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The role of ApoCI, LPL and CETP in plasma lipoprotein metabolism - studies in mice

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Fenofibrate Increases HDL Cholesterol by Reducing the Expression of Cholesteryl Ester Transfer Protein

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Submitted

Objective - While fenofibrate reduces triglycerides (TG) both in humans and mice, fenofibrate increases HDL-cholesterol only in humans. Since humans express the cholesteryl ester transfer protein (CETP), whereas mice do not, we investigated whether the fenofibrate-induced increase in HDL-cholesterol depends on the expression of CETP.

Methods and Results - *APOE*3-Leiden (E3L)* mice, with a human-like lipoprotein profile, and *CETP.E3L* littermates were fed a Western-type diet with or without 0.04% fenofibrate. In male mice, fenofibrate decreased plasma TG in *E3L* and *CETP.E3L* mice (-59% and -60%; $P < 0.001$), caused by a strong reduction in VLDL. Whereas fenofibrate did not affect HDL-cholesterol in *E3L* mice, HDL-cholesterol was strongly increased (+91%) in *CETP.E3L* mice. Similar effects were observed in female mice. Fenofibrate did not affect the turnover of HDL-CE, indicating that fenofibrate causes a higher steady-state HDL-cholesterol level without altering the HDL-cholesterol flux through plasma. In *CETP.E3L* mice, fenofibrate reduced hepatic CETP mRNA (-72%; $P < 0.01$) and tended to reduce plasma CETP mass (-8%) and activity (-9%), which reached significance when adjusted for HDL-cholesterol (-42%; $P < 0.01$ and -42%; $P < 0.01$). In female mice, plasma CETP mass (-35%, $P < 0.05$) and activity (-32%, $P < 0.01$) were decreased even without adjusting for HDL-cholesterol.

Conclusion - Fenofibrate increases HDL-cholesterol in *CETP.E3L* mice by reducing the CETP-dependent transfer of HDL-cholesterol to (V)LDL, as related to reduced hepatic CETP expression and a reduced plasma (V)LDL pool.

High plasma triglyceride (TG) levels are correlated with an increased risk for cardiovascular disease. Fibrates are widely used to reduce hypertriglyceridemia, thereby generating a less atherogenic lipid phenotype. Fibrates constitute their actions through activation of peroxisome proliferator-activated receptor alpha (PPAR α).^{1,2} Activated PPAR α heterodimerizes with retinoid X receptor (RXR) and subsequently binds to specific peroxisome proliferator response elements (PPREs) in target genes to alter their transcription.^{1,3} Fibrates thus lower TG levels by inhibiting hepatic TG production through increased hepatic β -oxidation and inhibition of *de novo* fatty acid synthesis, increasing LPL-mediated lipolysis, and providing a higher affinity of remnants for the LDL receptor (LDLR).²

A meta-analysis of 53 clinical studies using fibrates enrolling 16,802 subjects indicated that apart from a 36% reduction in plasma TG, fibrates increase HDL-cholesterol levels by about 10% in humans.⁴ Studies *in vitro* and in (transgenic) mice showed that fibrates increase the hepatic transcription of human *APOA1*⁵ and *APOA2*,⁶ decrease hepatic SR-BI protein,⁷ increase the scavenger receptor B type I (SR-BI)-mediated⁸ and adenosine triphosphate-binding cassette transporter A1 (ABCA1)-mediated⁹ cholesterol efflux from human macrophages, and increase plasma phospholipid transfer protein (PLTP) activity.^{10,11} All of these effects may thus potentially contribute to the increase in HDL-cholesterol as observed in humans.

In contrast to humans, fibrates do not affect or even decrease HDL-cholesterol levels in mice.^{5,7,10,12} This effect may be attributed to the fact that, in contrast to the human *APOA1* promoter, which contains a functional positive PPRE leading to increased *APOA1* transcription, the murine *apoa1* promoter contains a nonfunctional PPRE.⁵ However, another major difference between both species is that, in contrast to humans,¹³ mice do not express the cholesteryl ester (CE) transfer protein (CETP).¹⁴ CETP is a hydrophobic plasma glycoprotein that is involved in the exchange of CE and TG between HDL and apoB-containing lipoproteins (*e.g.* VLDL and LDL), resulting in the net transfer of CE from HDL to apoB-containing lipoproteins.¹⁵ Accordingly, CETP deficiency in humans is associated with elevated HDL-cholesterol levels¹⁶ and inhibition of CETP activity by small-molecule inhibitors increased HDL-cholesterol levels.¹⁷⁻²⁰ In addition, bezafibrate,^{21,22} fenofibrate,²³ and ciprofibrate²⁴ increased HDL-cholesterol in subjects with hyperlipidemia with a concomitant reduction in plasma CETP activity. In the latter study, plasma apoAI levels were not affected, which indicates that fibrates may increase HDL-cholesterol levels via apoAI-independent mechanisms, including a potential effect of fibrates on CETP expression.

Therefore, our aim was to investigate whether the fibrate-induced increase in HDL-cholesterol depends on CETP expression. Hereto, *APOE*3-Leiden (E3L)* mice, with a human-like lipoprotein profile,^{25,26} were crossbred with mice expressing human *CETP* under control of its natural flanking regions.²⁷ *CETP.E3L* and *E3L* littermates were fed a cholesterol-rich (0.25%, w/w) diet with or without 0.04% fenofibrate. Fenofibrate did not affect HDL levels in *E3L* mice after two weeks of drug administration, but increased HDL-cholesterol in *CETP.E3L* mice. In addition, fenofibrate reduced hepatic *CETP* mRNA expression, and plasma CETP mass and activity. From these studies we

conclude that fenofibrate increases HDL-cholesterol by reducing CETP-dependent transfer of CE from HDL to apoB-containing lipoproteins.

Materials and Methods

Animals

Hemizygous human *CETP* transgenic (*CETP*) mice, expressing a human *CETP* mini-gene under the control of its natural flanking sequences²⁷ were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and crossbred with hemizygous *E3L* mice²⁸ at our Institutional Animal Facility to obtain *E3L* and *CETP.E3L* littermates. Mice were housed under standard conditions in conventional cages and had free access to food and water. At the age of 8 weeks, mice were fed a semi-synthetic cholesterol-rich diet, containing 0.25% (w/w) cholesterol and 15% (w/w) fat (Western-type diet) (Hope Farms, Woerden, The Netherlands) for three weeks. Upon randomization according to total plasma cholesterol (TC) levels, mice received Western-type diet with or without 0.04% (w/w) fenofibrate (Sigma, St. Louis, MO, USA). Experiments were performed after 4 h of fasting at 12:00 pm with food withdrawn at 8:00 am, unless indicated otherwise. The institutional Ethical Committee on Animal Care and Experimentation has approved all experiments.

Plasma lipid and lipoprotein analysis

Plasma was obtained via tail vein bleeding as described²⁹ and assayed for TC and TG, using the commercially available enzymatic kits 236691 and 11488872 (Roche Molecular Biochemicals, Indianapolis, IN, USA), respectively. The distribution of lipids over plasma lipoproteins was determined by fast-performance liquid chromatography (FPLC) using a Superose 6 column as described previously.²⁹

CETP activity and mass determination

CETP activity in plasma was measured as the transfer of [³H]cholesteryl oleate ([³H]CO) from exogenous LDL to HDL as described elsewhere.³⁰ CETP activity was calculated as $\mu\text{mol CE transfer per ml plasma per h}$. Plasma CETP mass was analyzed by a two-antibody sandwich immunoassay as described previously.³¹

Plasma apoAI concentration

Plasma apoAI concentrations were determined using a sandwich ELISA. Hereto, rabbit anti-mouse apoAI polyclonal antibody (ab20453; Abcam plc, Cambridge, UK) was coated overnight onto Costar strips (Costar, Inc., New York, NY, USA) (3 $\mu\text{g/ml}$) at 4°C and incubated with diluted mouse plasma (dilution 1:400,000) for 90 min at 37°C. Subsequently, goat anti-mouse apoAI antibody (600-101-196; Rockland Immunochemicals, Inc., Gilbertsville, PA, USA; dilution 1:3000) was added and incubated for 90 min at 37°C. Finally, horse radish peroxidase (HRP)-conjugated rabbit anti-goat IgG antibody (605-4313; Rockland; dilution 1:15000) was added and incubated for 90 min at 37°C. HRP was detected by incubation with tetramethylbenzidine (Organon Teknika, Boxtel,

The Netherlands) for 15 min at room temperature. Purified mouse apoAI (A23100m; Biodesign International, Saco, Maine, USA) was used as a standard.

Radiolabeling of autologous HDL

One mouse from each experimental group was killed by cervical dislocation and blood was drawn from the retro-orbital vein. Sera were collected and HDL was isolated after density ultracentrifugation in a SW 40 Ti rotor (Beckman Instruments, Geneva, Switzerland) (4°C; 40,000 rpm; overnight).³² HDL (0.4 μmol HDL-cholesterol) was radiolabeled by incubation (37°C; 24 h) with [³H]cholesteryl oleyl ether ([³H]COEth)-labeled egg yolk phosphatidylcholine vesicles (40 μCi; 0.5 mg phosphatidylcholine) in the presence of lipoprotein deficient serum (1 ml) from *CETP.E3L* mice. Subsequently, HDL was re-isolated after density ultracentrifugation (12°C; 40,000 rpm; 24 h).

In vivo clearance of autologous HDL

After 6 weeks of diet, mice were injected via the tail vein with a trace of autologous [³H]COEth-labeled HDL (0.2x10⁶ cpm in PBS) at 8:00 am. At the indicated time points after injection, blood was collected to determine the serum decay of [³H]COEth by scintillation counting (Packard Instruments, Downers Grove, IL, USA). The total plasma volumes of the mice were calculated from the equation $V \text{ (ml)} = 0.04706 \times \text{body weight (g)}$, as determined from previous ¹²⁵I-BSA clearance studies.³³ The fractional catabolic rate (FCR) was calculated from the serum decay curves as described previously.³⁴ Briefly, curves were fitted using GraphPad Prism software, giving the best fit for one-phase exponential decay curves, described by the formula $Y = \text{span} \times \exp(-k \times x) + \text{plateau}$. Subsequently the FCR was calculated as $\text{span} / (\text{Area Under the Curve})$.

Hepatic mRNA expression, SR-BI protein, and lipid analysis

Livers were isolated after cervical dislocation. Total RNA was isolated using the NucleoSpin[®] RNA II kit (Macherey-Nagel, Düren, Germany) as recommended by the manufacturer. RNA expression was determined in duplicate by real-time PCR on a MyiQ Single-Color real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Primers for *CETP*³⁵ and *Sr-b1*³⁶ have been described previously. Primers for *Abca1*, *Apoa1*, *Cyp7A1*, and *Pltp* are listed in table 1. Expression levels were normalized using HPRT and cyclophilin as housekeeping genes.^{36,37} Hepatic SR-BI protein was determined by immunoblot analysis as described previously.³⁸ Liver lipids were determined by homogenizing liver samples in water (ca. 10% wet w/v) using a mini-beadbeater (Biospec Products, Inc., Bartlesville, OK, U.S.A.; 20 sec; 5000 rpm), followed by lipid-extraction as described by Bligh and Dyer.³⁹ Extracts were assayed for TC as described above. Protein was determined according to the method of Lowry *et al.*⁴⁰

Statistical analysis

All data are presented as means ± SD unless indicated otherwise. Data were analyzed using the unpaired Student's *t* test. *P*-values less than 0.05 were considered statistically significant.

Table 1. Primers for quantitative real-time PCR analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>Abca1</i>	CCCAGAGCAAAAAGCGACTC	GGTCATCATCACTTTGGTCCTTG
<i>Apoa1</i>	GGAGCTGCAAGGGAGACTGT	TGCGCAGAGAGTCTACGTGTGT
<i>Cyp7a1</i>	CAGGGAGATGCTCTGTGTTCA	AGGCATACATCCCTTCCGTGA
<i>Pltp</i>	TCAGTCTGCGCTGGAGTCTCT	AAGGCATCACTCCGATTGC

Abca1, ATP-binding cassette transporter a1; *Apoa1*, apolipoprotein a1; *Pltp*, phospholipid transfer protein

Results

Fenofibrate increases HDL-cholesterol in CETP.E3L mice

To study the effect of fenofibrate on plasma lipid levels in hyperlipidemic mice, *E3L* and *CETP.E3L* mice were fed a cholesterol-rich diet with or without 0.04% fenofibrate for two weeks. On such a cholesterol-rich diet, female mice have higher plasma TG and cholesterol levels (Fig. 1) than male mice, as reflected by increased VLDL levels (Fig. 2). This difference is caused by a higher VLDL-production.⁴¹ In *E3L* mice, fenofibrate decreased plasma TG levels both in females (-68%; $P < 0.001$) (Fig. 1A) and in males (-59%; $P < 0.001$) (Fig. 1B). Concomitantly, plasma cholesterol levels were decreased in females (-70%; $P < 0.001$) (Fig. 1C), whereas only a trend towards reduction was observed in males (Fig. 1D). These changes in plasma lipid levels were reflected by a strong reduction in VLDL-TG (not shown). Upon fenofibrate administration to *E3L* mice, cholesterol was strongly decreased in VLDL, intermediate-density lipoprotein (IDL) and LDL in females (-85%; Fig. 2A) and males (-91%; Fig. 2B), whereas HDL-cholesterol was not affected. Apart from typical IDL/LDL (fractions 9-13) and HDL (fractions 17-22), an additional particle was observed at fractions 14-16. This particle has previously been characterized as large apoE-rich HDL₁²⁵ and was also not affected by fenofibrate feeding.

Administration of fenofibrate to *CETP.E3L* mice had similar effects on total plasma lipid levels as compared to *E3L* mice. Plasma TG levels were decreased in females (-71%; $P < 0.01$) (Fig. 1A) and males (-60%; $P < 0.01$) (Fig. 1B), and plasma TC levels were reduced in females (-86%; $P < 0.01$), while a trend towards reduction was observed in males (Fig. 1D). Similarly as in *E3L* mice, fenofibrate caused a strong reduction in VLDL-TG (not shown) as well as in VLDL/IDL/LDL-cholesterol in females (-88%; Fig. 2C) and males (-93%; Fig. 2D). However, whereas HDL-cholesterol levels were not increased in *E3L* mice, fenofibrate treatment of *CETP.E3L* mice resulted in strongly elevated HDL-cholesterol levels both in females (+54%; Fig. 2C) and in males (+91%; Fig. 2D).

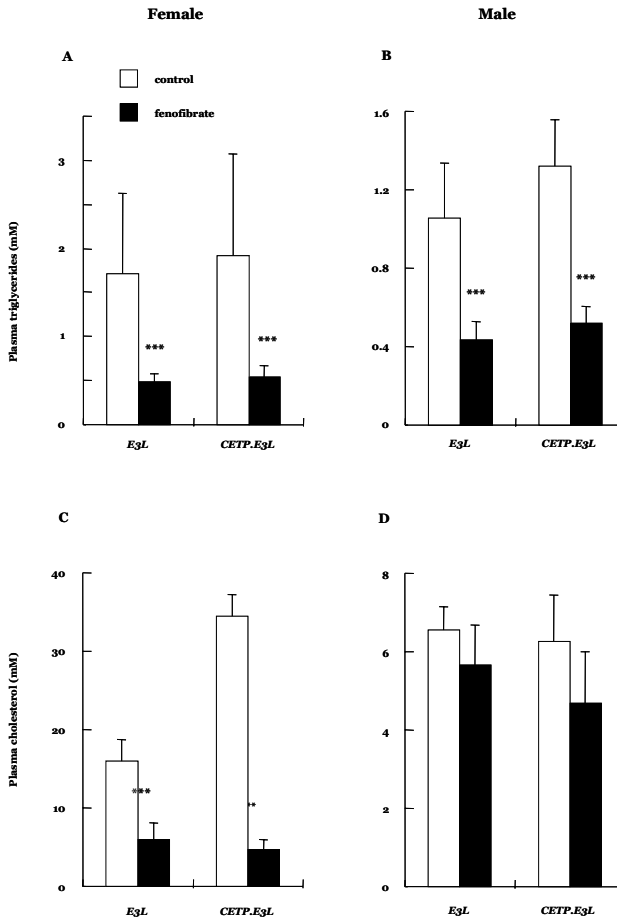


Figure 1. Effect of fenofibrate on plasma triglycerides and cholesterol. *E3L* and *CETP.E3L* female (A,C) and male (B,D) mice received a Western-type diet with (closed bars) or without (open bars) fenofibrate for two weeks. Plasma triglycerides (A,B) and cholesterol (C,D) were determined. Values are means \pm SD (n=6 per group). *** P <0.001 compared to control.

Fenofibrate increases the steady-state plasma HDL level without affecting HDL turnover in CETP.E3L mice

To examine the mechanism underlying the fenofibrate-induced increased HDL-cholesterol in *CETP.E3L* mice, male *E3L* and *CETP.E3L* mice were injected with autologous [3 H]COEth-labeled HDL and the serum decay was determined (Fig. 3). The expression of CETP *per se* appeared to accelerate the serum decay, as reflected by an increased fractional catabolic rate (FCR) as calculated pools of HDL-CE cleared per hour (+65%; P <0.01; Table 2). In *E3L* mice, fenofibrate administration did not affect the clearance of HDL-CE (Fig. 3A; Table 2). In contrast, fenofibrate decreased the FCR of HDL in *CETP.E3L* mice (-27%; P <0.01). However, the FCR as calculated as mM HDL-CE cleared per hour, was not affected by the expression of CETP in *E3L* mice, nor by

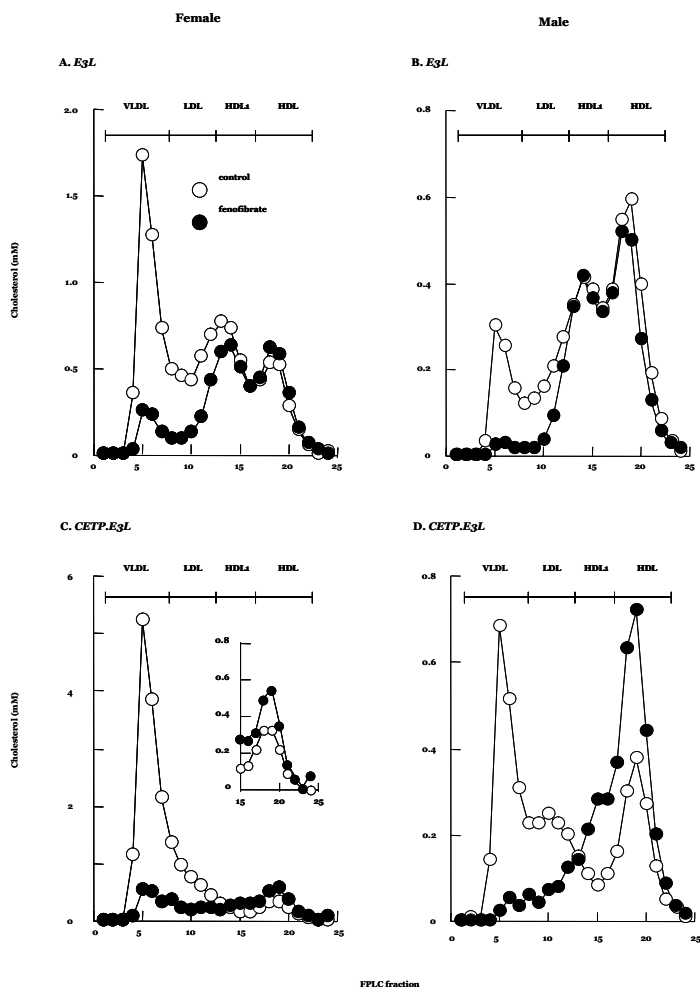


Figure 2. Effect of fenofibrate on the distribution of cholesterol over lipoproteins. Female (A,C) and male (B,D) *E3L* (A,B) and *CETP.E3L* (C,D) mice received a Western-type diet with (closed circles) or without (open circles) fenofibrate for 2 weeks. Plasmas of the various mouse groups were pooled (n=6 per group). Lipoproteins were separated by FPLC and fractions were analyzed for cholesterol.

fenofibrate feeding of either *E3L* or *CETP.E3L* mice (Table 2). This indicates that CETP expression and fenofibrate feeding alter the steady-state plasma HDL-cholesterol level without affecting the net HDL-cholesterol flux through plasma.

Fenofibrate decreases hepatic CETP mRNA expression and plasma CETP mass and activity

Since differences in genes encoding proteins that are crucially involved in HDL metabolism may account for the increase in HDL-cholesterol in *CETP.E3L* mice upon

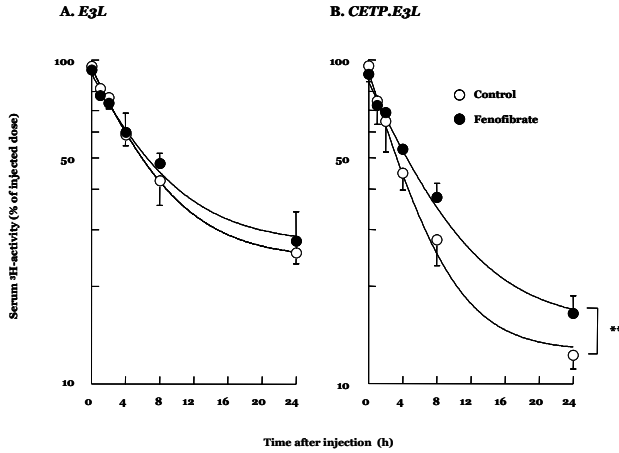


Figure 3. Effect of fenofibrate on the plasma clearance of HDL. *E3L* (A) and *CETP.E3L* (B) male mice received a Western-type diet with (closed circles) or without (open circles) fenofibrate for 6 weeks. Mice were injected with autologous [³H]COEth-labeled HDL and serum ³H-activity was determined at the indicated time points. Values are means ± SD (n=5 per group). ***P*<0.01 compared to control.

Table 2. Effect of fenofibrate on the FCR of HDL-CE in *CETP.E3L* and *E3L* mice

	Control	Fenofibrate
FCR (pools HDL-CE per h)		
<i>E3L</i>	0.067±0.003	0.057±0.004
<i>CETP.E3L</i>	0.111±0.006	0.081±0.003*
FCR (mM HDL-CE per h)		
<i>E3L</i>	0.166±0.008	0.162±0.011
<i>CETP.E3L</i>	0.142±0.008	0.162±0.007

E3L and *CETP.E3L* male mice were fed a Western-type diet with or without fenofibrate. After 6 weeks, mice were injected with autologous [³H]COEth-labeled HDL. The data from figure 3 were used to calculate the fractional catabolic rate (FCR) as pools of HDL-CE or mM HDL-CE cleared per hour. Values are expressed as means ± S.E.M. relative to control mice (n=5 mice per group). **P*<0.01 compared to control.

Table 3. Effect of fenofibrate on hepatic mRNA expression in *CETP.E3L* and *E3L* mice

	<i>E3L</i> mice		<i>CETP.E3L</i> mice	
	Control	Fenofibrate	Control	Fenofibrate
<i>Cyp7a1</i>	1.00±0.18	0.50±0.07*	1.00±0.20	0.43±0.08*
<i>Pltp</i>	1.00±0.18	3.25±0.36**	1.00±0.22	2.65±0.45*
<i>Abca1</i>	1.00±0.15	0.50±0.08*	1.00±0.06	0.77±0.04*
<i>Sr-b1</i>	1.00±0.14	0.52±0.04*	1.00±0.12	0.58±0.10*
<i>Apoa1</i>	1.00±0.20	0.51±0.02*	1.00±0.21	0.59±0.04*
<i>CETP</i>	n.d.	n.d.	1.00±0.12	0.28±0.05**

E3L and *CETP.E3L* mice were fed a Western-type diet with or without fenofibrate. After 6 weeks, livers were collected to determine mRNA expression. Values are expressed as means ± S.E.M. relative to control mice (n=4 per group). n.d., not detectable. * $P < 0.05$; ** $P < 0.01$ compared to controls.

fenofibrate treatment, we examined the effect of fenofibrate on their hepatic expression (Table 3). *Pltp* was increased in *E3L* (3.5-fold; $P < 0.01$) and *CETP.E3L* mice (4.6-fold; $P < 0.05$), consistent with previously reported effects of fenofibrate.^{10,11} The expression of the *Abca1*, involved in HDL formation, was also decreased in *E3L* (-50%; $P < 0.05$) and *CETP.E3L* (-33%; $P < 0.05$) mice. Likewise, *Sr-b1* was decreased in *E3L* (-48%; $P < 0.05$) and *CETP.E3L* (-42%; $P < 0.05$) mice to a similar extent, as reflected by similar reductions in hepatic SR-BI protein levels (approximately -25%) for *E3L* ($P = 0.06$) and *CETP.E3L* mice ($P < 0.05$) (Fig. 4). *Apoa1* expression was decreased in *E3L* (-49%; $P < 0.05$) and *CETP.E3L* (-41%; $P < 0.05$) mice without affecting the plasma apoAI level (approximately 80 mg/dl in all groups).

The expression of *Pltp*, *Abca1*, *Sr-b1*, and *Apoa1* are thus similarly affected by fenofibrate in *E3L* and *CETP.E3L* mice, and can thus not explain the differentially raised HDL in *CETP.E3L* mice as compared to *E3L* mice. Therefore, the HDL-raising effect of fenofibrate in *CETP.E3L* mice is likely to be a direct consequence of CETP modulation. Indeed, fenofibrate strongly decreased CETP expression in *CETP.E3L* mice (-72%; $P < 0.01$). Since the liver X receptor (LXR) strongly regulates the expression of *CETP*,⁴² we determined whether fenofibrate feeding would decrease the cholesterol content in the liver. Indeed, fenofibrate reduced the hepatic cholesterol content in *E3L* (4.9±2.6

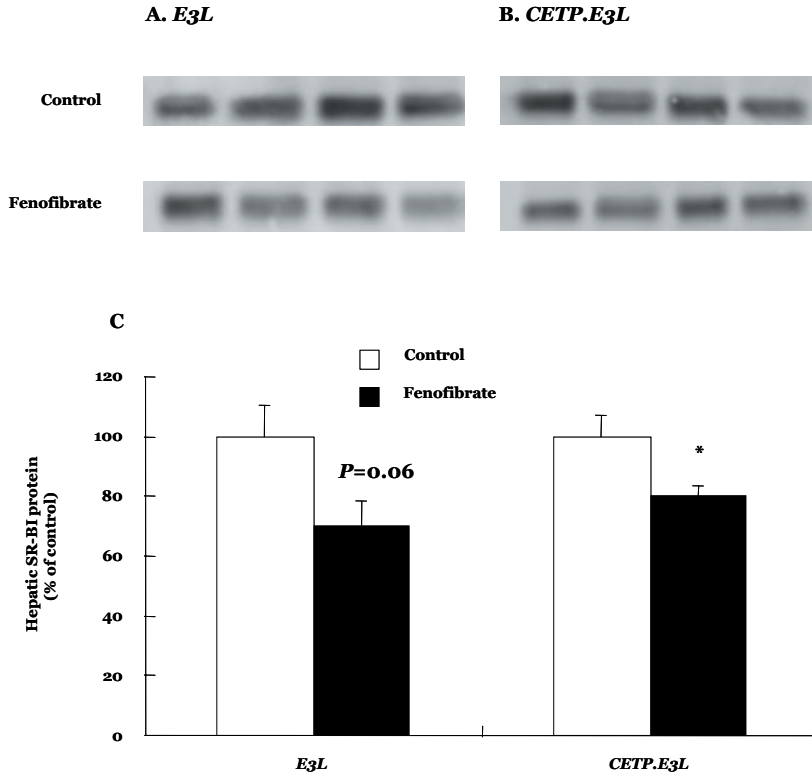


Figure 4. Effect of fenofibrate on hepatic SR-BI protein levels. *E3L* (A) and *CETP.E3L* (B) male mice received a Western-type diet with (closed bars) or without (open bars) fenofibrate for 6 weeks. Livers were isolated after cervical dislocation. SR-BI protein was determined by immunoblot analysis. Intensity of bands were determined by pixel counting and calculated relative to the control mice (C). Values are means \pm S.E.M. (n=4 per group). * P <0.05 compared to controls.

vs. 9.6 ± 3.7 μ g TC/mg protein) and *CETP.E3L* mice (3.6 ± 1.0 vs. 13.0 ± 3.7 μ g TC/mg protein; P <0.05).

The fenofibrate-induced reduction in hepatic *CETP* expression was accompanied by a trend towards reduction in plasma *CETP* mass (23 ± 5 vs. 25 ± 8 μ g/ml; -8%) and activity (0.57 ± 0.13 vs. 0.63 ± 0.18 μ mol CE/ml/h; -9%). Apart from hepatic mRNA expression, plasma *CETP* levels are also determined by the plasma HDL-cholesterol level.⁴³ Indeed, *CETP* activity was predominantly found on HDL, since similar *CETP* activities were measured in plasma with and without prior precipitation of the apoB-containing lipoproteins (0.52 ± 0.08 vs. 0.56 ± 0.19 μ mol CE/ml/h). Adjustment for HDL-cholesterol resulted in a significant reduction in the *CETP* mass (11 ± 2 vs. 20 ± 6

$\mu\text{g CETP}/\mu\text{mol HDL-C}$); -42% ; $P < 0.01$) and CETP activity (0.29 ± 0.06 vs. 0.50 ± 0.14 $\mu\text{mol CE}/\text{h}/\mu\text{mol HDL-C}$); -42% , $P < 0.01$). In female mice, plasma CETP mass (84 ± 31 vs. 130 ± 30 $\mu\text{g}/\text{ml}$); -35% , $P < 0.05$) and activity (1.07 ± 0.27 vs. 1.58 ± 0.01 $\mu\text{mol CE}/\text{ml}/\text{h}$; -32% , $P < 0.01$) were decreased even without adjusting for HDL-cholesterol.

Discussion

In addition to reducing plasma TG levels in humans and mice, fenofibrate increases HDL-cholesterol levels in humans,⁴ but not in mice.^{5,7,10,12} Since humans express CETP,¹³ whereas mice do not,¹⁴ we investigated whether CETP might play a role in the fenofibrate-induced increase in HDL-cholesterol. Here we show that fenofibrate increases HDL-cholesterol in *CETP.E3L* mice, as paralleled by a reduction in hepatic *CETP* mRNA and plasma CETP activity, whereas such an effect was not observed in *E3L* mice.

We have previously shown that *E3L* mice are highly susceptible to dietary interventions with respect to modulating plasma lipid levels, and that these mice show a human-like response to drug interventions aimed at reducing plasma levels of apoB-containing lipoproteins, including statins (atorvastatin⁴⁴ and rosuvastatin⁴⁵) and fibrates (gemfibrozil⁴⁶). This is in sheer contrast with wild-type mice^{5,12} and more conventional hyperlipidemic mice such as apoE-deficient^{12,47} or LDL receptor-deficient⁴⁸ mice, which show either an adverse or no response to such interventions. In particular, administration of fenofibrate to wild-type¹² and apoE-deficient^{12,47} mice showed an unexpected increase in plasma TG and TC levels caused by elevated levels of lipoprotein remnants, with a concomitant reduction in HDL-cholesterol. In the present paper, we demonstrate that *E3L* mice also show a human-like response to fenofibrate with respect to decreasing TG and cholesterol in apoB-containing particles, albeit that HDL-cholesterol was not increased. We reasoned that introduction of human CETP in these *E3L* mice, which permits cholesteryl ester exchange between HDL and apoB-containing lipoproteins, would thus result in an excellent mouse model to study the effects of fenofibrate on HDL metabolism.

Indeed, we demonstrate that while *CETP.E3L* mice retain their ability to respond to fenofibrate with respect to a similar reduction of VLDL-TG and VLDL-cholesterol as compared with *E3L* mice, they now also respond by an increase in HDL-cholesterol level. Apparently, the fact that mice normally do not express CETP prevents a human-like response to HDL-modulating drug interventions, like fibrate treatment. In agreement with this hypothesis, we have previously observed that treatment of *E3L* mice with statins also did not increase HDL-cholesterol albeit that VLDL reductions of as much as 60% were achieved.^{44,46,49} HDL-cholesterol levels can theoretically be modulated by several key proteins involved in HDL metabolism, including ABCA1,⁹ SR-BI,⁸ PLTP,^{10,22} apoAI,^{2,5,50-52} and CETP.^{21,23,24} Therefore, we examined the potential contribution of each of these factors in the fenofibrate-induced increase of HDL-cholesterol in *CETP.E3L* mice.

The HDL-cholesterol level in mice is largely determined by the hepatic *Abca1* expression, which results in the export of hepatic cholesterol towards nascent HDL in

plasma.⁵³ In fact, it has been reported that treatment of chow-fed rats with ciprofibrate increased their hepatic *Abca1* expression, concomitant with an increase in plasma HDL-cholesterol levels.⁵⁴ However, fenofibrate did not increase hepatic *Abca1* expression in either *E3L* or *CETP.E3L* mice. On the contrary, a strong trend towards reduction of *Abca1* mRNA was observed in both genotypes. Since a reduction of ABCA1 is linked with reduced HDL-cholesterol levels,⁵³ this indicates that the effect of fenofibrate on HDL-cholesterol in *CETP.E3L* mice can thus not be explained by altered *Abca1* expression.

Whereas bezafibrate did not increase the plasma PLTP mass and activity levels in humans,²² fenofibrate has been shown to increase the hepatic *Pltp* expression in mice, which was associated with increased plasma PLTP activity and HDL size, at least in human *APOA1* transgenic mice.¹⁰ Accordingly, we found that fenofibrate induced the hepatic *Pltp* expression both in *E3L* and *CETP.E3L* mice. However, the relative increase was even more pronounced in *E3L* mice as compared to *CETP.E3L* mice, while HDL-cholesterol was not affected in *E3L* mice. In this respect, it is also of note that adenovirus-mediated hepatic expression of *PLTP* results in a dose-dependent reduction of HDL-cholesterol levels, instead of increasing HDL-cholesterol, in both wild-type and human *APOA1*-transgenic mice.⁵⁵ Therefore it is unlikely that the induction of PLTP is the cause for the increase in HDL-cholesterol as observed in *CETP.E3L* mice.

In mice, hepatic SR-BI represents the most important pathway for the selective clearance of HDL-associated cholesteryl esters from plasma.⁵⁶ It has been shown that fenofibrate can down-regulate hepatic SR-BI protein in wild-type mice, independent of *Sr-b1* expression, via a post-transcriptional mechanism, which was correlated with an increased HDL size based on FPLC profiling.⁷ We found that fenofibrate treatment did result in a similar reduction of *Sr-b1* expression in *E3L* (-48%) and *CETP.E3L* mice (-42%), with a concomitant reduction in hepatic SR-BI protein levels (-20-30%). However, whereas we did observe an increase in HDL₁ cholesterol levels (+69%) after prolonged administration of fenofibrate in *E3L* mice (*i.e.* six weeks), as has also been shown for wild-type mice by Mardones *et al.*,⁷ this was not observed in *CETP.E3L* mice. Instead, we found increased levels of cholesterol in regularly sized HDL in *CETP.E3L* mice. Since similar reductions in SR-BI were observed in *E3L* and *CETP.E3L* mice, whereas the increase in HDL-cholesterol was observed only in *CETP.E3L* mice, SR-BI reduction can be ruled out as a causal factor for HDL-cholesterol elevation in *CETP.E3L* mice.

In human *APOA1* transgenic mice, human *APOA1* hepatic mRNA and plasma protein levels were increased after fenofibrate treatment,⁵ probably by the binding of PPAR α to a positive PPRE in the human *APOA1* gene promoter.⁵² Given the tight relation between HDL-cholesterol and apoAI levels in humans, it could thus be expected that upregulation of apoAI expression would be the main causal factor for increasing HDL-cholesterol levels in humans. Fenofibrate treatment has an opposite effect on murine apoAI (*i.e.* reduction of expression and plasma levels),⁵ which could theoretically thus easily explain why fenofibrate does not increase HDL-cholesterol in mice. However, although we do observe a reduction of hepatic *Apoa1* expression upon fenofibrate treatment of *E3L* (-49%) and *CETP.E3L* mice (-41%). HDL-cholesterol is never-

theless markedly increased in *CETP.E3L* mice. The fact that plasma apoAI was not affected may thus be explained by increased lipidation of apoAI, thereby preventing the clearance of apoAI.

Collectively, these data thus suggest that down-regulation of *CETP* expression is the predominant cause of the fenofibrate-induced elevation of HDL-cholesterol. Expression of *CETP* in *E3L* mice decreased the HDL-cholesterol level (approximately -35%) and increased the FCR of HDL-CE as calculated as pools of HDL-CE cleared per hour (+65%; $P < 0.01$), consistent with previous findings on *CETP* expression in rats.⁵⁷ However, the HDL-CE turnover as calculated as mM HDL-CE cleared per hour, was not affected by the expression of *CETP* in *E3L* mice. This indicates that *CETP* expression does not affect the net HDL-cholesterol flux through plasma, but nevertheless decreases the steady-state plasma cholesterol level. Likewise, Kee *et al.*⁵⁸ showed that *CETP* inhibition in rabbits does not compromise the HDL-CE clearance from plasma, while increasing HDL-cholesterol. Treatment of *CETP.E3L* mice with fenofibrate resulted in an increased HDL-cholesterol level, strongly decreased hepatic *CETP* expression levels, and reduced plasma *CETP* mass and activity levels. The increase in HDL-cholesterol may thus be caused by the combination of reduced hepatic *CETP* expression and reduced levels of apoB-containing lipoproteins as CE acceptors, thereby inhibiting the *CETP*-mediated transfer of CE from HDL to (V)LDL.

It is tempting to speculate about the mechanism(s) underlying the effect of fenofibrate on hepatic *CETP* expression. Dietary cholesterol has been shown to increase *CETP* mRNA expression in *CETP* transgenic mice,²⁷ possibly via an LXR responsive element in the *CETP* promoter.⁴² Conversely, a decrease in hepatic *CETP* mRNA expression might thus be the consequence of a reduction in LXR signaling. Fenofibrate treatment indeed decreased both plasma and hepatic cholesterol, which is likely to reduce the level of oxysterols, the natural ligands of LXR α . In addition, PPAR α -activation by fenofibrate might be directly responsible for the inhibition of LXR α by binding to a PPRE in its regulatory region. Downregulation of LXR α is supported by a concomitant decrease in the expression of *Cyp7a1*, another LXR-target gene.⁵⁹ This is in accordance with previous data of Post *et al.*,⁶⁰ who showed a 65% reduction in hepatic *Cyp7a1* mRNA upon ciprofibrate administration to wild-type mice. Nevertheless, it should be mentioned that *Cyp7a1* is also regulated directly by fibrates via a negative PPRE in its promoter sequence.⁶¹ In line with the findings of Repa *et al.*,⁶² a reduction in LXR α might also explain the reduction of *Abca1* expression. Besides these mechanisms explaining reduced *CETP* expression by fenofibrate, Cheema *et al.*⁶³ recently identified a potential PPRE in the promoter region of *CETP*, which provides the possibility for direct regulation of *CETP* by PPAR α agonists, albeit that it is unclear as yet whether this potential PPRE is functional.

Fibrate treatment has been associated with a reduction of cardiovascular disease.⁴ The recent FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) study, which assessed the effects of fenofibrate on cardiovascular risk in subjects with type 2 diabetes mellitus in a long-term, controlled trial, showed a reduction in total cardiovascular events, but did not reveal a reduced risk of the primary outcome of coronary events.⁶⁴ Nevertheless, the authors suggested that a more beneficial outcome might

have been masked by a larger portion of statin treatment in the placebo group as compared to the fenofibrate group. Even though the benefit of an increase in HDL-cholesterol by CETP-inhibition is still under debate,⁶⁵⁻⁶⁸ raising HDL-cholesterol levels is generally considered anti-atherogenic. Besides the ability of fibrates to potently reduce plasma TG, their concomitant effect on increasing HDL by reduction of CETP activity may thus be an additional advantageous anti-atherogenic property. It may be speculated that combination therapies of fibrates (*i.e.* reducing CETP expression) with small molecule CETP inhibitors (*i.e.* reducing plasma CETP activity) may help to further reduce cardiovascular risk.

Taken together, our data show that fenofibrate increases HDL-cholesterol by reducing the CETP expression and activity in *CETP.E3L* mice. Therefore, we postulate that the increase in HDL that is found in subjects after fibrate administration is caused by reduced CETP activity, probably acting in concert with apoAI, ABCA1, PLTP, and possibly CD36- and LIMPII-analogous 1 (CLA1), the human homologue of SR-BI. Studies using *CETP.E3L* mice can provide valuable information of the benefit that may be expected from combination therapy involving administration of fibrates and CETP inhibitors.

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