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

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8 Atorvastatin Increases HDL Cholesterol by Reducing Cholesteryl Ester Transfer Protein

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Objective - In addition to lowering low-density lipoprotein (LDL)-cholesterol, statins modestly increase high-density lipoprotein (HDL)-cholesterol in humans. This increase is not seen in mice, a species without cholesteryl ester transfer protein (CETP) expression. Therefore, our aim was to determine whether the increase in HDL depends on CETP expression.

Methods and Results - *APOE*3-Leiden* (*E3L*) mice, with a human-like lipoprotein profile and a human-like responsiveness to statin treatment, were crossbred with *CETP* transgenic mice. Whereas atorvastatin-treatment (0.01% in diet) reduced VLDL-cholesterol in both *E3L* and *CETP.E3L* mice (by >80%), HDL-cholesterol increased only in *CETP.E3L* mice (+52%). Atorvastatin down-regulated hepatic *CETP* expression in *CETP.E3L* mice (-57%; $P<0.01$), and reduced plasma CETP mass (-45%; $P<0.05$) and activity (-57%; $P<0.01$), the latter two when adjusted for HDL-cholesterol. Hepatic expression levels of genes involved in HDL metabolism, such as *Pltp*, *Abca1*, *Sr-b1*, and *Apoa1*, were not differently affected by atorvastatin as compared to those in *E3L* mice. Finally, a dose escalation study showed that atorvastatin decreased plasma CETP mass and activity, and increased HDL-cholesterol in a dose-dependent manner.

Conclusion - Atorvastatin 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.

Epidemiological studies have established that a high level of low-density lipoprotein (LDL)-cholesterol is a major cardiovascular risk factor.[1] In the past decades, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (*i.e.* statins) have been successfully used to reduce LDL-cholesterol. Statins inhibit this rate-determining enzyme of cholesterol synthesis, which results in hepatic depletion of cholesterol.[2,3] As a consequence, VLDL production is reduced and the hepatic expression of the LDL receptor (LDLr) is upregulated, leading to decreased plasma cholesterol levels in apoB-containing lipoproteins (*i.e.* VLDL and LDL).[4,5] Indeed, a meta-analysis of 25 studies indicated that statins reduce LDL-cholesterol levels by 20-40%.[6] In addition, statins elevate high-density lipoprotein (HDL)-cholesterol levels by typically 5-15%.[7-9]

Low HDL-cholesterol has been confirmed as a strong and independent risk factor for cardiovascular disease in a meta-analysis of four prospective studies. An increase in HDL-cholesterol of 1 mg/dl resulted in a 2-3% decrease in cardiovascular risk.[10] One of the key players in HDL-metabolism is cholesteryl ester transfer protein (CETP), a hydrophobic plasma glycoprotein. CETP transfers neutral lipids (*e.g.* triglycerides [TG] and cholesteryl esters [CE]) between lipoproteins, resulting in the net flux of CE from HDL towards apoB-containing lipoproteins in exchange for TG.[11,12] Accordingly, CETP-deficient subjects display increased HDL-cholesterol levels[13] and also inhibition of CETP activity by small-molecule inhibitors leads to increased HDL-cholesterol levels.[14-17]

Treatment of patients with combined hyperlipidemia with atorvastatin resulted in increased levels of relatively CE-rich large HDL_{2a} with a concomitant decrease in CE-poor small HDL_{3c}.[18] This was associated with a minor reduction in CETP mass and a decrease in total CETP-mediated CE transfer from HDL to apoB-containing lipoproteins.[18] Simvastatin treatment of normolipidemic subjects also resulted in an increase in HDL-cholesterol (+8.3%), with a concomitant reduction in CETP concentration (-26%).[19] Likewise, in type 2 diabetic subjects carrying the CETP TaqIB polymorphism, the increase in HDL-cholesterol (+7.2%) after atorvastatin treatment is correlated with the reduction in CETP mass (-18%).[20] Although these results indicate that the effects of statin treatment on HDL-cholesterol levels are related to a reduction in CETP-mediated transfer of CE, a causal relationship between statin-induced reduced CETP activity and increased HDL-cholesterol levels has not been proven as yet.

*APOE*3-Leiden* (*E3L*) transgenic mice are an established model for hyperlipidemia and atherosclerosis[21,22] and display a human-like lipoprotein profile.[23,24] In contrast to treatment of wild-type and other hyperlipidemic mouse lines,[25-27] administration of atorvastatin to *E3L* mice resulted in reductions in total cholesterol (TC) levels, by lowering apoB-containing lipoproteins, as observed in humans.[28] However, in contrast to humans, in *E3L* mice HDL-cholesterol levels were not increased by atorvastatin treatment.[28,29] Of note is that *E3L* mice, like other mice, do not express CETP,[30] whereas humans do.[31] Therefore, the aim of this study was to evaluate whether the effect of statin treatment on HDL-cholesterol levels would depend on CETP expression. Hereto, *E3L* mice were crossbred with transgenic mice expressing human *CETP* under control of the natural flanking regions (*E3L.CETP* mice).[32] Whereas HDL-cholesterol was not affected in *E3L* mice, atorvastatin indeed increased HDL-cholesterol levels in *CETP*. *E3L* mice. In addition, hepatic *CETP* mRNA expression, and plasma CETP mass and

activity were reduced. From these results we conclude that atorvastatin increases HDL-cholesterol by reducing CETP expression and activity.

Materials and Methods

Animals

Hemizygous human *CETP* transgenic (*CETP*) mice, expressing a human *CETP* minigene under the control of natural flanking sequences[32] were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and crossbred with hemizygous *E3L* mice[22] at our Institutional Animal Facility to obtain *E3L* and *CETP.E3L* littermates (C57Bl/6J background). Mice were housed under standard conditions in conventional cages and had free access to food and water. Mice were fed a semi-synthetic diet containing 15% [w/w] fat (Hope Farms, Woerden, The Netherlands), supplemented with either 0.1% or 0.25% (w/w) cholesterol (Sigma, St. Louis, MO, USA) for two weeks. Subsequently, the mice received the same diet with or without atorvastatin (Lipitor®20, Pfizer B.V., Capelle a/d IJssel, The Netherlands). 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 described[33] and assayed for total cholesterol (TC) using the enzymatic kit 236691 (Roche Molecular Biochemicals, Indianapolis, IN, USA). The distribution of lipids over plasma lipoproteins was determined by fast-performance liquid chromatography (FPLC) as described previously.[33]

CETP Activity and Mass Determination

CETP activity in plasma was measured as the transfer of [^{3}H]cholesteryl oleate ([^{3}H]CO) from exogenous LDL to HDL as described elsewhere.[34] CETP activity was calculated as μmol CE transfer per ml plasma per h. Plasma CETP mass was analyzed by a two-antibody sandwich immunoassay as described previously.[35]

ApoAI Plasma 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 μg/ml) at 4°C and incubated with diluted mouse plasma (dilution 1:400000) 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, and 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.

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*[36] and *Sr-b1*[37] have been described previously. Primers for *Abca1*, *Apoa1*, *Hmgcoa reductase*, and *Pltp* are listed in table 1. Expression levels were normalized, using HPRT and cyclophilin as housekeeping genes.[37,38] Hepatic SR-BI protein was determined by immunoblot analysis as described previously.[39]

Table 1. Primers for quantitative real-time PCR analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Hmgcoa reductase	CCGGCAACAACAAGATCTGTG	ATGTACAGGATGGCGATGCA
Abca1	CCCAGAGCAAAAAGCGACTC	GGTCATCATCACTTTGGTCCTTG
Apoa1	GGAGCTGCAAGGGAGACTGT	TGCGCAGAGAGTCTACGTGTGT
Pltp	TCAGTCTGCGCTGGAGTCTCT	AAGGCATCACTCCGATTTGC

Abca1, ATP-binding cassette transporter a1; *Apoa1*, apolipoprotein a1; *Hmgcoa reductase*, hydroxymethylglutaryl coenzyme A reductase; *Pltp*, phospholipid transfer protein

Statistical Analysis

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

Results

Atorvastatin Increases HDL-Cholesterol in Mice Expressing CETP

Treatment of male *E3L* mice, on a diet containing 0.25% (w/w) cholesterol, with atorvastatin (0.01%, w/w) caused a reduction in TC by -25% (3.8±1.2 vs. 5.1±0.9 mM) (data not shown). This effect was reflected by a strong decrease in (V)LDL-cholesterol (-86%), whereas HDL-cholesterol was not affected (Fig. 1A). Atorvastatin induced a similar decrease in TC in *CETP.E3L* mice by -31% (2.9±1.0 vs. 4.3±0.8 mM; $P<0.05$). In *CETP.E3L* mice, atorvastatin also caused a strong reduction in (V)LDL-cholesterol (-88%; Fig. 1B). Moreover, whereas HDL-cholesterol levels were unaffected in *E3L* mice, atorvastatin administration increased HDL-cholesterol (+52%; Fig. 1B) in *CETP.E3L* mice.

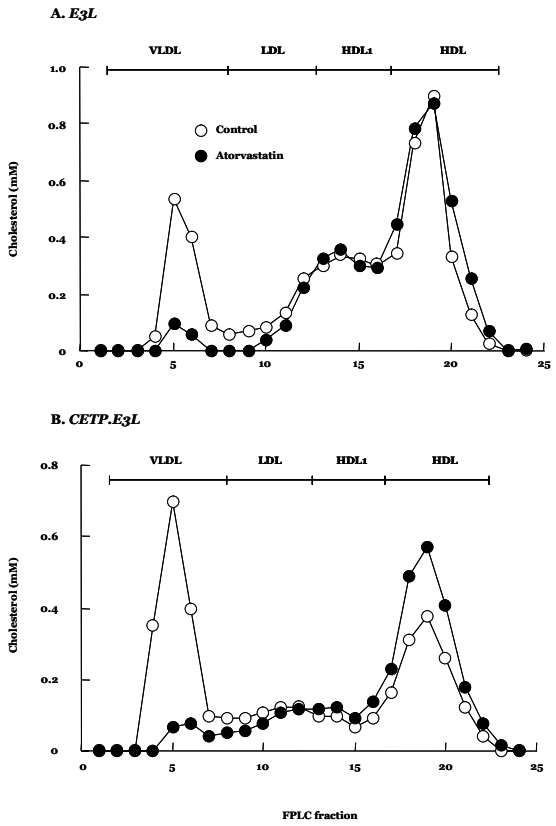

Figure 1. Effect of atorvastatin on the distribution of cholesterol over lipoproteins. Male *E3L* (A) and *CETP.E3L* (B) mice received a diet containing 0.25% (w/w) cholesterol without (open circles) or with (closed circles) 0.01% (w/w) atorvastatin for 6 weeks. Plasmas of the various mouse groups were pooled (n=6 per group). Lipoproteins were separated by FPLC, and fractions were analyzed for cholesterol.

Atorvastatin Decreases Hepatic CETP *mRNA Expression and Plasma CETP Mass and Activity*

In line with previous observations in *E3L* mice,[40] atorvastatin increased the expression of *Hmgcoa reductase* both in *E3L* (2.5-fold; $P<0.05$) and in *CETP.E3L* mice (2.8-fold; $P<0.05$) (Table 2). This is probably caused by an attempt to compensate for the decrease in cholesterol formation upon statin administration.

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 atorvastatin treatment, we examined the effect of atorvastatin on hepatic expression of *Pltp*, *Abca1*, *Sr-b1*, *Apoa1*, and *CETP* (Table 2).

The expression of *Pltp*, involved in transfer of phospholipids between lipoproteins, was slightly but not significantly increased in both *E3L* (+34%) and *CETP.E3L* (+69%) mice upon treatment. In addition, the expression of *Abca1*, which is an important determinant for HDL formation, was reduced in *E3L* (-59%; $P<0.05$) and in *CETP.E3L* (-45%; $P<0.05$) mice. Since increased plasma PLTP activity[41,42] and reduced hepatic ABCA1 levels[43] are associated with decreased HDL-cholesterol levels, these effects on mRNA expression cannot contribute to the increase in HDL-cholesterol in *CETP.E3L* mice.

Table 2. Effect of atorvastatin on hepatic mRNA expression in *E3L* and *CETP.E3L* transgenic mice

	E3L mice		*CETP.E3L* mice	
	Control	Atorvastatin	Control	Atorvastatin
Hmgcoa reductase	1.00±0.24	2.46±0.32*	1.00±0.18	2.80±0.52*
Pltp	1.00±0.18	1.34±0.25	1.00±0.22	1.69±0.65
Abca1	1.00±0.15	0.41±0.10*	1.00±0.06	0.55±0.10*
Sr-b1	1.00±0.14	0.70±0.16	1.00±0.12	0.73±0.07
Apoa1	1.00±0.20	0.87±0.10	1.00±0.21	0.99±0.07
CETP	n.d.	n.d.	1.00±0.12	0.43±0.09**

E3L and *CETP.E3L* male mice were fed a cholesterol-containing diet (0.25%) with or without 0.01% (w/w) atorvastatin. 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 control.

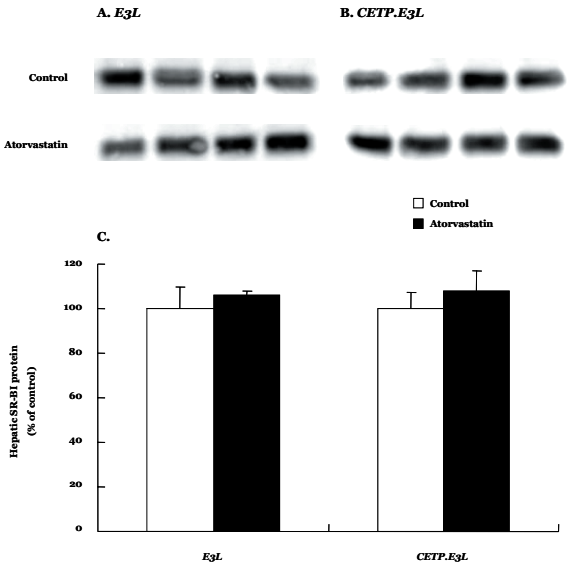

Figure 2. Effect of atorvastatin on hepatic SR-BI protein levels. *E3L* and *CETP.E3L* male mice received a diet containing 0.25% (w/w) cholesterol with or without 0.01% (w/w) atorvastatin for 6 weeks. Livers were isolated after cervical dislocation. SR-BI protein was determined by immunoblot analysis in *E3L* (A) and *CETP.E3L* (B) mice. Intensity of bands were determined by pixel counting and calculated relative to the control mice (C). Values are means ± S.E.M. (n=4 per group).

SR-BI is involved in the selective uptake of HDL-CE, and a reduction might thus result in increased HDL-cholesterol.[44] Atorvastatin tended to reduce hepatic *Sr-b1* expression in both *E3L* (-30%; n.s.) and *CETP.E3L* mice (-27%; n.s.). Since *Sr-b1* expression does not correlate well with protein mass,[45] hepatic SR-BI protein levels were also determined. Immunoblot analysis showed that SR-BI protein was not affected by atorvastatin as compared to control mice (Fig. 2). Therefore, the atorvastatin-mediated increase in HDL-cholesterol in *CETP.E3L* mice are not explained by differences in SR-BI expression.

Increased levels of apoAI are positively correlated with HDL-cholesterol.[46] However, mRNA levels of *Apoa1* were not affected in both types of mice as compared to their controls. In addition, atorvastatin did not increase apoAI plasma levels in *E3L* and in *CETP.E3L* mice (Table 3), thereby excluding a role of apoAI in the atorvastatin-mediated increase in HDL-cholesterol.

Altogether, atorvastatin caused similar effects on the expression of these genes in both *E3L* and *CETP.E3L* mice. The main discriminatory factor between both types of

Table 3. Effect of atorvastatin on plasma apoAI protein levels and plasma CETP mass and activity levels in *E3L* and *CETP.E3L* transgenic mice

	E3L mice		*CETP.E3L* mice	
	Control	Atorvastatin	Control	Atorvastatin
ApoAI				
(mg/dl)	77±41	85±42	75±25	49±14
CETP mass				
(µg/ml)	n.d.	n.d.	25±8	22±8
(µg CETP/µmol HDL-cholesterol)	n.d.	n.d.	20±6	11±4*
CETP activity				
(µmol CE/ml/h)	n.d.	n.d.	0.63±0.18	0.45±0.11
(µmol CE/h/µmol HDL-cholesterol)	n.d.	n.d.	0.44±0.15	0.19±0.08**

E3L and *CETP.E3L* male mice were fed a cholesterol-containing diet (0.25%) with or without 0.01% (w/w) atorvastatin. After 6 weeks, plasma apoAI levels and plasma CETP mass and activity levels were determined. Values are expressed as means ± S.D. (n=5 per group). n.d., not detectable. *P<0.05; **P<0.01 compared to control.

mice after atorvastatin treatment is a -57% reduction in hepatic *CETP* expression in the *CETP.E3L* mice ($P<0.01$) (Table 2), whereas CETP expression could of course not be detected in *E3L* mice. The decrease in *CETP* expression was accompanied by a trend towards reduction in plasma CETP mass (-12%) and activity (-29%) (Table 3). Apart from mRNA, also plasma HDL-cholesterol is a determinant of CETP levels.[12] CETP activity was predominantly found on HDL, since precipitation of the apoB-containing lipoproteins did not affect CE transfer activities in atorvastatin-treated mice (0.37±0.15 μmol CE/ml/h) and in controls (0.56±0.19 μmol CE/ml/h). Therefore, CETP was adjusted for HDL-cholesterol, which led to significant reductions of -45% in CETP mass ($P<0.05$) and -57% in CETP activity ($P<0.01$) (Table 3).

Atorvastatin Dose-Dependently Decreases CETP Mass and Activity

To determine whether the effects of atorvastatin on HDL-cholesterol and CETP levels are dose-dependent, female *CETP.E3L* mice were fed a diet containing 0.1% (w/w) for two weeks, randomized according to plasma cholesterol levels, and successively received the diet supplemented with 0.001% and 0.01% of atorvastatin (w/w) for two weeks. Atorvastatin dose-dependently decreased plasma cholesterol up to -71% ($P<0.01$) at the highest concentration (Fig. 3A). This was accompanied by a dose-dependent increase in HDL-cholesterol up to 176% (Fig. 3B) and dose-dependent reductions in plasma CETP mass up to -57% ($P<0.05$) (Fig. 3C) and CETP activity up to -61% ($P<0.05$) (Fig. 3D).

Discussion

Statins do not affect[27] or even increase[26] plasma total cholesterol levels in apoE-deficient mice, and LDL receptor deficient mice also hardly show a response to statin treatment.[25] In contrast, *E3L* mice respond to statin treatment with respect to lowering of apoB-containing lipoproteins and reduction of atherosclerosis development similarly as humans.[40] To investigate whether the statin-induced elevation of HDL-cholesterol in humans would depend on CETP expression, we crossbred *E3L* mice with *CETP* transgenic mice. We found that atorvastatin increased HDL-cholesterol in *CETP.E3L* mice, which was not observed in *E3L* littermates. This was accompanied by decreased hepatic *CETP* mRNA expression levels with concomitant reductions in plasma CETP mass and activity.

Although steady-state HDL-cholesterol was not affected by atorvastatin in *E3L* mice, it was increased in *CETP.E3L* mice. Apparently, lack of CETP expression in mice[30] prevents the atorvastatin-induced increase in HDL-cholesterol. However, several additional key players in HDL-metabolism might be affected differently in *CETP.E3L* as compared to *E3L* mice, and thus participate in the HDL-cholesterol raising effect.

ApoAI is a prerequisite for the formation of HDL particles. *Apoa1* deficient mice have reduced HDL levels[47] and inversely, human *APOA1* transgenic mice show an increase in HDL.[46] Therefore, an increase in *Apoa1* expression might account for the increased HDL-cholesterol levels. However, atorvastatin did not increase hepatic *Apoa1* expression or plasma apoAI levels either in *E3L* mice nor in *CETP.E3L* mice. Lipid-

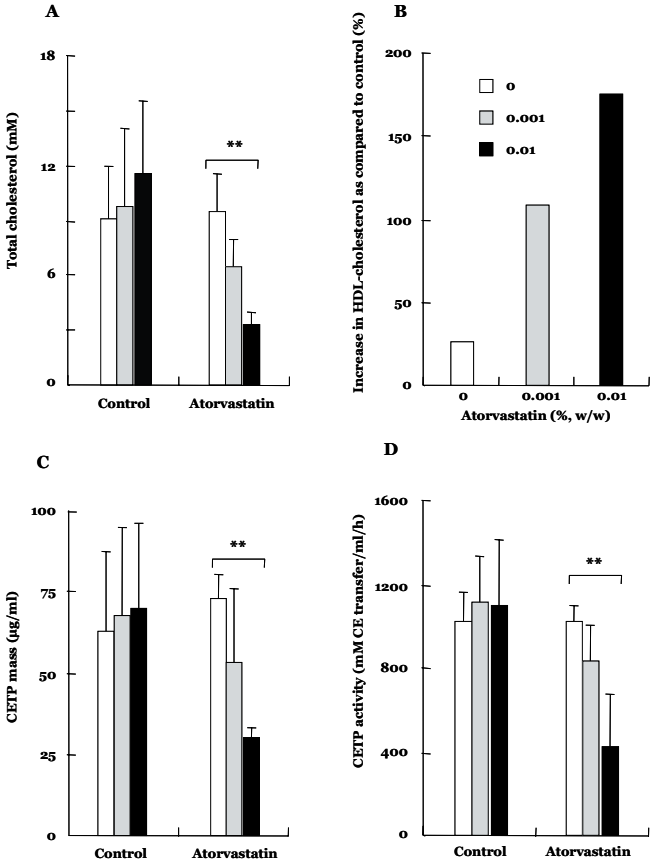

Figure 3. Effect of atorvastatin on plasma cholesterol, CETP mass, and CETP activity. Female *CETP.E3L* mice received a diet containing 0.1% (w/w) cholesterol (open bars; n=6) or this diet supplemented with 0, 0.001, or 0.01% (w/w) atorvastatin (closed bars; n=5) for 2 weeks. Plasma cholesterol (A), the increase in HDL-cholesterol as compared to control mice (B), CETP mass (C), and CETP activity (D) were determined. Statistical analysis was performed using univariate trend analysis (**P<0.01).

poor apoAI is subsequently lipidated via ABCA1. Since overexpression of human *ABCA1* increases HDL-cholesterol levels in mice,[48] whereas the disruption of *Abca1* gene leads to a deficiency in HDL-cholesterol,[49] the decreased *Abca1* expression in *E3L* and *CETP.E3L* mice as we observed upon atorvastatin treatment cannot explain the elevation of HDL in *CETP.E3L* mice. PLTP plays an important role in the remodeling of HDL. A slight but non-significant increase in *Pltp* expression was observed both in *E3L* and in *CETP.E3L* mice. Since adenoviral mediated gene-transfer of *PLTP* to the liver results in a dose-dependent reduction of HDL-cholesterol,[41,42] this excludes *Pltp* expression as a cause for the increased HDL levels upon atorvastatin treatment in *CETP.E3L* mice.

Finally, hepatic SR-BI forms the most important pathway for selective HDL-CE uptake from plasma in mice.[44] We found that atorvastatin did not affect hepatic SR-BI protein levels in both types of mice. Taken these results together, the raise in HDL-cholesterol in *CETP.E3L* mice can not be explained by atorvastatin-mediated effects on apoAI, ABCAI, PLTP, or SR-BI.

Thus, our data indicate that the reduction in hepatic *CETP* mRNA is the primary cause of the statin-induced increase in HDL-cholesterol. Both the decrease in plasma CETP activity and the reduction in the available CE-acceptor particles (*i.e.* VLDL) can account for a reduction in CETP transfer activity, which in its turn causes the increase in HDL-cholesterol. In addition to its transfer activity, CETP was implicated in the direct[50] and in the SR-BI-mediated[51] HDL-CE uptake by hepatocytes. Inhibition of these uptake pathways may also contribute to the increase in HDL-cholesterol.

The atorvastatin-induced down-regulation of *CETP* expression may be caused by a reduction in plasma cholesterol levels. Since cholesterol feeding of *CETP* transgenic mice increases hepatic *CETP* mRNA expression[32] via an LXR responsive element in the *CETP* promoter,[52] the mechanism underlying the atorvastatin-induced down-regulation of *CETP* expression might conversely be related to a reduction in LXR signaling, as the reduction in plasma cholesterol may result in a decrease in oxysterols, the natural ligands of LXRα. In addition, the CETP promoter activity is affected by several other regulatory transcription factors,[12] which alone or in combination with others could be responsible for decreased transcription.

Clinical studies have established that statins improve the survival rate of patients with hypercholesterolemia and coronary artery disease by lowering LDL-cholesterol in addition to pleiotropic anti-inflammatory effects.[53,54] However, a high residual cardiovascular risk still remains.[55,56] Even with aggressive atorvastatin treatment in the PROVE-IT (Pravastatin or Atorvastatin Evaluation and Infection Therapy) study, the risk remained 60-70% despite greater protection against death or major cardiovascular events.[8] A more pronounced increase in HDL levels might further reduce the events. Therefore a combination therapy of atorvastatin and a small-molecule CETP inhibitor, torcetrapib, is currently tested in humans.[14] The results so far are promising since combination therapy in subjects with low HDL-cholesterol (<1 mM), increased HDL-cholesterol by +61% and decreased LDL-cholesterol by -17%. Studies on the effect of combination therapy on atherosclerosis development in *CETP.E3L* mice might provide valuable and timely evidence about the benefit that can be expected from such a therapy, while results from long-term clinical studies using cardiovascular disease endpoints are awaited.

In conclusion, our results show that atorvastatin increases HDL-cholesterol in *CETP.E3L* mice by reducing the hepatic *CETP* expression and plasma CETP activity. We postulate that the increase in HDL after atorvastatin treatment in humans is also caused by a reduction in CETP activity. Further reduction of CETP activity beyond that achieved by statins might result in a more pronounced increase in HDL and provide additional beneficial effects regarding reduction of cardiovascular risk. *CETP.E3L* mice constitute a useful model to test such strategies.

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