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

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Abstract **Peroxisome proliferator-activated receptors (PPARs) control the transcription of genes involved in lipid metabolism. Activation of PPARδ may have antiatherogenic effects through the increase of plasma HDL, theoretically promoting reverse cholesterol transport from peripheral tissues toward the liver for removal via bile and feces. Effects of PPARδ activation by GW610742 were evaluated in wild-type and Abca1-deficient (*Abca1*$^{-/-}$) mice that lack HDL. Treatment with GW610742 resulted in an ~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 *3-hydroxy-3-methylglutaryl-coenzyme A 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. PPARδ activation is associated with increased 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.**—van der Veen, J. N., J. K. Kruit, R. Havinga, J. F. W. Baller, G. Chimini, S. Lestavel, B. Staels, P. H. E. Groot, A. K. Groen, and F. Kuipers. **Reduced cholesterol absorption upon PPARδ activation coincides with decreased intestinal expression of NPC1L1. *J. Lipid Res.* 2005.** 46: **526–534.**

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Plasma levels of HDL-cholesterol are inversely related to the development of atherosclerosis (1). 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.

ABCA1 is considered to be essential in RCT (2). ABCA1 is ubiquitously expressed and probably involved in the formation of preβ-HDL particles and the efflux of cholesterol from peripheral tissues toward HDL (3). HDL is considered a major source for bile-destined cholesterol (4). However, we recently demonstrated that, despite the absence of HDL, hepatobiliary cholesterol flux and fecal sterol excretion are not affected in Abca1-deficient (*Abca1*$^{-/-}$) mice (5, 6). 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 (7, 8).

Several genes involved in the control of cholesterol me-

Abbreviations: *Abca1*$^{-/-}$, Abca1-deficient; FPLC, fast-protein liquid chromatography; Hmgr, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; Mdr2, multidrug resistance P-glycoprotein 2; NPC1L1, Niemann-Pick C1-like 1; Pdk4, pyruvate dehydrogenase kinase isoenzyme 4; PPAR, peroxisome proliferator-activated receptor; RCT, reverse cholesterol transport; Sr-b1, scavenger receptor B1.

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tabolism are transcriptionally regulated by nuclear receptors. Peroxisome proliferator-activated receptors (PPARs) constitute a subgroup of the nuclear receptor superfamily, designated PPARα (NR1C1), PPARδ/β (NR1C2), and PPARγ (NR1C3), all of which serve functions in lipid homeostasis and energy metabolism (9). 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 antiatherogenic effects (10). Whether or not PPARδ activation, like PPARα activation (11, 12), is associated with altered bile formation and fecal sterol loss is not known.

This study shows that PPARδ activation in mice increased 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 the downregulation of intestinal gene expression of the very recently identified cholesterol absorption protein Niemann-Pick C1-like 1 (NPC1L1) (13).

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'Arbesle, 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 the use of experimental animals.

PPARδ agonist

GW610742 (GlaxoSmithKline Pharmaceuticals, Stevenage, UK) (**Fig. 1**) is a high-affinity ligand for PPARδ. The specificity of GW610742, as evaluated by ligand binding studies, revealed EC_{50} values of 28 nM for murine PPARδ versus 8,900 nM and >10,000 nM for murine PPARα and PPARγ, respectively. For human PPARδ, PPARα, and PPARγ, the EC_{50} values are 1, 1,200, and 4,100 nM, respectively (14) (L. Patel, personal communication). The specificity of GW1516 has been described previously (10, 14).

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 g, 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 7–8, 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 min 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.

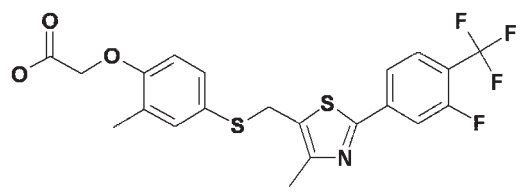

Fig. 1. Chemical structure of the peroxisome proliferator-activated receptor δ (PPARδ)-specific agonist GW610742.

Analytical methods

Livers were homogenized and hepatic and biliary lipids were extracted (15). Hepatic, biliary, and plasma concentrations of cholesterol, triglycerides, and phospholipids were determined as previously described (6). 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 real-time PCR (Taqman)

RNA isolation, cDNA synthesis, and real-time quantitative PCR were performed as described by Plösch et al. (6). Primer and probe sequences for *Abca1*, *Abcg5*, *Abcg8*, *Acat2*, *3-hydroxy-3-methylglutaryl-coenzyme A reductase* (*Hmgr*), *liver X receptor α*, *multidrug resistance P-glycoprotein 2* (*Mdr2*), and *scavenger receptor B1* (*Sr-b1*) (6) as well as for *β-actin* (11) have been published. Furthermore, the following primers/probes were used. For *Pparδ*, sense, 5′-AGA 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 *pyruvate dehydrogenase kinase isoenzyme 4* (*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 CTT CA-3′; antisense, 5′-CCC GGG AAG TTG GTC ATG-3′; and probe, 5′-ACT CCA GCA AAC ACC GCA CTG CC-3′ (accession number, AY437866). For *36b4*, sense, 5′-GCT TCA TTG TGG GAG CAG ACA-3′; antisense, 5′-CAT GGT GTT CTT GCC CAT CAG-3′; and probe, 5′-TCC AAG CAG ATG CAG CAG ATC CGC-3′ (accession number, NM_007475).

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. (16). 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 (17). DBA/1 wild-type mice (n = 5 per group) received a diet with or without 0.017% (w/w) GW610742. After 6 days, mice received an intravenous injection of 1.1 μCi of [^{3}H]cholesterol dissolved in intralipid and an oral dose of 1.0 μCi of [^{14}C]cholesterol dissolved in medium-chain triglyceride oil. At 24, 48, and 72 h after administration, blood samples were taken by retro-orbital puncture and feces was collected. At day 10, mice were anesthetized and bile was collected for 30 min. ^{14}C and ^{3}H activity in plasma, bile, and feces was measured by liquid scintillation counting. Blood samples obtained 72 h 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 polyethylene membrane inserts (23.1

TABLE 1. Animal characteristics and plasma and hepatic lipid concentrations in untreated and GW610742-treated DBA/1 wild-type and *Abca1*$^{-/-}$ mice

Variable	Untreated		GW610742 Treated	
	Wild Type	*Abca1*$^{-/-}$	Wild Type	*Abca1*$^{-/-}$
Body weight (g)	20.9 ± 0.8	21.4 ± 0.9	20.0 ± 1.5	21.4 ± 0.9
Liver weight (% of body weight)	4.2 ± 0.3	4.8 ± 0.5	5.7 ± 0.5[a]	5.9 ± 0.4[a]
ALAT (U/l)	94 ± 43	225 ± 181[b]	76 ± 23	146 ± 159
LDH (U/l)	653 ± 241	841 ± 335	603 ± 312	653 ± 404
Plasma cholesterol (mM)	1.9 ± 0.3	0.5 ± 0.1[b]	2.5 ± 0.3[a]	0.5 ± 0.2[b]
Plasma triglycerides (mM)	0.8 ± 0.2	1.2 ± 0.6	0.7 ± 0.3	1.1 ± 0.4
Liver cholesterol (nmol/mg liver)	3.9 ± 0.4	4.4 ± 0.8	3.7 ± 0.5	4.2 ± 0.3
Liver phospholipids (nmol/mg liver)	20.6 ± 3.5	23.0 ± 6.8	23.4 ± 1.7	22.3 ± 3.7
Liver triglycerides (nmol/mg liver)	9.7 ± 3.1	9.3 ± 1.1	8.8 ± 2.6	13.1 ± 3.6[a]

Abca1$^{-/-}$, Abca1-deficient; ALAT, aspartate aminotransferase; LDH, lactate dehydrogenase. Values are expressed as means ± SD (n = 6 in all groups).

[a] Significant difference between GW610742-treated and untreated mice with the same genotype.

[b] Significant difference between wild-type and *Abca1*$^{-/-}$ mice with the same treatment.

mm, 3 μm pore size) were obtained from Becton Dickinson (Le Pont de Claix, France). Caco-2 cells were routinely grown in plastic flasks (ATGC Biotechnologie, Marne la Vallée, France) under a humidified atmosphere containing 10% CO_2 at 37°C in Dulbecco's modified essential medium containing 25 mM glucose and Glutamax, supplemented with penicillin-streptomycin (100 IU/ml and 100 g/ml, respectively), 1% nonessential amino acids, and 20% heat-inactivated fetal calf serum.

To establish the intestinal barrier model for the assay, Caco-2 cells (between passages 40 and 45) were plated at a density of 0.25×10^6 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 fetal calf serum in the lower compartment and serum-free medium in the upper compartment. Media were changed every day. Three weeks later, cells were activated with ligands for PPARα (Wy14643 at 50 μM), PPARγ (rosiglitazone at 100 nM), or PPARδ (GW1516 at 100 nM) for 24 h in the upper compartment. After incubation, cell layers were briefly rinsed twice with ice-cold PBS (10 mM phosphate buffer, pH 7.5, 2.7 mM KCl, and 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). cDNAs were quantified by real-time PCR on a MX 4000 apparatus (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 $MgCl_2$, the Brilliant Quantitative PCR Core Reagent Kit mix as recommended by the manufacturer (Stratagene), and 0.33× SYBR Green (Sigma-Aldrich, Saint Quentin Fallavier, France). The conditions were 95°C for 10 min followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. NPC1L1 mRNA levels were subsequently normalized to 28S mRNA (sense, 5′-AAA CTC TGG 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 experiment was performed in triplicate.

Immunohistochemistry

Histology of livers and small intestines was examined after hematoxylin/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 Ki67 polyclonal antibody (1:500; Novo Castra, Newcastle, UK).

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. $P < 0.05$ was considered statistically significant.

RESULTS

Animal characteristics

Table 1 shows that body weights of DBA/1 wild-type and *Abca1*$^{-/-}$ mice were similar and not influenced by treatment with GW610742. Liver weights of untreated wild-type and *Abca1*$^{-/-}$ 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

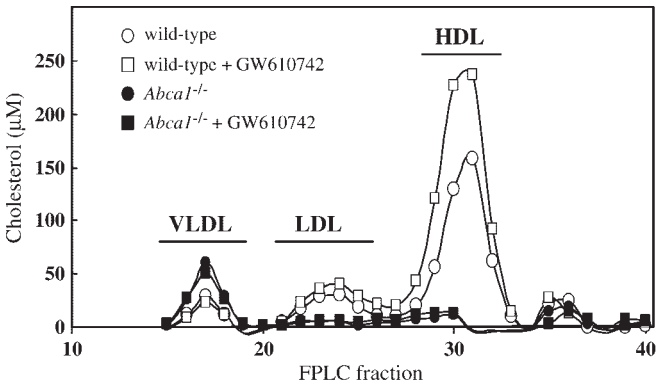

Fig. 2. Fast-protein liquid chromatography (FPLC) separation of plasma lipoproteins of untreated and GW610742-treated DBA/1 wild-type and Abca1-deficient (*Abca1*$^{-/-}$) mice. DBA/1 wild-type mice (open symbols) and *Abca1*$^{-/-}$ mice (closed symbols) were treated with solvent (circles) or with GW610742 (squares) for 8 days (n = 6 per group). Plasma from all individual mice per group was pooled and subjected to gel filtration using Superose 6 columns. Cholesterol content in each fraction was measured.

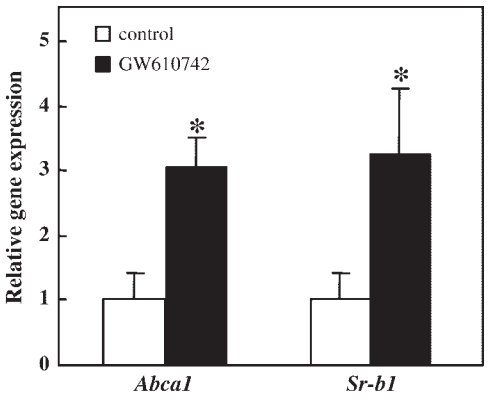

Fig. 3. Gene expression in the peritoneal macrophages of untreated (open bars) and GW610742-treated (closed bars) DBA/1 wild-type mice, measured by real-time PCR. Data are presented as means of three assays performed in duplicate ± SD. Expression values are normalized to *36b4*, and expression in untreated mice was set to 1.00 (n = 3 per group). * Significant differences by Student's *t*-test ($P < 0.05$).

liver sections of treated animals (data not shown). Treatment with GW610742 did not induce liver injury, as indicated by unaffected plasma lactate dehydrogenase (LDH) and aspartate aminotransferase (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*$^{-/-}$ mice remained extremely low. FPLC analysis (**Fig. 2**) confirmed the complete lack of HDL-cholesterol in *Abca1*$^{-/-}$ mice and revealed an ~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 ~3-fold upregulated upon PPARδ activation (**Fig. 3**), demonstrating that GW610742 did induce systemic effects.

Hepatic concentrations of cholesterol, phospholipids, and triglycerides (Table 1) were similar in wild-type and *Abca1*$^{-/-}$ mice and were not affected by PPARδ activation in wild-type mice, whereas hepatic triglycerides were slightly increased upon treatment in *Abca1*$^{-/-}$ mice.

Biliary secretion rates of bile salts, cholesterol, and phospholipids were similar in untreated *Abca1*$^{-/-}$ mice compared with wild-type mice (**Table 2**), in accordance with published data (5). Treatment with GW610742 did not significantly affect biliary secretion rates in wild-type or *Abca1*$^{-/-}$ mice. This is in accordance with the absence of any effect on the hepatic expression of several genes involved in cholesterol metabolism and transport (**Fig. 4**, left panels). Only hepatic *Abcb4* (*Mdr2*) 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 *Pdk4* (19) was measured. Hepatic expression of this gene was ~6-fold upregulated upon PPARδ activation in both strains of mice.

Fecal excretion of neutral sterols is induced upon PPARδ activation

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

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

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, NPC1L1 was identified as a critical component of the intestinal cholesterol absorption machinery (13). Therefore, we measured mRNA levels of *Npc1l1* along the length of the small intestine of untreated and

TABLE 2. Bile flow and biliary secretion rates in untreated and GW610742-treated DBA/1 wild-type and *Abca1*$^{-/-}$ mice

	Untreated		GW610742 Treated	
Variable	Wild Type	*Abca1*$^{-/-}$	Wild Type	*Abca1*$^{-/-}$
Bile flow (μl/min/g liver)	2.4 ± 0.6	2.1 ± 0.7	2.2 ± 0.4	2.0 ± 0.5
Bile salts (nmol/min/100 g body weight)	445 ± 203	483 ± 169	440 ± 165	428 ± 121
Cholesterol (nmol/min/100 g body weight)	4.9 ± 1.4	6.9 ± 3.2	7.5 ± 2.9	7.7 ± 2.2
Phospholipids (nmol/min/100 g body weight)	40.9 ± 8.3	55.0 ± 15.0	64.1 ± 18.6	57.8 ± 18.6
Cholesterol/phospholipid ratio	0.12 ± 0.02	0.12 ± 0.05	0.13 ± 0.04	0.13 ± 0.01

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

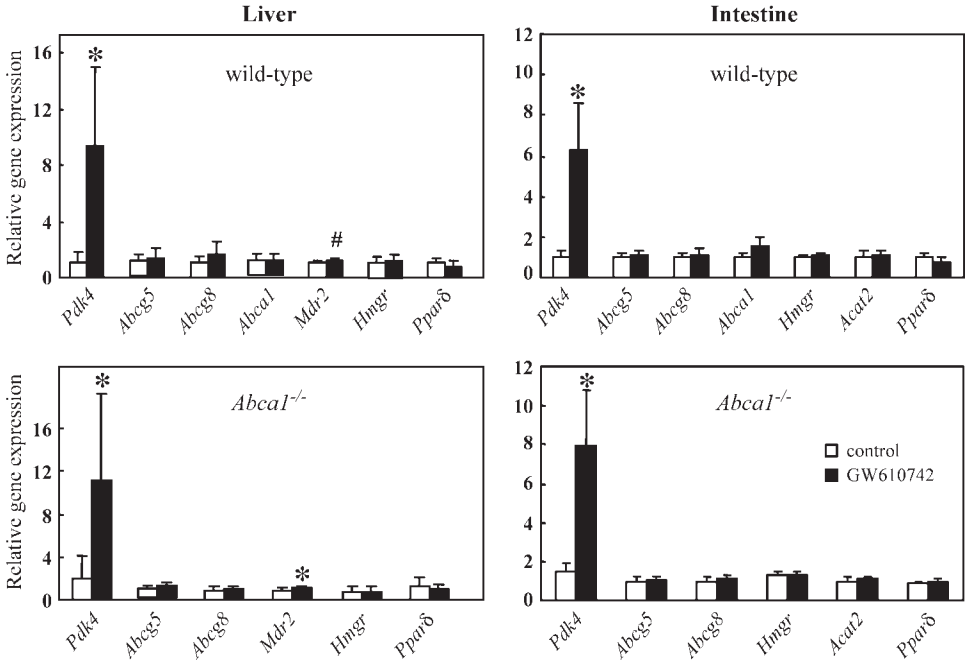

Fig. 4. Gene expression in the livers and intestines of untreated (open bars) and GW610742-treated (closed bars) DBA/1 wild-type and *Abca1*$^{-/-}$ mice, measured by real-time PCR. Data are presented as means of six assays performed in duplicate ± SD. Expression values are normalized to *β-actin*, and expression in untreated wild-type mice was set to 1.00. *,# Significant differences by Student's *t*-test ($P < 0.05$ and $P < 0.01$, respectively). Hmgr, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; Mdr2, multidrug resistance P-glycoprotein 2; Pdk4, pyruvate dehydrogenase kinase isoenzyme 4.

treated mice. Expression of the gene was decreased by 35% in the jejunum upon PPARδ activation and was also lower in ileal sections of treated animals (**Fig. 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 **Fig. 8A**, showing 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]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]cholesterol loss is larger then 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 (20). Conversion of labeled cholesterol into bile salts was not affected by PPARδ activation, as shown in Fig. 8C. Fecal excretion of ^{14}C-labeled bile salts (Fig. 8D) was somewhat lower in the treated mice, probably because of the lower efficiency of cholesterol absorption in these animals.

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

Repression of Npc1l1 expression is specific for PPARδ agonist in Caco2 cells

To assess the specificity of the observed effects on *Npc1l1* expression, we evaluated the consequences of PPARα,

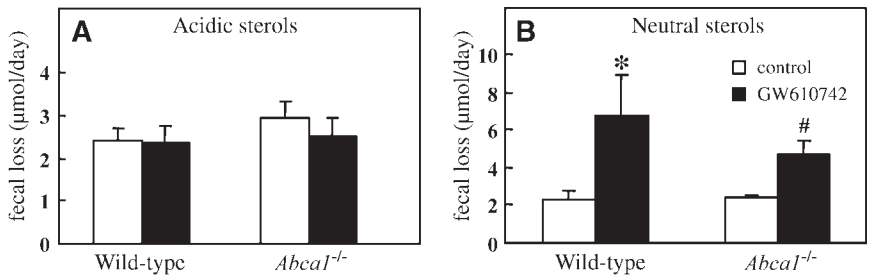

Fig. 5. Fecal loss of acidic sterols (A) and neutral sterols (B) in untreated and GW610742-treated DBA/1 wild-type and *Abca1*$^{-/-}$ mice. Feces of untreated (open bars) and treated (closed bars) wild-type and *Abca1*$^{-/-}$ mice were collected during the last 24 h of the experiment. *,# Significant differences by Student's *t*-test ($P < 0.005$ and $P < 0.001$, respectively). Values represent means ± SD.

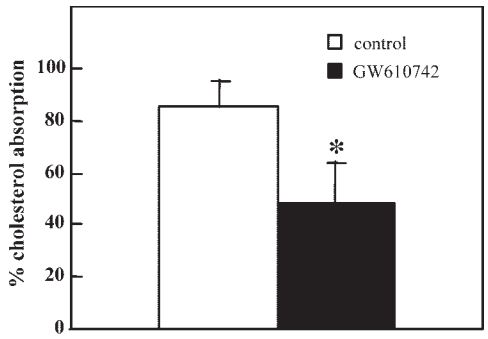

Fig. 6. Cholesterol absorption in untreated (open bars) and GW610742-treated (closed bars) DBA/1 wild-type mice. Cholesterol absorption in treated and untreated mice was measured using the plasma dual-isotope method (n = 5 per group). After 6 days of treatment, mice received an intravenous injection of [^{3}H]cholesterol and an oral dose of [^{14}C]cholesterol. Plasma samples obtained 72 h after administration were used for the calculation of fractional cholesterol absorption. Values represent means ± SD. * Significant differences by Student's *t*-test ($P < 0.005$).

PPARγ, and PPARδ activation by specific agonists in polarized Caco2 cells. **Figure 9** shows that both the PPARα agonist Wy14643 and the PPARγ agonist rosiglitazone had no effect on *Npc1l1* expression, whereas the PPARδ agonist GW1516 exhibited a clear (−33%) reduction in *Npc1l1* expression. This demonstrates that the reduced expression of *Npc1l1* is specific for PPARδ activation.

DISCUSSION

This study shows that activation of PPARδ results in increased plasma HDL levels in DBA/1 wild-type mice. Although increased 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 PPARδ activation in wild-type mice. In fact, excretion rates were highly similar in wild-type and

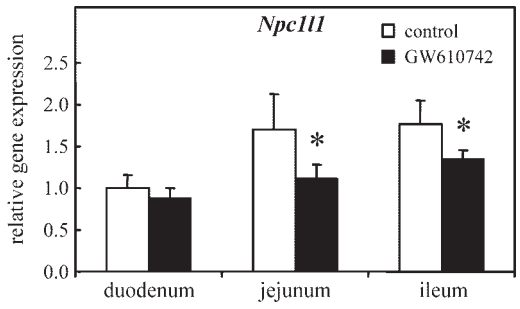

Fig. 7. Expression of Niemann-Pick C1-like 1 (*Npc1l1*) along the length of the small intestine of untreated (open bars) and GW610742-treated (closed bars) DBA/1 wild-type mice, measured by real-time PCR. Data are presented as means of six assays performed in duplicate ± SD. Expression values are normalized to *β-actin*, and expression in duodenum of untreated mice was set to 1.00. * Significant differences by Student's *t*-test ($P < 0.05$).

Abca1$^{-/-}$ mice, confirming that HDL is not an essential source of biliary cholesterol in mice (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. PPARδ 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 (21): intestinal expression of the recently described potential target of ezetimibe, *Npc1l1* (13), appeared to be decreased upon PPARδ activation.

Analogous to the situation described in obese and hyperlipidemic rhesus monkeys (10), PPARδ 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 *Abca1*$^{-/-}$ mice, supporting the essential role of ABCA1 in HDL formation. The question of by which mechanism PPARδ activation increases HDL-cholesterol concentrations remains to be answered. Induced expression of *Abca1* as well as *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 (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 (23). Yet, we did not observe the induction of hepatic *Abca1* expression. The reason for the discrepancy in PPARδ-mediated effects on *Abca1* expression between macrophages and liver remains to be established.

HDL-cholesterol is considered a preferential source of biliary cholesterol (4). However, despite the marked differences in plasma HDL-cholesterol levels, no differences in biliary cholesterol excretion were observed between untreated wild-type and *Abca1*$^{-/-}$ mice or between treated and untreated wild-type mice. These observations are consistent with earlier work (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 PPARδ activation in both wild-type and *Abca1*$^{-/-}$ mice, in spite of the fact that biliary cholesterol excretion was not induced. This could theoretically be attributable to a higher intestinal cholesterol synthesis. This parameter has not been measured directly, but intestinal expression of *Hmgr* was not affected upon PPARδ activation, which strongly suggests unaltered intestinal cholesterol synthesis. Most cholesterol is synthesized in the peripheral tissues in mice (24), and peripheral synthesis may have been enhanced upon PPARδ activation to maintain total body cholesterol balance. It is also highly un-

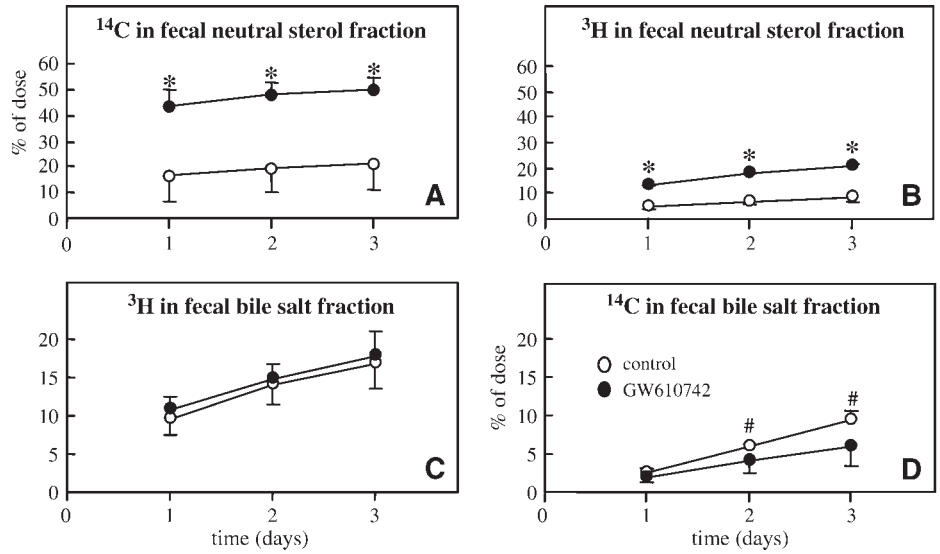

Fig. 8. Fecal loss of intravenously and orally administered radiolabeled cholesterol in untreated and GW610742-treated DBA/1 wild-type mice. A and B show fecal loss of ^{14}C-labeled and ^{3}H-labeled neutral sterols, respectively, in treated (closed symbols) and untreated (open symbols) mice. C and D show fecal loss of ^{14}C-labeled and ^{3}H-labeled bile salts, respectively. Values are presented as percentages of administered dose ± SD (n = 5 per group). *,# Significant differences by Student's *t*-test ($P < 0.01$ and $P < 0.05$, respectively).

likely that accelerated intestinal cell turnover was the cause of enhanced fecal sterol loss: Ki67 staining of intestinal sections revealed no differences between GW610742-treated and untreated mice.

Cholesterol absorption efficiency was clearly reduced upon PPARδ activation in wild-type mice. Because the amounts of bile salts and phospholipids excreted into the intestinal lumen, important for efficient cholesterol absorption (25, 26), as well as *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 the intestinal expression of *Abcg5* and *Abcg8*. Our data suggest that PPARδ 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) (21). Very recently, Altmann et al. (13) proposed NPC1L1 to be critical for intestinal cholesterol absorption and to represent a target of ezetimibe. Our results show that PPARδ activation clearly reduced intestinal expression of *Npc1l1*, predominantly in the jejunal part of the small intestine, where most of the cholesterol absorption takes place. Our in vitro results show that this reduced expression of *Npc1l1* is highly specific for PPARδ activation. There was no effect of selective PPARα and PPARγ agonists on *Npc1l1* expression in Caco-2 cells, but there was a clear suppression by the PPARδ agonist, indicating that human *Npc1l1* is also responsive to PPARδ activation.

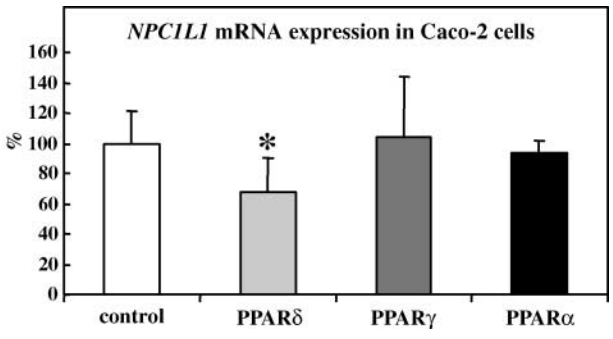

Fig. 9. Expression of *Npc1l1* in polarized Caco2 cells treated with PPARα, PPARγ, or PPARδ agonists, as measured by real-time PCR. Data are presented as means of three assays ± SD. Expression values are normalized to 28S, and expression in control cells was set to 1.00. * Significant differences by one-tailed Mann-Whitney *U* test ($P < 0.05$).

No data are available yet on the factors involved in *Npc1l1* transcription regulation: whether PPARδ controls intestinal *Npc1l1* expression by direct or indirect means remains to be established. Because the amount of NPC1L1 protein is clearly reduced in enterocytes of heterozygous *Npc1l1*$^{+/-}$ mice (13), it is likely that the ~40% reduction in jejunal *Npc1l1* mRNA levels was associated with reduced amounts of the protein. Upon oral administration of [^{14}C]cholesterol, absorption in chow-fed *Npc1l1*$^{+/-}$ mice into plasma and liver appeared to be reduced by ~40% compared with wild-type mice, although this difference failed to reach statistical significance (27). A significant reduction in fractional cholesterol absorption in heterozygote mice compared with wild-type controls was noted after feeding a diet containing 0.1% sodium cholate (13).

It has been proposed that PPARδ might induce antiatherogenic actions (10). Our data support this notion, because PPARδ activation resulted in increased HDL-cholesterol levels. However, potential antiatherogenic effects

would not be expected to be achieved by induction of the "classical" pathway of RCT, because 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. (20), as suggested by the unexpectedly rapid fecal excretion of intravenously administered ^{3}H-labeled neutral sterols in GW610742-treated mice (Fig. 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 hypercholesterolemic humans (28) and prevention of atherosclerosis development in *Apolipoprotein E*$^{-/-}$ mice (29) 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 humans by an unidentified mechanism of action. Thus, PPARδ is a promising target for the development of novel drugs aimed at preventing atherosclerosis.

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