Novel insights in cholesterol excretion
van der Velde, A.E.

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Pparδ activation leads to increased transintestinal cholesterol efflux

A.E. van der Velde, C.L.J. Vrins, K. van den Oever, R.P.J. Oude Elferink, F. Kuipers, A.K. Groen

In preparation
ABSTRACT

Background & aims: Peroxisome Proliferator-Activated Receptors (PPARs) are involved in the regulation of energy homeostasis and lipid metabolism. PPARδ activation has been shown to increase fecal neutral sterol secretion. This phenomenon cannot be explained by an increase in hepatobiliary cholesterol secretion, nor, sufficiently, by reduction of cholesterol absorption. Therefore we hypothesize that PPARδ activation leads to stimulation of transintestinal cholesterol efflux (TICE). Methods: to establish whether activation of PPARδ leads to an increased rate of TICE, intestine perfusions were performed in GW610742 treated FVB mice. To correct for a possible effect of PPARδ activation on cholesterol absorption, ezetimibe and ezetimibe/GW610742 treated mice were also evaluated. Results: PPARδ agonist treatment had little effect on plasma lipid levels but stimulated both fecal neutral sterol excretion and TICE. GW610742 decreased Npc1l1 expression but had no effect on the cholesterol exporters Abcg5/Abcg8. Interestingly, expression of Rab9, a protein known to be involved in intracellular cholesterol trafficking increased. Treatment of the mice with ezetimibe alone had no effect on TICE, but in mice that received both ezetimibe and GW610742, ezetimibe inhibited the effect of GW610742 on TICE. Conclusions: Activation of PPARδ, by GW610742 treatment stimulates cholesterol excretion in mice. The effect seems primarily due to a twofold increase in TICE, suggesting that this pathway provides an interesting target for development of drugs aiming at the prevention of atherosclerosis.

INTRODUCTION

The Peroxisome Proliferator-Activated Receptor (PPAR) family belongs to the superfamily of nuclear receptors. The three known isoforms of PPARs are PPARα, PPARβ/δ and PPARγ (1). PPARα influences lipid metabolism (2-5) and is highly expressed in heart, liver, kidney, intestine, and brown fat, tissues that demonstrate high rates of fatty acid β oxidation (6). PPARγ is expressed mainly in adipose tissue and affects energy metabolism (7-9) and in particular the isomer PPARy2, plays an important role in adipose tissue (10). PPARδ, however, is ubiquitously expressed. High levels of PPARδ transcripts are detected in the brain, kidney, small intestine, and Sertoli cells (11). The role of PPARδ remained elusive for a long time. This changed with the generation of Pparδ-/- mice. Two independent groups have created Pparδ-/- mouse lines (12;13). Pparδ-/- mice were smaller from
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the fetal stage to the postnatal period, and showed an increased prenatal death rate due to placental defects. Brain defects were observed in Pparδ-/- mice in the form of altered myelination of the corpus callosum. Adipose stores were smaller than normal and enhanced hyperplasia of the epidermis has been detected in these mice. The studies with Pparδ-/- mice suggest that PPARδ is involved in embryonal development, myelination of the corpus callosum, epidermal cell proliferation, and lipid metabolism.

Shortly after the publication of the Pparδ-/- mice studies, it turned out that PPARδ plays a prominent role in fatty acid oxidation, energy dissipation and mitochondrial respiration (14; 15). Adipose tissue specific overexpression of PPARδ in mice protected these mice against high fat induced obesity. Even db/db mice did not develop obesity after activation of PPARδ, by agonist GW501516 (16). Overexpression of PPARδ in skeletal muscle of mice led to a remarkable increase in energy endurance capacity (17). PPARδ activation of rat myotubes caused a prominent upregulation of mRNA expression levels of pyruvate dehydrogenase kinase 4 (Pdk4). Treatment with a PPARδ agonist also improved insulin resistance induced by a high-fat diet (15). So, apparently, PPARδ couples fatty acid metabolism with glucose metabolism. Takahashi et al. even suggested that the physiological role of PPARδ may be a direct switch from glucose metabolism to fatty acid metabolism (18).

Besides the roles PPARδ fulfills in fatty acid and glucose homeostasis, PPARδ activation is also known to affect reverse cholesterol transport (RCT). PPARδ activation led in some cases to increased levels of HDL cholesterol and enhanced apolipoprotein A-I specific cholesterol efflux (19-21). Van der Veen et al. (21) showed that PPARδ activation resulted in increased fecal neutral sterol output, whereas hepatobiliary cholesterol secretion was unaltered. PPARδ activation in wild-type DBA1 mice led to reduced cholesterol absorption. Although cholesterol absorption was reduced, it could not fully explain the increase in fecal neutral sterol secretion. Recently, our group showed that direct transintestinal cholesterol efflux (TICE) is a major pathway for the excretion of cholesterol, at least in mice (22). Since, PPARδ activation led to an increase in fecal neutral sterol secretion, that could not be accounted for by an increase in hepatobiliary cholesterol secretion, nor solely by reduction of cholesterol absorption, we hypothesize that PPARδ activation leads to stimulation of TICE.
MATERIALS AND METHODS

Animals and Diet

Male FVB mice (2 - 4 months) were purchased from Harlan. Mice, n=7 for each group, received a reference diet (20 % casein, no 4068.02, Arie Blok BV, The Netherlands), a PPARδ agonist diet (reference diet supplemented with 0.017 % (w/w) GW610742), an ezetimibe diet (reference diet supplemented with Ezetrol, Schering-Plough, Utrecht, the Netherlands; 10 mg/kg.day), or a reference diet supplemented with ezetimibe (10 mg/kg.day) and PPARδ agonist (GW610742; 0.017 % (w/w)) for 2 weeks. GW610742 was a generous gift from GlaxoSmithKline (Les Ulis, France). The specificity of GW610742 has been described previously (21). The ezetimibe, GW610742 and ezetimibe + GW610742 diets were made by mixing powdered ezetimibe and/or GW610742 with powdered reference diet. Reference diet contained 0.006 % cholesterol. Food and water were supplied ad libitum. Mice were maintained on a 24 h light-dark cycle and weighed each morning. All experiments were performed with the approval of the local Ethical Committee for Animal Experiments.

Cholesterol intake and output measurements

Daily food intake and fecal neutral sterol excretion of all mice were measured for a period of 4 days. Mice were housed in normal mouse cages, 1 to 5 mice per cage, to mimic their natural situation as much as possible. On day 1, the mice and diet were weighed. On day 3, the mice and remaining diet were weighed and feces were collected. Fecal neutral sterols were determined as described below.

Intestine perfusion procedures

Mice were anaesthetized intraperitoneal with 0.1 ml FFD {Hypnorm (fentanyl/fluanisone; 1 ml/kg) and diazepam (10 mg/kg)}/ 5 g body weight and placed on a heat pad to maintain body temperature. The bile duct was cannulated via the gallbladder to divert biliary cholesterol. Bile flow was determined gravimetrically assuming a density of 1 g/ml. The first 15 min fraction was used to measure biliary cholesterol secretion. Proximal small intestines (first 10 cm) were perfused, because under normal conditions the highest TICE rate is observed in the proximal part of the small intestine (22). Perfusions were performed as previously described (22). Perfusate fractions were stored at -20°C until further analysis. At the end of the perfusion period, blood was collected by cardiac
puncture and serum was obtained by means of centrifugation (3000 rpm, 5 min). Serum was stored at -80°C. The perfused intestinal segment was cut into three equal pieces and those pieces were cut in two equal parts. The intestinal segments were snap-frozen in liquid nitrogen. Intestinal segments and serum were stored at -80°C before further analysis.

**Perfusion fluid composition**
Perfusions were carried out with a modified Krebs solution (119.95 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4·7H2O, 15 mM HEPES, 1.3 mM CaCl2·2H2O and 10 mM L-glutamine; final pH 7.4) supplemented with 10 mM taurocholate (TC) : 2 mM phosphatidylcholine (PC). TC/PC mixtures were made as follows: taurocholate (Sigma) dissolved in methanol and egg yolk L-α-phosphatidylcholine (Sigma) dissolved in chloroform were mixed and solvents were evaporated under a mild stream of nitrogen at 45°C. After evaporation, films were lyophilized overnight. Lyophilized samples were stored under nitrogen gas at -20°C until the day of the intestine perfusions. Before the start of the intestine perfusions, the micelles were dissolved in perfusion buffer (room temperature).

**Determination of mRNA Levels**
Total RNA was isolated from perfused intestinal segments using the Trizol reagent according to the manufacturer’s protocol (Invitrogen). Purified RNA was treated with RQ1 RNase-free DNase (1 units/2 μg of total RNA, Promega) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) according to the protocols supplied by the manufacturers. Gene expression analysis was performed by use of SYBR green. Quantitative gene expression analysis was performed on a Biorad MyIQ. PCR primers were designed on the basis of Primer Express 1.7 software with the manufacturer’s default settings (Applied Biosystems) or found by consulting the PrimerBank website (http://pga.mgh.harvard.edu/primerbank) and validated for identical efficiencies (see table 1 for primer sequences). Hypoxanthine-guanine phosphoribosyl transferase (HPRT), cyclophilin, and 36B4 were used as standard housekeeping genes.
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Table 1. Realtime PCR primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Size</th>
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<tr>
<td>36B4</td>
<td>NM_007475</td>
<td>GGACCCGAGAAGACCTCCTT</td>
<td>GCACATCCTCAGAATTTCAATGG</td>
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<td>Abca1</td>
<td>NM_013454</td>
<td>GTTGTGAGATGTATACATAGTG</td>
<td>TTCCCCGAAAAGCAGAGTC</td>
<td>96</td>
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<tr>
<td>Abcg1</td>
<td>AF323659</td>
<td>CCTTCTCAGATCATCAGGCC</td>
<td>CCGATCCCAATGTGCGA</td>
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</tr>
<tr>
<td>Abcg5</td>
<td>NM_031884</td>
<td>TGGCCCTGGTCAGCATCT</td>
<td>ATTTTTAAGGAATGGGCACTCTT</td>
<td>81</td>
</tr>
<tr>
<td>Abcg8</td>
<td>NM_026180</td>
<td>CGCTCGTCAGATTTCCAATGA</td>
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<tr>
<td>Cyclophilin</td>
<td>M60456</td>
<td>TCGAGGCGAATAGAAGGT</td>
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<tr>
<td>Hmg-CoA red</td>
<td>M62766</td>
<td>TCTGCGAGTCAGTGGGATTT</td>
<td>CCTCGCTCTGTGACCAATTT</td>
<td>69</td>
</tr>
<tr>
<td>HPRT</td>
<td>J00423</td>
<td>TTGTCCTGAGTCTGAAAGGA</td>
<td>AGCAGTCAGCAAGAAACTTATAG</td>
<td>91</td>
</tr>
<tr>
<td>Limp-2</td>
<td>NM_007644</td>
<td>AGAAGGCGGTAGACCCAGGC</td>
<td>GTAGGGGAGTCTGACTTGGA</td>
<td>159</td>
</tr>
<tr>
<td>Npc1fl</td>
<td>AY437866</td>
<td>GAGAAGCCAAGATGCTACTACTG</td>
<td>CCCCGAAGGGTGCTGGTCA</td>
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</tr>
<tr>
<td>Pdk4</td>
<td>NM_013743</td>
<td>GGGGTGGACCTGGTGAATTT</td>
<td>GCCGCTTGCTCTAGTGCA</td>
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<tr>
<td>Ppara</td>
<td>NM_011144</td>
<td>TGAACAAAGCAGGGGATG</td>
<td>TCAACCTGGGTCGCAGTCA</td>
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<td>Pparγ</td>
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<td>CATCAGTTTGGAAGATGGCAAG</td>
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<td>Rab8</td>
<td>NM_023126</td>
<td>GTAGGAGGACCCCTGCTGTCCTG</td>
<td>CGTGAATTGGCTGGAAACCAG</td>
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<tr>
<td>Rab9</td>
<td>NM_019773</td>
<td>TACCGAGATGTGAAAGAGCCT</td>
<td>AAAGTAGGATAGTCGCGCCGTTG</td>
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<td>Scd-1</td>
<td>NM_009127</td>
<td>TACTACAAAGCCGGCCTCCC</td>
<td>CAGCAGTACCAGGGCACCA</td>
<td>65</td>
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</table>

**FPLC**

Lipoproteins in serum were separated by FPLC (Pharmacia fine chemicals) using a superose 6 column. Lipoproteins were separated by injecting 110 µl pooled serum supplemented with PBS to a total injecting volume of 200 µl. Lipoproteins were separated using a flowrate of 0.5 ml/min. Serum was separated into 40 fractions.

**Analytical procedures**

Lipids of perfusate and bile were extracted using the Bligh & Dyer method (23). Biliary, perfusate and hepatic cholesterol were measured by a fluorescent method as described previously (24). Total cholesterol concentrations of complete serum samples, as well as serum fractions separated by FPLC, were determined using the total cholesterol RTU kit from (Marcy-l’Etoile, France). Triglycerides in serum and liver homogenates were determined using the Triglycerides Ecoline S+ kit (Diagnostic Systems GmbH, Holzheim, Germany). Biliary and hepatic phospholipids were measured enzymatically by a method described previously (25). Bile acids in bile were measured by a method described earlier by Turley and Dietschy (26). For fecal neutral sterol analysis, 1-day fecal samples were collected,
lyophilized and grinded. Samples were prepared for fecal neutral sterol analysis by gas chromatography as described previously (27).

RESULTS

In order to establish the effect of PPARδ activation on cholesterol metabolism, 4 groups of FVB mice received different dietary treatments. The mice received: a semi purified reference diet; this diet supplemented with ezetimibe; or PPARδ agonist (GW610742, 0.017 % (w/w)) diet or a combined ezetimibe and GW610742 diet. Mice were given these diets for 2 weeks. After the first week, food intake and fecal neutral sterol output were measured and after the second week the proximal small intestine was perfused.

TICE was measured in the proximal small intestine, using a modified Krebs-Ringer perfusion buffer supplemented with mixed micelles composed of 10 mM TC and 2 mM PC. The results are depicted in figure 1. PPARδ activation by GW610742 treatment stimulated TICE (2.39 ± 0.56 versus 1.37 ± 0.53 nmol cholesterol/min.100 g body wt), with ezetimibe, the stimulatory effect of GW610742 on TICE was not significant (1.64 ± 0.80 versus 1.12 ± 0.34 nmol cholesterol/min.100 g body wt). Interestingly, ezetimibe treatment alone did not affect TICE.

Figure 2A shows that PPARδ treatment, not only led to stimulation of TICE, but also, in a similar fashion, affected fecal neutral sterol excretion. Food intake was monitored during the same period as fecal neutral sterol excretion was measured. Surprisingly, the GW610742 treated mice ate 1.7 fold more than control mice (fig. 2B). Interestingly, this effect was abolished in the presence of ezetimibe.

GW610742 treatment caused a 50 to 60 % increase in liver weight as compared to the liver weights of the corresponding control treated mice (table 2). Yet, cholesterol, phospholipids and triglyceride levels did not change when expressed per mg liver protein. The increase in liver weight is presumably caused by proliferation of peroxisomes. Macroscopic analysis of the livers from treated animals indicated that the livers were not fatty (data not shown).

GW610742 treatment led to a significant increase in bile flow and bile salt secretion rate but had no effect on biliary cholesterol secretion or phospholipid secretion (table 3). Ezetimibe abolished the effect on bile salt secretion.
Figure 1. Effect of GW610742 treatment on transintestinal cholesterol efflux. FVB mice received reference diet, a GW610742 diet (0.017 % (w/w)), an ezetimibe diet (10 mg/kg.day) or an ezetimibe + GW610742 diet for 2 weeks. Proximal small intestinal perfusions were performed, using TC/PC (10:2 mM) as cholesterol acceptor. Values are expressed as mean ± SD. * Indicates a significant effect of GW610742 in comparison to control treated animals.

Table 1

<table>
<thead>
<tr>
<th>Diet</th>
<th>FIEE (mmol/min.100 g body wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref. Diet</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>GW610742</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>Ezetimibe</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Ezetimibe + GW610742</td>
<td>1.5 ± 0.4</td>
</tr>
</tbody>
</table>

Figure 2. Effect of GW610742 on fecal neutral sterol secretion and food intake. FVB mice (n=7 per group) were fed for 1 week a reference diet, a GW610742 diet (0.017 % (w/w)), an ezetimibe diet (10 mg/kg.day) or an ezetimibe + GW610742 diet. (A) Fecal neutral sterol secretion was measured during a 3 day period. (B) Food intake was measured in the same period as fecal neutral sterol secretion was evaluated. Values are expressed as mean ± SD. * Indicates a significant effect of GW610742 or ezetimibe in comparison to control treated animals.
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### Table 2. Liver parameters of the mice.

<table>
<thead>
<tr>
<th></th>
<th>Ref. Diet</th>
<th>GW610742</th>
<th>Ezetimibe</th>
<th>Ezetimibe + GW610742</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight (% of body wt)</td>
<td>4.5 ± 0.2</td>
<td>6.7 ± 0.4$^a$</td>
<td>4.3 ± 0.4</td>
<td>6.7 ± 0.4$^a$</td>
</tr>
<tr>
<td>Hepatic triglycerides (nmol/mg liver)</td>
<td>9.4 ± 6.2</td>
<td>5.2 ± 2.7</td>
<td>13.6 ± 5.1</td>
<td>8.0 ± 4.4</td>
</tr>
<tr>
<td>Hepatic cholesterol (nmol/mg liver)</td>
<td>7.5 ± 0.7</td>
<td>6.1 ± 2.2</td>
<td>7.8 ± 1.3</td>
<td>7.1 ± 1.9</td>
</tr>
<tr>
<td>Hepatic phospholipids (nmol/mg liver)</td>
<td>24.5 ± 3.0</td>
<td>24.4 ± 8.6</td>
<td>23.7 ± 2.3</td>
<td>29.3 ± 5.5</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD.

$^a$Indicates a significant difference between the GW610742 treated animals and their corresponding controls.

### Table 3. Biliary parameters of the mice.

<table>
<thead>
<tr>
<th></th>
<th>Ref. Diet</th>
<th>GW610742</th>
<th>Ezetimibe</th>
<th>Ezetimibe + GW610742</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile flow (μl/min. g liver)</td>
<td>0.9 ± 0.6</td>
<td>2.7 ± 0.6$^a$</td>
<td>1.2 ± 0.5</td>
<td>2.6 ± 0.8$^a$</td>
</tr>
<tr>
<td>Bile salts (nmol/min.100 g body wt)</td>
<td>69 ± 28</td>
<td>163 ± 79$^a$</td>
<td>191 ± 74</td>
<td>132 ± 79</td>
</tr>
<tr>
<td>Cholesterol (nmol/min.100 g body wt)</td>
<td>0.9 ± 0.3</td>
<td>0.8 ± 0.4</td>
<td>0.9 ± 0.3</td>
<td>0.5 ± 0.2$^a$</td>
</tr>
<tr>
<td>Phospholipids (nmol/min.100 g body wt)</td>
<td>8.3 ± 1.3</td>
<td>8.7 ± 4.4</td>
<td>12.2 ± 3.9</td>
<td>7.7 ± 4.0</td>
</tr>
<tr>
<td>Cholesterol / phospholipid ratio</td>
<td>0.11 ± 0.02</td>
<td>0.10 ± 0.04</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.02</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD.

$^a$Indicates a significant difference between the GW610742 treated animals and their corresponding controls.

Total serum cholesterol and triglyceride levels did not change upon GW610742 treatment (fig. 3A and B). To evaluate whether GW610742 treatment resulted in changes in cholesterol concentrations in the different lipoproteins, FPLC was performed (fig. 3C). GW610742 induced a shift of cholesterol from HDL towards LDL.
Figure 3. Serum analysis of GW610742 treated mice. FVB mice received reference diet, a GW610742 diet (0.017 % (w/w)), an ezetimibe diet (10 mg/kg day) or an ezetimibe + GW610742 diet for 2 weeks. Blood was collected and serum was obtained by centrifugation. (A) Total serum cholesterol levels and (B) serum triglyceride levels were measured. (C) Lipoproteins were separated by FPLC and total cholesterol concentration in the FPLC fractions was measured.
Intestinal gene expression analysis was performed to establish the role of PPARδ activation on the expression of genes which are involved in cholesterol transport as well as known PPARδ target genes (fig. 4). The classic PPARδ target Pdk4 was induced about tenfold but stearoyl-CoA desaturase 1 (Scd1) even more; about 20fold. Ezetimibe decreased the effect of GW610742 on both genes. The expression of the cholesterol efflux promoter Abca1 increased but Npc1l1 expression was reduced upon PPARδ activation, as has been described previously (21). Sr-b1 expression was not altered upon GW610742 treatment, but ezetimibe decreased Sr-b1 expression significantly. Abcg5, Abcg8 and also Hmg-CoA reductase were not affected by GW610742, nor did ezetimibe effect the expression of these genes. Since the Npc1l1 mediated cholesterol transport involves intracellular vesicle trafficking (28) we also determined expression of known genes involved in this pathway (29; 30). Interestingly, expression of Rab9 and Limp-2 increased upon GW610742 treatment. In contrast, expression of Rab8 was not changed. Pparα expression increased but Pparγ was not effected by GW610742 treatment. Ezetimibe decreased expression of Pparδ.
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C. **Abca1**

D. **NPC1L1**

E. **Sr-b1**

F. **Abcg5**

G. **Abcg8**

H. **Hmg-CoA red**

*Ref. Diet GW610742 Ezetimibe Ezetimibe + GW610742*
Figure 4. Intestinal gene expression analysis of GW610742 treated mice. FVB mice received reference diet, a GW610742 diet (0.017 % (w/w)), an ezetimibe diet (10 mg/kg.day) or an ezetimibe + GW610742 diet for 2 weeks. Proximal small intestinal perfusions were performed, using TC/PC (10:2 mM) as cholesterol acceptor. At the end of the 2 hour perfusion period, intestines were collected cut into 3 equal segments. The second segment was analysed. Intestinal Pdk4, Scd1, Abca1, Npc1l1, Sr-b1, Abcg5, Abcg8, Hmg-CoA red, Rab9, Limp-2, Ppara and Ppary expression levels were measured. As control genes HPRT, cyclophilin and 36B4 were used.

DISCUSSION
In this study we show for the first time that PPARδ activation by GW610742 not only leads to increased fecal neutral sterol output in mice (21), but also stimulates TICE. In an earlier study van der Veen et al. (21) demonstrated that part of the increased neutral sterol excretion induced by GW610742 could be explained by decreased cholesterol absorption due to an inhibition of Npc111. To abrogate the influence of Npc111 we carried out the experiments with and without ezetimibe, the
established inhibitor of \( \text{Npc1l1} \). Ezetimibe treatment had a substantial effect on neutral sterol excretion without affecting biliary cholesterol secretion. Part of this effect is caused by the inhibition of cholesterol absorption. Yet, to compensate for the loss of cholesterol from the body one would expect to see an increase in biliary cholesterol secretion which was not observed. Similar results have been reported by Yu et al. (31). Despite, the strong increase of fecal neutral sterol excretion ezetimibe treatment did not affect TICE. On the one hand this result shows that \( \text{Npc1l1} \) does not play an (inhibiting) role in TICE. On the other hand, TICE is not involved in the ezetimibe induced increase in neutral fecal sterol excretion and another pathway must be responsible.

Food intake of the mice was measured in the same period that feces was collected for fecal neutral sterol analyses. GW610742 treated animals ate significantly more than control treated animals, whereas they do not differ in body weight when compared to their corresponding controls (not shown). An increased food consumption has also been observed in LDLR-/- mice treated with PPAR\( \delta \) agonist GW610742 (32). In this study, however, the GW610742 treated LDLR-/- mice showed a significant increase in body weight as compared to LDLR-/- mice that did not have PPAR\( \delta \) activation. Muscle specific PPAR\( \delta \) transgenic animals had enhanced physical activity, whereas they did not differ in body weight as compared to wild-type mice (17). An effect of PPAR\( \delta \) activation on energy expenditure might explain why GW610742 treated mice had an increased food intake without a concomitant increase in body weight. Surprisingly, the stimulating effect of PPAR\( \delta \) activation by GW610742 treatment on food consumption was not observed when ezetimibe was given at the same time. We speculate that ezetimibe inhibits the uptake of GW610742 or promotes its metabolism.

Van der Veen et al. (21) suggested that PPAR\( \delta \) activation might lead to increased TICE, since the increased fecal neutral sterol output could not be completely explained by the reduction in cholesterol absorption. To test this hypothesis, proximal small intestines of the different treated animals were perfused. Since both reduced cholesterol absorption, as well as, reduced Npc1l1 expression were observed in GW610742 treated animals (21), ezetimibe and ezetimibe + GW610742 treated mice were included, to exclude potential re-uptake of intestinally secreted cholesterol (33). Interestingly, there was no difference in TICE between the control and ezetimibe treated mice, suggesting that uptake of intestinally secreted cholesterol is very low under the used conditions in the
Effect PPAR\(\delta\) activation on TICE

intestinal perfusion set up. GW610742 treatment led to an increased TICE rate (fig. 2), however when ezetimibe was given at the same time, the effect decreased and no longer reached statistical significance again pointing to interference of ezetimibe with the efficacy of the agonist.

PPAR\(\delta\) activation led to a higher bile flow and a higher bile salt secretion rate, both with and without ezetimibe treatment (table 3). This has not been observed previously in DBA1 wild-type mice (21). Cholesterol and other biliary parameters were unaffected upon GW610742 treatment as has been reported previously (21).

Total serum cholesterol and triglyceride levels were unaffected upon PPAR\(\delta\) activation (fig. 3A) as has been observed previously in 60mg GW610742/kg.day treated LDLR\(-/-\) mice (32). Subsequent analysis of the lipoprotein profile revealed a shift of cholesterol from HDL to LDL in both GW610742 treated groups. Whether this change in serum cholesterol distribution might affect TICE needs to be investigated.

To get more insight in the genes that could be involved in the upregulation of TICE, intestinal gene expression analysis was performed (fig. 4). Ezetimibe treatment decreased the effect of GW610742 on its known target gene Pdk4, suggesting that indeed the compound influences uptake of the agonist. Confirming earlier results the expression of Npc1l1 decreased. Conversely, expression of Abca1 increased whereas Sr-b1 was not affected. Nor was expression of the cholesterol effluxing heterodimer Abcg5/g8 influenced. This came not as a surprise since we have shown previously that these genes do not control the rate of TICE under the conditions used in this study. To investigate why PPAR\(\delta\) activation led to increased TICE, less known cholesterol metabolism effectors were investigated, such as intracellular cholesterol trafficking key players like Rab8, Rab9 and Limp-2. Rab8 is involved in the regulatory machinery that leads to ABCA1-dependent removal of cholesterol from endocytic circuits (29). Rab9 plays a role in cholesterol trafficking from late endosomes to the trans golgi network (34). Intestinal Rab8 expression was unaltered upon PPAR\(\delta\) activation, Rab9 and Limp-2 expression, however, significantly increased upon GW610742 treatment. The involvement of the proteins encoded by these genes in TICE is currently under investigation.

PPAR\(\delta\) activation has been shown to accelerate RCT, not only in mice (21) but also in primates (19) and man (20). PPAR\(\delta\) activation leads to increased cholesterol efflux from macrophages (19-21), without affecting biliary cholesterol
secretion rates (21). Here, we show, in mice, that PPARδ activation stimulates TICE. The development of drugs that stimulate TICE by PPARδ activation might be a promising target for preventing atherosclerosis.
REFERENCES


