.9 ± 0.8</td>
<td>21.4 ± 0.9</td>
<td>20.0 ± 1.5</td>
<td>21.4 ± 0.9</td>
</tr>
<tr>
<td>Liver weight (% of bw)</td>
<td>4.2 ± 0.3</td>
<td>4.8 ± 0.5</td>
<td>5.7 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.9 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALAT (units/l)</td>
<td>94 ± 43</td>
<td>225 ± 181&lt;sup&gt;a&lt;/sup&gt;</td>
<td>76 ± 23</td>
<td>146 ± 159</td>
</tr>
<tr>
<td>LDH (units/l)</td>
<td>653 ± 241</td>
<td>841 ± 335</td>
<td>603 ± 312</td>
<td>653 ± 404</td>
</tr>
<tr>
<td>Plasma cholesterol (mM)</td>
<td>1.9 ± 0.3</td>
<td>0.5 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma triglycerides (mM)</td>
<td>0.8 ± 0.2</td>
<td>1.2 ± 0.6</td>
<td>0.7 ± 0.3</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>Liver cholesterol (nmol/mg liver)</td>
<td>3.9 ± 0.4</td>
<td>4.4 ± 0.8</td>
<td>3.7 ± 0.5</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>Liver phospholipids (nmol/mg liver)</td>
<td>20.6 ± 3.5</td>
<td>23.0 ± 6.8</td>
<td>23.4 ± 1.7</td>
<td>22.3 ± 3.7</td>
</tr>
<tr>
<td>Liver triglycerides (nmol/mg liver)</td>
<td>9.7 ± 3.1</td>
<td>9.3 ± 1.1</td>
<td>8.8 ± 2.6</td>
<td>13.1 ± 3.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abca<sup>-/-</sup>: Abca1-deficient; ALAT, aspartate aminotransferase; LDH, lactate dehydrogenase. Values are expressed as mean ± SD (n=6 in all groups)

<sup>a</sup> Significant difference between wild-type and Abca<sup>-/-</sup> mice with the same treatment.

<sup>b</sup> Significant difference between GW610742 treated and untreated mice with the same genotype.

**RESULTS**

**Animal characteristics**

Table 1 shows that body weights of DBA/1 wild-type and Abca<sup>-/-</sup> mice were similar and not influenced by treatment with GW610742. Liver weights of untreated wild-type and Abca<sup>-/-</sup> mice did not differ, but treatment with the PPARδ agonist resulted in slightly increased liver weights in both strains. This was probably related to peroxisome proliferation, as revealed...
by enhanced catalase staining in liver sections of treated animals (data not shown). Treatment with GW610742 did not induce liver injury as indicated by unaffected plasma LDH and ALAT levels.

**PPARδ activation increases plasma HDL and induces Abca1 expression in macrophages, but has no effect on hepatobiliary cholesterol excretion**

In accordance with previous reports\(^5,18\), plasma cholesterol levels were ~75% lower in Abca1\(^{-/-}\) mice than in wild-type mice (Table 1). Treatment with GW610742 increased total plasma cholesterol by ~30% in wild-type mice, whereas cholesterol levels in Abca1\(^{-/-}\) remained extremely low. FPLC analysis (Figure 2) confirmed the complete lack of HDL-cholesterol in Abca1\(^{-/-}\) mice and revealed a ~50% increase in HDL-cholesterol levels in wild-type mice upon PPARδ activation.

Expression levels of both Abca1 and Sr-b1 in thioglycollate-elicited peritoneal macrophages of wild-type mice were approximately 3-fold upregulated upon PPARδ activation (Figure 3), demonstrating that GW610742 did induce systemic effects.
Hepatic concentrations of cholesterol, phospholipids and triglycerides (Table 1) were similar in wild-type and Abca1<sup>−/−</sup> mice and were not affected by PPARδ activation in wild-type mice, whereas hepatic triglycerides were slightly increased upon treatment in Abca1<sup>−/−</sup> mice.

Biliary secretion rates of bile salts, cholesterol and phospholipids were similar in untreated Abca1<sup>−/−</sup> mice compared to wild-type mice (Table 2), in accordance with published data<sup>5</sup>. Treatment with GW610742 did not significantly affect biliary secretion rates in wild-type or Abca1<sup>−/−</sup> mice. This is in accordance with the absence of any effect on hepatic expression of several genes involved in cholesterol metabolism and transport (Figure 4, left hand panels). Only hepatic Abcb4 (<i>Mdr2</i>) expression was slightly but significantly induced upon treatment but this did not affect biliary phospholipid secretion. As a positive control, expression of the PPARδ target gene <i>Pdk4</i><sup>19</sup>, encoding pyruvate dehydrogenase kinase isoenzyme 4, was measured. Hepatic expression of this gene was ~6-fold upregulated upon PPARδ activation in both strains of mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild-type</th>
<th>Abca1&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Wild-type</th>
<th>Abca1&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>GW610742</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile flow (μl/min/g liver)</td>
<td>2.4 ± 0.6</td>
<td>2.1 ± 0.7</td>
<td>2.2 ± 0.4</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>Bile salts (nmol/min/100g bw)</td>
<td>445 ± 203</td>
<td>483± 169</td>
<td>440 ± 165</td>
<td>428 ± 121</td>
</tr>
<tr>
<td>Cholesterol (nmol/min/100 g bw)</td>
<td>4.9 ± 1.4</td>
<td>6.9 ± 3.2</td>
<td>7.5 ± 2.9</td>
<td>7.7 ± 2.2</td>
</tr>
<tr>
<td>Phospholipids (nmol/min/100g bw)</td>
<td>40.9 ± 8.3</td>
<td>55.0 ± 15.0</td>
<td>64.1 ± 18.6</td>
<td>57.8 ± 18.6</td>
</tr>
<tr>
<td>Cholesterol/phospholipid ratio</td>
<td>0.12 ± 0.02</td>
<td>0.12 ± 0.05</td>
<td>0.13 ± 0.04</td>
<td>0.13 ± 0.01</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD (n=6 in all groups)

Figure 4. Gene expression in the livers and intestines of untreated (open bars) and GW610742-treated (closed bars) DBA/1 wild-type and Abca1<sup>−/−</sup> mice, measured by real-time PCR. Data are presented as the mean of 6 assays performed in duplicate ± SD. Expression values are normalized to β-actin and expression in untreated wild-type mice was set to 1.00. * and # indicate significant differences (Student’s t test, <i>p</i> < 0.05 and <i>p</i> < 0.01 respectively).
**Fecal excretion of neutral sterols is induced upon PPARδ activation**

Fecal excretion of acidic sterols (bile salts) was similar in all groups (Figure 5A). However, fecal excretion of neutral sterols, 80% of which comprised of cholesterol, was 2 to 3-fold increased upon PPARδ activation in wild-type and Abca1−/− mice (Figure 5B). Since hepatobiliary efflux of cholesterol was not induced upon treatment, increased sterol excretion might be directly mediated by intestinal adaptations. Figure 4 (right hand panels) shows that intestinal expression levels of Abca1, Abcg5 and Abcg8 were not affected upon treatment with GW610742. Expression of Acat2, responsible for esterification of cholesterol in enterocytes and crucial for cholesterol absorption, and of the gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme in cholesterol synthesis, were also unaffected.

To investigate whether increased intestinal cell proliferation may have contributed to increased fecal cholesterol excretion through accelerated cell shedding, a Ki67 staining was performed on intestinal sections. This, however, did not show any sign of accelerated proliferation upon PPARδ activation (data not shown).

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**PPARδ activation decreases cholesterol absorption, accelerates fecal excretion of plasma-derived cholesterol and reduces intestinal Npc1l1 expression**

Figure 6 shows that PPARδ activation led to a 43% reduction of cholesterol absorption efficiency in DBA/1 wild-type mice, despite the unaffected expression levels of Abcg5 and Abcg8. Recently, Niemann-Pick C1 Like 1 protein has been identified as a critical component of the intestinal cholesterol absorption machinery13.
Therefore, we measured mRNA levels of *Npc1l1* along the length of the small intestine of untreated and treated mice. Expression of the gene was decreased by 35% in the jejunum upon PPAR\(\delta\) activation and was also lower in ileal sections of treated animals (Figure 7). A similar decrease in intestinal *Npc1l1* expression (i.e., -40%) was observed in *Abca1*\(^{-/-}\) mice upon treatment with GW610742.

Reduced cholesterol absorption is also apparent from Figure 8A, showing a markedly increased fecal recovery of orally administered \(^{14}\)C-cholesterol in GW610742-treated mice. Figure 8B shows that fecal excretion of intravenously injected \(^{3}\)H-labeled cholesterol was higher upon treatment with GW610742, which is likely attributable in part to less efficient reabsorption of biliary \(^{3}\)H-cholesterol. However, the 2.5-fold increase in fecal \(^{3}\)H-

![Figure 7. Expression of *Npc1l1* along the length of the small intestine of untreated (open bars) and GW610742-treated (closed bars) DBA/1 wild-type mice, measured by real-time PCR. Data are presented as the mean of 6 assays performed in duplicate ± SD. Expression values are normalized to \(\beta\)-actin and expression in duodenum of untreated mice was set to 1.00. * indicate significant differences (Student’s \(t\) test, \(p < 0.05\)).](image)

![Figure 8. Fecal loss of intravenously and orally administered radiolabeled cholesterol in untreated and GW610742-treated DBA/1 wild-type mice. Panel A and B show fecal loss of \(^{14}\)C-labeled and \(^{3}\)H-labeled neutral sterols respectively in treated (closed symbols) and in untreated (open symbols) mice. Panel C and D show fecal loss of \(^{14}\)C-labeled and \(^{3}\)H-labeled bile salts, respectively. Values are presented as % of administered dose (\(n = 5\) per group). * and # indicate significant differences (Student’s \(t\) test, \(p < 0.01\) and \(p < 0.05\) respectively).](image)
cholesterol loss is larger than expected on the basis of a 40% reduction in cholesterol absorption efficiency. These data suggest that cholesterol may partly be excreted directly from plasma into the intestinal lumen\textsuperscript{20}. Conversion of labeled cholesterol into bile salts was not affected by PPAR\(\delta\) activation, as shown in figure 8D. Fecal excretion of \[^{14}C\]-labeled bile salts (Figure 8C) was somewhat lower in the treated mice, probably due to the lower efficiency of cholesterol absorption in these animals.

Total fat absorption was not affected by treatment with the PPAR\(\delta\) agonist, as indicated by similar fecal fat excretion rates in both groups (data not shown).

**Repression of Npc1l1 expression is specific for PPAR\(\delta\) agonist in CaCo2 cells**

To assess the specificity of the observed effects on Npc1l1 expression, we have evaluated the consequences of PPAR\(\alpha\), PPAR\(\gamma\) and PPAR\(\delta\) activation by specific agonists in polarized CaCo2 cells. Figure 9 shows that both the PPAR\(\alpha\) agonist Wy14643 and the PPAR\(\gamma\) agonist rosiglitazone had no effect on NPC1L1 expression, whereas the PPAR\(\delta\) agonist GW1516 exhibited a clear (-33\%) reduction in NPC1L1 expression. This demonstrates that the reduced expression of NPC1L1 is specific for PPAR\(\delta\) activation.

**DISCUSSION**

This study shows that activation of PPAR\(\delta\) results in increased plasma HDL levels in DBA/1 wild-type mice. Although elevated HDL levels might theoretically deliver more cholesterol to the liver for excretion into bile, hepatobiliary excretion of cholesterol and bile salts was not affected upon PPAR\(\delta\) activation in wild-type mice. In fact, excretion rates were highly similar in wild-type and Abca1\(^-\) mice, confirming that HDL is not an essential source for biliary cholesterol in mice\textsuperscript{5,6}. In spite of the unaltered biliary excretion rates, fecal loss of neutral sterols was doubled in both strains after treatment with GW610742, which could not be ascribed to either increased intestinal cholesterol synthesis or accelerated intestinal cell proliferation. PPAR\(\delta\) activation reduced cholesterol absorption efficiency in wild-type mice without any change in intestinal Abca1, Abcg5 or Abcg8 expression, but did not affect total fat absorption. Reduction of cholesterol absorption without changes in Abcg5 and Abcg8 expression has also been reported upon treatment of mice with the cholesterol absorption-
reducing drug ezetimibe\textsuperscript{21}: intestinal expression of the recently described potential target of ezetimibe, \textit{Npc1l1}\textsuperscript{13}, appeared to be decreased upon PPAR\textgreek{d} activation.

Analogous to the situation described in obese and hyperlipidemic rhesus monkeys\textsuperscript{10}, PPAR\textgreek{d} activation beneficially altered plasma lipid profiles in wild-type mice by increasing HDL-cholesterol concentrations. This increase in HDL-cholesterol levels was not observed in \textit{Abca1}\textsuperscript{-/-} mice, supporting the essential role of ABCA1 in HDL formation. The question by which mechanism PPAR\textgreek{d} activation elevates HDL-cholesterol concentrations remains to be answered. Induced expression of \textit{Abca1} as well as \textit{Sr-b1} in peritoneal macrophages isolated from GW610742-treated wild-type mice suggests that induction of these efflux mediators may contribute. However, bone-marrow transplantation studies\textsuperscript{22} indicate that the contribution of macrophage-derived cholesterol to plasma HDL levels is limited in mice: plasma HDL-cholesterol levels probably reflect ABCA1-mediated efflux events in all peripheral organs and tissues. ABCA1-mediated cholesterol efflux appeared to be a major source of plasma HDL-cholesterol in mice\textsuperscript{23}. Yet, we did not observe induction of hepatic \textit{Abca1} expression. The reason for the discrepancy in PPAR\textgreek{d}-mediated effects on \textit{Abca1} expression between macrophages and liver remains to be established.

HDL-cholesterol is considered a preferential source of biliary cholesterol\textsuperscript{4}. However, despite the marked differences in plasma HDL-cholesterol levels no differences in biliary cholesterol excretion were observed between untreated wild-type and \textit{Abca1}\textsuperscript{-/-} mice or between treated and untreated wild-type mice. These observations are consistent with earlier work\textsuperscript{5,6}, indicating that delivery of HDL-cholesterol to the liver is not rate-controlling for biliary cholesterol secretion in mice.

Surprisingly, fecal excretion of neutral sterols was 2 to 3-fold increased upon PPAR\textgreek{d} activation in both wild-type and \textit{Abca1}\textsuperscript{-/-} mice, in spite of the fact that biliary cholesterol excretion was not induced. This could theoretically be due to a higher intestinal cholesterol synthesis. This parameter has not been measured directly, but intestinal expression of \textit{Hmgr} was not affected upon PPAR\textgreek{d} activation which strongly suggests unaltered intestinal cholesterol synthesis. Most cholesterol is synthesized in the peripheral tissues in mice\textsuperscript{24} and peripheral synthesis may have been enhanced upon PPAR\textgreek{d} activation to maintain total body cholesterol balance. It is also highly unlikely that accelerated intestinal cell turnover was the cause of enhanced fecal sterol loss: Ki67 staining on intestinal sections revealed no differences between GW610742-treated and untreated mice.

Cholesterol absorption efficiency was clearly reduced upon PPAR\textgreek{d} activation in wild-type mice. Since the amounts of bile salts and phospholipids excreted into the intestinal lumen, important for efficient cholesterol absorption\textsuperscript{25,26}, as well as \textit{Acat2} expression were unaffected, these factors can be excluded as the cause of the reduced cholesterol absorption. Surprisingly, reduced cholesterol absorption was not associated with any change in intestinal expression of \textit{Abcg5} and \textit{Abcg8}. Our data suggests that PPAR\textgreek{d} may reduce cholesterol absorption by interference with cellular uptake, i.e., by a mechanism that is related to the mode of action of the cholesterol absorption inhibitor ezetimibe\textsuperscript{21}. Very recently, Altmann \textit{et al.}\textsuperscript{13} proposed Niemann-Pick C1 Like 1 protein to be critical for intestinal cholesterol absorption and to represent a target of ezetimibe. Our results show that PPAR\textgreek{d} activation clearly reduced intestinal expression of \textit{Npc1l1}, predominantly in the jejunal part of the small intestine where most of the cholesterol absorption takes place. Our \textit{in vitro} results show that this reduced expression of \textit{Npc1l1} is highly specific for PPAR\textgreek{d} activation. There was no effect of selective PPAR\textgreek{a} and PPAR\textgreek{y} agonists on \textit{Npc1l1}
expression in Caco-2 cells, but a clear suppression by the PPARδ agonist, indicating that the human NPC1L1 is also responsive to PPARδ activation.

No data are available yet on factors involved in Npc1l1 transcription regulation: whether PPARδ controls intestinal Npc1l1 expression by direct or indirect means remains to be established. Since the amount of Npc1l1 protein is clearly reduced in enterocytes of heterozygous Npc1l1+/− mice, it is likely that the ~40% reduction in jejunal Npc1l1 mRNA levels were associated with reduced amounts of the protein. Upon oral administration of 14C-cholesterol, absorption in chow-fed Npc1l1+/− mice into plasma and liver appeared to be reduced by ~40% when compared to wild-type mice, although this difference failed to reach statistical significance. A significant reduction in fractional cholesterol absorption in heterozygote mice compared to wild-type controls was noted after feeding a diet containing 0.1% sodium cholate.

It has been proposed that PPARδ might induce anti-atherogenic actions. Our data support this notion, since PPARδ activation resulted in elevated HDL-cholesterol levels. However, potential anti-atherogenic effects would not be expected to be achieved by induction of the “classical” pathway of RCT since fecal cholesterol loss was enhanced without stimulation of hepatobiliary cholesterol excretion. Activation of PPARδ may stimulate direct excretion of plasma-derived cholesterol via the intestine, a mechanism that has been described by Kruit et al., as suggested by the unexpectedly rapid fecal excretion of i.v. administered [3H]-labeled neutral sterols in GW610742-treated mice (Figure 8B). In addition, enhanced fecal neutral sterol loss as a consequence of impaired intestinal cholesterol absorption upon PPARδ activation, which in effect increases RCT, can be considered a beneficial action. Indeed, studies have shown a 20% reduction of LDL levels in hypercholesterolaemic humans and prevention of atherosclerosis development in Apoe−/− mice upon inhibition of cholesterol absorption by ezetimibe. Our results suggest that reduction of cholesterol absorption upon treatment with GW610742 is, at least in part, mediated by reduced intestinal expression of Npc1l1, a proposed target of ezetimibe. Interestingly, ezetimibe was also shown to increase plasma HDL-cholesterol in mice and in humans by a so far unidentified mechanism of action. Thus, PPARδ is a promising target for development of novel drugs aimed at prevention of atherosclerosis.

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