Fenofibrate reduces intestinal cholesterol absorption via PPARα-dependent modulation of NPC1L1 expression in mouse

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Abstract  Fibrates, including fenofibrate, exert their biological effects by binding peroxisome proliferator-activated receptor α (PPARα), a member of the nuclear receptor superfamily of ligand-activated transcription factors. Treatment with PPARα agonists enhances fatty acid oxidation, decreases plasma triglycerides, and may promote reverse cholesterol transport. In addition, fibrate administration can reduce intestinal cholesterol absorption in patients, although the molecular mechanism for this effect is unknown. Because Niemann-Pick C1-Like 1 (NPC1L1) is already known to be a critical protein for cholesterol absorption, we hypothesized that fenofibrate might modulate NPC1L1 expression to alter intestinal cholesterol transport. Here, we find that fenofibrate-treated wild-type mice have decreased fractional cholesterol absorption (35–47% decrease) and increased fecal neutral sterol excretion (51–83% increase), which correspond to decreased expression of NPC1L1 mRNA and protein (38–66% decrease) in the proximal small intestine. These effects of fenofibrate are dependent on PPARα, as Ppara-knockout mice fail to respond like wild-type littermates. Fenofibrate affects the ezetimibe-sensitive pathway and retains the ability to decrease cholesterol absorption and NPC1L1 mRNA expression in chow-fed liver X receptor α/β-double-knockout mice and high-cholesterol- or cholic acid-fed wild-type mice. These data demonstrate that fenofibrate specifically acts via PPARα to decrease cholesterol absorption at the level of intestinal NPC1L1 expression.—Valasek, M. A., S. L. Clarke, and J. J. Repa. Fenofibrate reduces intestinal cholesterol absorption via PPARα-dependent modulation of NPC1L1 expression in mouse. J. Lipid Res. 2007. 48: 2725–2735.

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Peroxisome proliferator-activated receptor α (PPARα, NR1C1) is a member of the nuclear receptor superfamily and acts as a ligand-activated transcription factor in response to a variety of fatty acids (1, 2). As such, PPARα regulates the expression of genes involved in lipid metabolism, especially fatty acid oxidation and transport. Fibric acid derivatives, including fenofibrate and gemfibrozil, function by binding and activating PPARα. Fibrates are especially useful in treating hypertriglyceridemia but can also slightly decrease plasma total cholesterol and LDL-cholesterol concentrations while increasing HDL-cholesterol concentrations (3). Therefore, fibrate administration affords patients a more favorable plasma lipid composition, although the magnitude of positive impact on coronary event outcomes in various patient populations is uncertain (4–6). Whereas fibrates have been studied extensively with regard to their influence on many aspects of hepatic lipid metabolism, their potential impact on the molecular mechanisms governing intestinal cholesterol biology is not well understood.

Intestinal cholesterol absorption is defined as the transfer of cholesterol from the intestinal lumen to the lymph (7) and involves at least three initial phases: intraluminal, transmembrane, and intracellular (within enterocyte). Recent efforts have focused on elucidating the mechanisms responsible for moving cholesterol across the apical membrane of enterocytes (transmembrane phase); therefore, several candidate proteins have been proposed to function as intestinal cholesterol transporters, including scavenger receptor class B type I (SR-BI), ABCA1, ABCG5/8, aminopeptidase N, caveolin-1 (CAV1)/annexin-2, and Niemann-Pick C1-Like 1 (NPC1L1) (8–14). With the discovery of the specific and potent cholesterol absorption inhibitor ezetimibe (15, 16), new criteria can now be used to define and characterize a candidate intestinal cholesterol transporter (17). Not only should knockout animals
duced in the small intestine in a PPARα

NPC1L1 mRNA and protein expression are markedly re-

erated rate of fecal neutral sterol excretion. Moreover,

creased fractional cholesterol absorption and an accel-

erated rate of fecal neutral sterol excretion. Together,

these data suggest a central role for NPC1L1 in intestinal cholesterol absorption.

PPARα agonists may affect plasma LDL-cholesterol in part by decreasing cholesterol absorption (22–25). Therefore, we hypothesized that fenofibrate might act via PPARα to suppress the expression of NPC1L1, thereby limiting cholesterol absorption at the level of cholesterol transport across the apical membrane of the enterocyte. Here, we report that mice treated with fenofibrate show decreased fractional cholesterol absorption and an accelerated rate of fecal neutral sterol excretion. Moreover, NPC1L1 mRNA and protein expression are markedly reduced in the small intestine in a PPARα-dependent manner. These data clearly show that fenofibrate, acting via PPARα, reduces cholesterol absorption in part by altering the intestinal expression of NPC1L1.

MATERIALS AND METHODS

Animal experiments

Pparα-null mice on a 129S4/SvJae genetic background were kindly provided by Frank Gonzalez (National Cancer Institute, National Institutes of Health) (26). Lxrasα-double-knockout mice were generated as described previously (13, 27) and maintained on a mixed-strain background (C57Bl/6*129/SvJae), as were age- and gender-matched wild-type controls. Mice were fed a cereal-based rodent diet (Diet 7001; Teklad, Madison, WI) that contains 0.02% (w/w) cholesterol and ~4% total lipid. For most experiments, mice were fed the powdered form of this diet for a period of 7–10 days. In some experimental groups, the diet was supplemented with fenofibrate (2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid isopropyl ester) to provide an approximate dose of 800 milligrams per kilogram body weight (mpk)/day, ezetimibe (10 mpk/day), cholesterol (0.2%, w/w), or cholic acid (0.1%, w/w). The calculated quantities of dietary drug supplement use a food consumption rate of 160 g diet/day/kg body weight. For oral gavage dosing, fenofibrate or GW7647 was administered once daily as a suspension in 1% methylcellulose and 1% Tween-80. Fenofibrate and cholic acid were purchased from Sigma, ezetimibe was provided by Harry R. Davis, Jr. (Schering-Plough Research Institute), and GW7647 was provided by Timothy Wilson (GlaxoSmithKline Research). Mice were housed in a temperature-controlled environment with 12 h light/dark cycles with free access to food and water. Unless specified otherwise, mice were euthanized and tissues harvested at the end of the dark cycle; thus, mice were in a fed state at the time of study. Experiments were done in compliance with the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Com-

mittee of the University of Texas Southwestern Medical Center at Dallas.

Cholesterol balance measurements

Intestinal absorption. Fractional cholesterol absorption was measured by a fecal dual-isotope ratio method (28). Three days before euthanasia, mice received a single intragastric dose of medium-chain triglyceride oil containing [5,6-3H]sitostanol (2 μCi; American Radiolabeled Chemicals, Inc., St. Louis, MO) and [4-14C]cholesterol (1 μCi; Perkin-Elmer Life Sciences, Boston, MA). Stools were collected over the subsequent 3 days. Samples of the dosing mixture and aliquots of stool were extracted, and the ratio of 14C to 3H in each was determined to calculate percentage cholesterol absorption (28).

Fecal neutral sterol excretion. Stools were collected from individually housed mice during the final 3 days of each experiment. They were dried, weighed, and ground. An aliquot was saponified and solvent-extracted, and amounts of cholesterol, coprostanol, epico-

prostanol, and cholestanone were quantified by gas chromatogra-

phy (29). The measured sterols were added together to represent total neutral sterols and then adjusted to reflect the daily excretion (based on feces collected during 3 days) per 100 g body weight.

Preparation of samples for RNA and protein measurements

Mice were anesthetized and exsanguinated via the descending vena cava. Small intestines were removed, flushed with ice-cold PBS, and cut into three sections of equal length (the proximal third was used in these studies). The sections were slit length-

wise, and the mucosae were gently scraped, frozen in liquid nitrogen, and stored at ~85°C. Total RNA was isolated from tissue samples using RNA STAT-60 (Tel-Test, Inc.). Total protein was obtained from the organic phase remaining after RNA iso-

lation by precipitating with isopropanol, consecutively washing with 0.3 M guanidine hydrochloride in 95% ethanol and then ethanol, and finally solubilizing the protein pellet in 1% SDS and 50 mM Tris-Cl, pH 8.8 (30). RNA concentrations were determined by absorbance at 260 nm. Protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Quantitative real-time PCR

Quantitative real-time (qRT) PCR was performed using an Applied Biosystems Prism 7900HT sequence detection system as described (31). Briefly, total RNA was treated with DNase I (RNase-free; Roche Molecular Biochemicals) and reverse-transcribed with random hexamers using SuperScript II (Invitrogen) to generate cDNA. Primers for each gene were designed using Primer Express Software (Perkin-Elmer Life Sciences) and validated by analysis of template titration and dissociation curves. Primer se-

quences for all assayed genes are available upon request. Each qRT-PCR contained (in a final volume of 10 μl) 25 ng of reverse-

transcribed RNA, each primer at 150 nM, and 5 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems), and each sample was analyzed in triplicate. Results were evaluated by the comparative cycle number at threshold (Ct) method (User Bulletin No. 2; Perkin-Elmer Life Sciences) using cyclophilin as the invariant control gene. Similar results were obtained when villin was used as the housekeeping gene, suggesting that the changes observed were not the result of altered expression of our calibrator.

Western analysis

Total protein obtained from whole-cell lysates of the proximal third of the small intestine were size-fractionated on 6%
Fenofibrate modulates NPC1L1 expression in mouse small intestine via PPARα

To determine whether fenofibrate influences fractional cholesterol absorption, we treated male wild-type and Pparα-null mice by supplementation of the diet (800 mpk/day, 10 days). As shown in Fig. 1A, dietary fenofibrate decreases fractional cholesterol absorption by 35% in wild-type mice (vehicle vs. fenofibrate, 57% vs. 38%, respectively; P < 0.05). This finding is consistent with reports that administration of other PPARα agonists, such as WY-14643 ([4-chloro-6-(2,3-dimethylphenyl)amino]-2-pyrimidinyl)[thio]-acetic acid) and gemfibrozil, inhibits cholesterol absorption in mice and rats, respectively (22, 24). To our knowledge, these studies represent the first measurement of fractional cholesterol absorption in mice treated specifically with fenofibrate. In contrast to wild-type animals, Pparα-null mice fail to respond to fenofibrate. This finding is critical, as it distinguishes a PPARα-dependent mechanism and rules out potential cross-reactivity with PPARβ/δ, which was recently reported to regulate cholesterol absorption (33). The inhibition of cholesterol absorption was associated with an increase in fecal neutral sterol excretion (Fig. 1B) (83% increase; 10 vs. 19 μmol/day/100 g body weight, respectively; P < 0.05), which did not occur in Pparα-null mice.

We hypothesized that activation of PPARα might influence the expression of the putative intestinal cholesterol transporter NPC1L1, thereby limiting cholesterol absorption at the level of cholesterol uptake across the apical membrane of the enterocyte. To test this, we measured NPC1L1 mRNA expression by two methods: qRT-PCR and in situ hybridization. As shown in Fig. 1C, qRT-PCR analysis of the proximal small intestine revealed a reduction in the relative expression of NPC1L1 mRNA in fenofibrate-treated wild-type mice (55% reduction) but not Pparα-null mice. The housekeeping gene, cyclophilin, did not differ between treatment groups (data not shown). In situ hybridization was carried out on transverse and longitudinal sections of segments of jejunum (13–18 cm from the pyloric sphincter) using an antisense RNA probe directed at the 3′ untranslated region of NPC1L1 mRNA determined previously to be specific to NPC1L1 (without cross-reacting with NPC1) by Northern analysis (18). As shown in Fig. 1D, NPC1L1 displays a distribution pattern along the entire villus length consistent with expression in enterocytes, and fenofibrate-treated animals have markedly reduced expression. Sense controls give no signal above background (data not shown). Thus, activation of PPARα decreases both cholesterol absorption and NPC1L1 mRNA expression.

To exert a physiologically relevant effect on cholesterol absorption, the change in NPC1L1 mRNA must result in a change in protein level. Therefore, we prepared whole cell lysates from proximal small intestinal mucosae and immunoblotted them with polyclonal antisera for NPC1L1 and actin. Figure 2A depicts representative immunoblots of protein from one animal from each experimental group. Quantification of the NPC1L1 protein bands from four to six animals from each group (Fig. 2B) revealed marked, significant reductions of NPC1L1 protein levels by fenofibrate (66% reduction) in wild-type mice but not Pparα-null knockout mice.

PPARα-modulated gene expression in the proximal small intestine

To determine whether other target genes could be involved in the PPARα-dependent reduction of cholesterol absorption, we measured the relative mRNA levels in the proximal small intestine of several proteins thought to function in cholesterol absorption and homeostasis. First, we wanted to characterize the relative levels of PPAR and liver X receptor (LXR) isoforms in the proximal small intestine. In qRT-PCR, the cycle times inversely correlate with the quantity of mRNA in the sample, so that more abundant expressed genes have lower CT values (31, 34). Based on the average CT (wild-type vehicle-treated group; Fig. 3A), the rank order for abundance of PPAR and LXR isoforms in untreated proximal intestine is PPARβ > PPARα > PPARγ and LXRα > LXRβ. PPARα was the only isoform induced by fenofibrate (4.1-fold compared with vehicle). Note that the PPARα expression detected
in Pparα-knockout animals represents the recognition of an aberrant transcript that produces no functional protein. The classical PPARα target gene acyl-coenzyme A oxidase 1 (ACOX1) is induced by fenofibrate in a PPARα-dependent manner (2.7-fold). This establishes that PPARα is present in the proximal small intestine and responds to fenofibrate treatment by increasing the expression of known target genes, ACOX1 and PPARα itself (35, 36). In addition, PPARα activation has no effect on the mRNA levels of LXRα and LXRβ, transcription factors previously implicated in the regulation of cholesterol absorption (13).

As shown in Fig. 3B, ABCA1 mRNA levels are increased significantly (3.1-fold) by fenofibrate treatment in wild-type mice but not Pparα-knockout mice. This induction is not important for the regulation of cholesterol absorption by fenofibrate, however, as Abca1-knockout mice treated with fenofibrate show reduced fractional cholesterol absorption and expression of NPC1L1 mRNA, similar to wild-type controls (data not shown). In contrast to ABCA1, ABCG5 and ABCG8 show no change in mRNA expression level by fenofibrate administration (Fig. 3B). Neither SR-BI nor CAV1 mRNA expression is altered significantly by treatment (Fig. 3B);
cholesterol absorption and higher fecal neutral sterols. Interestingly, LXR deletion itself leads to lower basal excretion of fecal neutral sterols (Fig. 4B). An increase in fractional cholesterol absorption (Fig. 4A) and a decrease in NPC1L1 expression correlates with a decrease in cholesterol absorption. Therefore, we treated PPARα wild-type mice and Pparα/−/− (PPARα KO) mice with or without fenofibrate for 10 days. Whole cell protein was isolated from proximal small intestine, and 40 μg protein/lane was fractionated by SDS-PAGE. A: Representative immunoblot showing one individual animal from each treatment group. B: NPC1L1 protein was quantified using OptiQuant version 3.1. Values represent means ± SEM of data from four to six mice per group. Different letters indicate statistically different groups (P < 0.05) as assessed by ANOVA and described in Materials and Methods.

however, NPC1L1 mRNA is downregulated (55% decrease), which correlates with a decrease in cholesterol absorption (Fig. 1).

**Fenofibrate inhibits cholesterol absorption and NPC1L1 expression independent of LXRa**

As it has been proposed that PPARα activation may decrease cholesterol absorption by activating LXRs (22), we sought to determine whether LXRs were involved in the PPARα-mediated reduction of NPC1L1 expression and cholesterol absorption. Therefore, we treated Lxrα/β-double-knockout mice with or without fenofibrate for 7 days. **Figure 4** shows that NPC1L1 mRNA expression is reduced significantly by fenofibrate treatment in both wild-type mice and Lxrα/β-double-knockout mice. This decrease in NPC1L1 expression correlates with a decrease in fractional cholesterol absorption (Fig. 4A) and an increase in the excretion of fecal neutral sterols (Fig. 4B). Interestingly, LXR deletion itself leads to lower basal cholesterol absorption and higher fecal neutral sterol excretion. Thus, LXRα and LXRβ are both present in the proximal small intestine but are not required for the fenofibrate-induced modulation of NPC1L1 expression or cholesterol absorption.

**Fenofibrate affects the ezetimibe-sensitive pathway**

Ezetimibe is a plasma LDL-cholesterol-lowering drug that acts by blocking intestinal cholesterol absorption. It has also been shown to bind to NPC1L1 (20) and to block NPC1L1-mediated cholesterol uptake in cultured cells (21). To determine whether fenofibrate acts on the ezetimibe-sensitive cholesterol absorption pathway, we treated animals with 10 mpk/day ezetimibe [a dose well above the approximate EC50 of 0.5 mpk/day for mice (20)], 800 mpk/day fenofibrate [a dose often used in rodent experiments that we determined to induce PPARα target genes in liver and small intestine of mouse (37)] (Fig. 3; data not shown), or both for a period of 10 days. As shown in **Fig. 5A**, ezetimibe potently decreases cholesterol absorption relative to vehicle controls (~92% decrease, 45% to 4%; P < 0.05). This magnitude of decrease matches several other reports showing that ezetimibe decreases cholesterol absorption by >90% in various mouse models (8, 18, 38, 39). Fenofibrate moderately decreases fractional cholesterol absorption (42% decrease, 45% to 26%; P < 0.05), similar to other mouse studies presented here (Figs. 1, 5). There was no significant difference between ezetimibe-treated and dual-treated groups, although a trend toward lower values for the dual-treated group was observed (3.8% vs. 2.6%; not significant). This confirms that the ezetimibe-insensitive pathway seems to be a minor pathway comprising only a small fraction (<10%) of the total amount of cholesterol absorbed, as seen previously (8, 18, 38, 39), but also shows that fenofibrate does not substantially affect the ezetimibe-insensitive pathway.

Theoretically, this minor pathway could be protein-mediated or might reflect the passive diffusion of cholesterol across the apical membrane. To determine the impact of fenofibrate on the major (ezetimibe-sensitive) pathway, we subtracted the fractional cholesterol absorption for the ezetimibe-treated group from that for the vehicle-treated group. This difference represents the ezetimibe-sensitive fractional cholesterol absorption under basal conditions. Similarly, to determine the ezetimibe-sensitive fractional cholesterol absorption during fenofibrate treatment, we subtracted the fractional cholesterol absorption value for the dual-treated (ezetimibe and fenofibrate) group from that for the fenofibrate-treated group. This calculation shows that fenofibrate significantly reduces ezetimibe-sensitive fractional cholesterol absorption (43% reduction, 41% to 23%; P < 0.05). Thus, fenofibrate acts to reduce cholesterol absorption by modulating the major (ezetimibe-sensitive) pathway. Finally, because NPC1L1 mRNA expression was reduced (~40%) by fenofibrate administration, but not ezetimibe, this illustrates that each drug uses different molecular mechanisms to inhibit cholesterol absorption.
Fenofibrate inhibits cholesterol absorption and NPC1L1 mRNA expression in mice fed a high-cholesterol or cholic acid diet

PPARα may also play a role in the biliary secretion of cholesterol, at least in humans, as fibrates (e.g., clofibrate) can have negative effects on biliary lipid composition by increasing cholesterol concentrations (40). One way to minimize the effects of alterations in biliary cholesterol secretion on the fecal dual-isotope method of measuring cholesterol absorption is to provide animals with excess dietary cholesterol. We fed mice a “low-cholesterol” (0.02%, w/w) or “high-cholesterol” (0.2%, w/w) diet supplemented with or without fenofibrate for 10 days. On both the low- and high-cholesterol diets, fenofibrate reduces fractional cholesterol absorption compared with appropriate vehicle controls (Fig. 6A; 47% reduction on the 0.02% cholesterol diet, 46% on the 0.2% cholesterol diet). NPC1L1 mRNA expression displays a similar pattern as frac-

Fig. 3. PPARα specifically regulates NPC1L1 mRNA levels but not those of other sterol transport proteins. Three month old male Pparα+/+ (PPARα WT) and Pparα−/− (PPARα KO) mice were fed powdered basal diet (Veh) or diet supplemented with fenofibrate at 800 mpk/day (Feno) for a total of 10 days. A: Relative mRNA levels of PPAR and liver X receptor (LXR) isoforms and acyl-coenzyme A oxidase 1 (ACOX1), a PPARα target gene. Hatched bars represent recognition of an aberrant transcript in Pparα-null mice. B: Relative mRNA levels for various sterol transport proteins. Values represent means ± SEM of data from four to six mice per group. Statistical analysis was performed by two-way ANOVA (factors: genotype, diet); if a significant interaction was observed, all four groups were compared, and different letters indicate statistically different groups (P < 0.05). * Statistical evaluation of PPARβ expression revealed no significant interaction but a modest (P < 0.05) decrease in mRNA attributable to fenofibrate.

Fenofibrate inhibits cholesterol absorption and NPC1L1 mRNA expression in mice fed a high-cholesterol or cholic acid diet

Fig. 4. Fenofibrate inhibits cholesterol absorption and NPC1L1 expression independent of LXRs. Three month old female LXRα/β+/+ (LXR WT) and LXRα/β−/− (LXR DKO) mice were fed powdered basal diet (Veh; open bars) or diet supplemented with fenofibrate at 800 mpk/day (Feno; closed bars) for a total of 7 days. Fractional cholesterol absorption was decreased (A) and fecal neutral sterol excretion was increased (B) by fenofibrate in both wild-type and knockout mice. These changes correlate with the relative NPC1L1 mRNA expression in proximal small intestine (C). Values represent means ± SEM of data from six mice per group. Values with different letters are statistically different (P < 0.05) as assessed by ANOVA and described in Materials and Methods.
tional cholesterol absorption, with values being significantly reduced by fenofibrate on either low or high cholesterol (Fig. 6B). In this experiment, the addition of cholesterol slightly decreases NPC1L1 expression and fractional cholesterol absorption. Thus, high levels of dietary cholesterol may modestly regulate NPC1L1 mRNA expression.

In contrast, depletion of enterocyte cholesterol levels by ezetimibe treatment has no effect on NPC1L1 mRNA expression (Fig. 5C) (18). In addition, female mice treated with 800 mpk/day fenofibrate for 30 days show no increase in biliary cholesterol concentration; rather, they show a decrease (data not shown). These data indicate that the observed changes in fractional cholesterol absorption are not a result of diluting the radiolabeled cholesterol tracer by enhanced biliary secretion of cholesterol. To determine whether alterations in the bile acid pathway were responsible for the decrease in cholesterol absorption in fenofibrate-treated animals, we fed female wild-type and Pparα-null animals a diet containing 0.1% cholic acid and then supplemented it with fenofibrate (800 mpk/day) or no supplementation (Fig. 6C, D). In this experiment, all groups show higher levels of fractional cholesterol absorption compared with other experiments. This is expected as a result of the cholic acid supplementation of the diet, which solubilizes cholesterol, thereby enhancing its absorption. In this setting, fenofibrate significantly decreases fractional cholesterol absorption by 18% in wild-type mice but not Pparα-null mice.

Fig. 5. Fenofibrate affects the ezetimibe-sensitive pathway. Three month old male Pparα+/− and Pparα−/− mice were fed powdered basal diet (Veh) or diet supplemented with ezetimibe (10 mpk/day; Ezet), fenofibrate (800 mpk/day; FENO), or both for a total of 10 days. A: Fractional cholesterol absorption was decreased in all drug-treated groups relative to vehicle controls. B: Calculated ezetimibe-sensitive fractional cholesterol absorption was decreased by fenofibrate. C: Fecal neutral sterol excretion was increased in all drug-treated groups relative to vehicle controls. D: Relative NPC1L1 mRNA by qRT-PCR analysis. Values represent means ± SEM of data from five to six mice per group. Different letters indicate statistically different groups (P < 0.05) as determined by ANOVA, and the asterisk indicates statistical difference as indicated by Student’s t-test (P < 0.05).

Fig. 6. Fenofibrate inhibits cholesterol absorption and NPC1L1 mRNA expression in mice fed a high-cholesterol or cholic acid diet. A, B: Three month old female A129/StJae (PPARα WT) mice were fed powdered low-cholesterol basal diet (0.02%, w/w) or high-cholesterol diet (0.2%, w/w) for 10 days. In addition, these diets were either unsupplemented (Veh; open bars) or supplemented with fenofibrate at 800 mpk/day (Feno; closed bars). C, D: Four month old female Pparα+/− (PPARα WT) and Pparα−/− (PPARα KO) mice were fed a diet containing 0.1% (w/w) cholic acid with no additional supplement (open bars) or supplemented with fenofibrate at 800 mpk/day (closed bars) for a total of 10 days. Cholesterol absorption was determined by the fecal dual-isotope method, and NPC1L1 mRNA levels were measured in the proximal small intestine by qRT-PCR. Values represent means ± SEM of data from five to eight mice per group. Values with different letters are statistically different (P < 0.05) as assessed by ANOVA and described in Materials and Methods.
Because fenofibrate can specifically decrease cholesterol absorption in a PPARα-dependent manner even under conditions of high dietary cholic acid, it is likely that fenofibrate affects extralumenal events to decrease transport. The modest decrease in cholesterol absorption by fenofibrate in this experiment may be attributable to an enhanced efficiency of transport in spite of fewer molecules of NPC1L1 in the intestinal epithelium. Interestingly, the enhancement of cholesterol absorption by cholic acid has also been observed in Npc1l1 heterozygotes (9). In addition, fenofibrate may affect bile acid homeostasis in mouse; therefore, addition of cholic acid may mask those effects. However, because cholic acid fails to normalize cholesterol absorption in fenofibrate-treated mice, these data support the idea that fenofibrate modulates transmembrane and/or intracellular events in the intestine.

**Long-term PPARα activation is required to decrease NPC1L1 expression**

To begin to understand the molecular mechanism by which PPARα activation leads to a reduction in NPC1L1 expression, we determined the time course of PPARα agonist-mediated repression of NPC1L1 mRNA. Animals treated with PPARα agonists for 3, 7, or 10 days show reduced NPC1L1 mRNA levels relative to vehicle controls (Fig. 7). In contrast, 12 h of treatment with fenofibrate fails to reduce NPC1L1 expression in small intestine, although other target genes, such as ACOX1 and PPARα, are increased (data not shown). This delay in gene repression, in addition to a failure of fenofibrate to alter histone (H3) acetylation of the 5’ enhancer/promoter region of this gene (data not shown), suggests that PPARα acts indirectly to regulate NPC1L1 mRNA levels. Thus, relatively long-term activation of PPARα is necessary to inhibit the expression of NPC1L1 mRNA under these conditions.

**DISCUSSION**

Fenofibrate is known to bind PPARα to enhance fatty acid oxidation and decrease serum triglycerides in patients and can also decrease plasma total cholesterol and LDL-cholesterol levels while increasing HDL-cholesterol levels (3). Two small studies also suggest that other fibrates, namely clofibrate and gemfibrozil, can reduce cholesterol absorption efficiency in humans (23, 25). The mechanism of the effects of fibrates (and other PPARα agonists) on cholesterol absorption, however, is poorly understood. Using the mouse model, we have delineated a molecular mechanism by which specific activation of PPARα by fenofibrate decreases cholesterol absorption via an inhibitory effect on NPC1L1 expression in the proximal small intestine. The fenofibrate effect clearly requires PPARα and not other PPAR isoforms, as Ppara-knockout mice fail to respond similarly in terms of fractional cholesterol absorption, fecal neutral sterol excretion, or NPC1L1 mRNA and protein expression in the proximal small intestine (Figs. 1, 2).

The present studies are consistent with the idea that NPC1L1 is an intestinal sterol transporter, as modulation of its expression correlates with changes in cholesterol absorption. This was also observed in a report comparing Npc1l1-null, Npc1l1-heterozygous, and wild-type mice, in which absorption and small intestinal uptake of cholesterol correlated with NPC1L1 mRNA expression (19). It is possible, however, that other factors are involved in the PPARα-mediated reduction of cholesterol absorption. Analysis of mRNA expression in proximal small intestine of fenofibrate-treated mice (Fig. 3) reveals that other known players in cholesterol metabolism (including ABCA1, ABCG5/8, and SR-B1) are unlikely to contribute to PPARα action, as their expression levels are unchanged or changed in a direction opposite from those expected to decrease cholesterol absorption. Although initially considered to be a candidate for the cholesterol transporter (13), evidence suggests that ABCA1 is localized to the abluminal surface of the enterocyte (41) and may have minimal impact on fractional cholesterol absorption (42, 43). In addition, Abca1-knockout mice treated with fenofibrate show reduced fractional cholesterol absorption and expression of NPC1L1 mRNA, similar to wild-type controls (data not shown). These data suggest that although PPARα activation results in an induction of ABCA1 expression in the small intestine, it is unlikely to substantially alter cholesterol absorption and is not required for the PPARα-mediated repression of cholesterol absorption.

In addition to the modulation of intestinal NPC1L1 expression in a PPARα-dependent manner, the observation that fenofibrate continues to reduce cholesterol absorption on a high-cholesterol diet (0.2%, w/w) indicates that the effect of fenofibrate is not primarily a product of changes in biliary cholesterol secretion but rather is a
result of alterations in enterocytic cholesterol transport. The cholesterol diet itself slightly reduces fractional cholesterol absorption (Fig. 5A), but not to the extent that fenofibrate does. Thus, biliary secretion of cholesterol would have to increase to a quantity >10-fold of the normal dietary cholesterol contribution to exert an effect similar to that of fenofibrate. However, in contrast to humans, biliary cholesterol is not changed in fenofibrate-treated mice (data not shown). In addition, hamsters treated with fenofibrate for 9 days have a decreased rate of hepatic cholesterol biosynthesis, as determined by reduced activities of HMG-CoA synthase and HMG-CoA reductase, and reduced incorporation of radiolabeled acetate into cholesterol (44). Another possibility that could contribute to a reduction in cholesterol absorption is a change in bile acid pool size or composition. However, it has been observed in rodents treated with fibrates that the rate of bile acid flow and secretion increases (37, 45), whereas the bile acid pool size is unchanged (46). In addition, cholate fails to fully normalize cholesterol absorption in fenofibrate-treated mice (Fig. 6). Therefore, these data support the idea that fenofibrate modulates transmembrane and/or intracellular events in the intestine in addition to intraluminal events.

Traditionally, the liver has been understood to be the primary target of fibrates. Here, we show not only that PPARα is expressed in small intestine but also that administration of fenofibrate, a PPARα agonist, alters target gene expression and function in that organ. Therefore, our results are consistent with the idea that activation of PPARα resident within the intestinal epithelium is responsible for the physiological changes we observed; however, they do not rule out the possibility that PPARα agonists may act at a distant site and then influence the intestine via humoral, metabolic, or other factors. Further investigation of the precise role(s) of PPARα in the intestine is warranted.

Fenofibrate affects the NPC1L1/ezetimibe pathway. Although fenofibrate has no significant effect on the minor (ezetimibe-insensitive) pathway, the major (ezetimibe-sensitive) pathway is significantly inhibited by fenofibrate. This is not surprising, as the ezetimibe-sensitive pathway constitutes ~90% of total fractional cholesterol absorption (Fig. 5) (8, 18, 38, 39). The reduction in cholesterol absorption by administration of ezetimibe closely matches a study in which Npc1l1-knockout mice were shown to have 86% reduced cholesterol absorption compared with wild-type mice (19). In our studies, changes in cholesterol absorption (35–47% reduction) consistently correspond to changes in NPC1L1 mRNA and protein expression (38–55% and 66% reduction, respectively). Thus, the finding that fenofibrate simultaneously alters both the ezetimibe-sensitive pathway and NPC1L1 expression is consistent with the idea that these pathways are one in the same. Indeed, it has been shown that Npc1l1-knockout mice are insensitive to ezetimibe (9) and that NPC1L1-expressing cells show specific binding to ezetimibe and exhibit ezetimibe-inhibited cholesterol uptake (20, 21).

Because NPC1L1 mRNA expression does not change upon administration of ezetimibe (Fig. 4D), it is unlikely that NPC1L1 mRNA levels respond to a decrease in cholesterol flux from the intestinal lumen. If the putative sterol regulatory element identified in the promoter of this gene (47) were responsive under these conditions, the ezetimibe-treated animals should have shown increased NPC1L1 expression in an attempt to enhance the import of cholesterol. Other known sterol-regulatory element binding protein target genes, such as HMG-CoA synthase and the low density lipoprotein receptor, are responsive under these cholesterol-depleting conditions (9, 18). Conversely, NPC1L1 mRNA is reduced slightly (~20%) by a high-cholesterol diet (0.2%, w/w) (Fig. 6B), whereas other studies show that supplementing cholesterol alone (2% or 0.12%, w/w) to the diet results in little (~20%) or no change, respectively, in NPC1L1 mRNA expression (48; M.A.V., unpublished observation). In contrast, when both cholesterol and cholic acid are added to the diet, NPC1L1 mRNA expression is decreased robustly (~75%) (19). Thus, dietary cholesterol alone only mildly affects NPC1L1 mRNA expression and may require other factors to enhance its regulation of NPC1L1 expression.

Although our data suggest that PPARα decreases cholesterol absorption by decreasing the expression of NPC1L1 in the intestine, they do not fully define the precise molecular mechanisms downstream of PPARα activation that lead to altered expression of NPC1L1. It was proposed previously that administration of a PPARα agonist could decrease cholesterol absorption by activating LXRs (22), which are themselves known to play a role in cholesterol efflux and absorption (49). Our data clearly indicate that fenofibrate is able to reduce cholesterol absorption and NPC1L1 expression independently of LXRs, as Lxrαβ-double-knockout mice continue to respond to fenofibrate in a similar manner as their wild-type controls. In addition, short-term treatment of wild-type mice with fenofibrate reveals no repression of NPC1L1 mRNA expression, whereas longer treatments of 3, 7, or 10 days show repression (Fig. 7). Thus, the impact of PPARα action on NPC1L1 expression is likely to be indirect, not only because nuclear receptors generally enhance the expression of direct targets (transactivation) but also because this process usually only requires several hours.

These studies support a role for PPARα in the regulation of NPC1L1, thus expanding the list of nuclear receptors known to influence NPC1L1 expression in the intestine. These include LXRα and PPARβ, which down-regulate NPC1L1, as LXR agonist treatment (T0901317, 5 days) of apolipoprotein E-KI mice on a Western diet reduces duodenal NPC1L1 mRNA expression by ~40% (50), whereas PPARβ agonist treatment [GW610742 (0.017%, w/w), 8 days] of DBA/1 wild-type mice reduces NPC1L1 mRNA expression in jejunum and ileum but not duodenum (33). Estrogen receptors may play a role in the upregulation of NPC1L1, as administration of high doses of 17β-estradiol (6 μg/day) to ovariectomized AKR or C57L mice increases NPC1L1 mRNA expression in duodenum and jejunum but not ileum (51). In each of these cases and in our studies, relatively long treatment durations were used to change NPC1L1 expression; there-
fore, the effects on NPC1L1 by nuclear receptor agonists could be secondary to other metabolic perturbations. Nevertheless, it is clear that nuclear receptors, including PPARs, can influence NPC1L1 gene expression.

The observation that fenofibrate causes PPARα-mediated repression of NPC1L1 has several important clinical implications. Whereas NPC1L1 expression is confined primarily to the small intestine in rodents, humans express similar (or greater) levels of NPC1L1 in liver compared with small intestine (9, 52). This raises the possibility that fenofibrate represses hepatic NPC1L1 expression in patients, thereby leading to a net increase in biliary secretion of cholesterol attributable to reduced reclamation of cholesterol across the canalicular membrane of hepatocytes, the proposed site of NPC1L1 protein in liver (21, 53). Indeed, it is well documented that fibrates can enhance biliary cholesterol secretion. Therefore, this particular effect of fibrates may be explained, at least in part, by changes in hepatic NPC1L1 expression. Because NPC1L1 also mediates the intestinal uptake of phytosterols (19), it would be interesting to test whether fenofibrate administration would decrease phytosterol absorption and plasma sterol levels in sitosterolemics or in Abcg5/8-knockout mice, a model of sitosterolemia, by decreasing the expression and function of NPC1L1. Indeed, the disease in mice and humans is ameliorated by blockade of sterol uptake by the administration of ezetimibe (54–56).

Importantly, our data suggest a paradigm shift in how we think about developing and implementing lipid-lowering therapies. In particular, the small intestine represents a tissue rich in targets for drug therapies, as it is a gatekeeper of homeostasis, regulating both the absorption and excretion of an exceedingly wide variety of biologically important compounds. Moreover, modulation of specific genes within tissues can be accomplished by small molecules. Here, we used a well-characterized drug (i.e., fenofibrate) to modulate a novel and specific gene target (i.e., NPC1L1) in the small intestine, thereby substantially altering intestinal cholesterol absorption. Clearly, the development of new therapeutic strategies that specifically target key genes may yield more powerful tools for physicians to better manage dyslipidemias and other diseases.

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