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## **Modulation of HDL metabolism : studies in APOE\*3-Leiden.CETP mice**

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# Chapter 3

## **ATORVASTATIN INCREASES HDL CHOLESTEROL BY REDUCING CETP EXPRESSION IN CHOLESTEROL-FED *APOE*\*3-LEIDEN.CETP MICE**

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### Abstract

**Objective:** In addition to lowering low-density lipoprotein (LDL)-cholesterol, statins modestly increase high-density lipoprotein (HDL)-cholesterol in humans and decrease cholesteryl ester transfer protein (CETP) mass and activity. 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 mice expressing human CETP under control of its natural flanking regions resulting in *E3L.CETP* mice. *E3L* and *E3L.CETP* mice were fed a Western-type diet with or without atorvastatin. Atorvastatin (0.01% in the diet) reduced plasma cholesterol in both *E3L* and *E3L.CETP* mice (-26% and -33%,  $P<0.05$ ), mainly in VLDL, but increased HDL-cholesterol only in *E3L.CETP* mice (+52%). Hepatic mRNA expression levels of genes involved in HDL metabolism, such as phospholipid transfer protein (*Pltp*), ATP-binding cassette transporter A1 (*Abca1*), scavenger receptor class B type I (*Sr-b1*), and apolipoprotein AI (*Apoa1*), were not differently affected by atorvastatin in *E3L.CETP* mice as compared to *E3L* mice. However, in *E3L.CETP* mice, atorvastatin down-regulated the hepatic CETP mRNA expression (-57%;  $P<0.01$ ) as well as the total CETP level (-29%) and CE transfer activity (-36%;  $P<0.05$ ) in plasma.

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

## Introduction

Epidemiological studies have established that a high level of low-density lipoprotein (LDL)-cholesterol is a major cardiovascular risk factor. In the past decades, statins have been successfully used to reduce LDL-cholesterol. Statins inhibit the rate-limiting enzyme of cholesterol synthesis, *i.e.* 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, resulting in hepatic depletion of cholesterol. 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).<sup>1</sup> Indeed, a meta-analysis of 25 studies indicated that statins reduce LDL-cholesterol levels by 20-40%.<sup>2</sup> In addition, statins elevate high-density lipoprotein (HDL)-cholesterol levels by typically 5-15%.<sup>3-5</sup> This effect is already observed at a low dose (20 mg/day) while higher doses (40 and 80 mg/day) have no additional elevating effects on HDL levels.<sup>3-5</sup>

Low HDL-cholesterol has been confirmed as a strong and independent risk factor for cardiovascular disease. An increase in HDL-cholesterol of 1 mg/dL results in a 2-3% decrease in cardiovascular risk.<sup>6</sup> One of the key players in HDL-metabolism is cholesteryl ester transfer protein (CETP). CETP is involved in the exchange of triglycerides (TG) and cholesteryl esters (CE) between lipoproteins, resulting in the net flux of CE from HDL towards apoB-containing lipoproteins (*e.g.* VLDL and LDL) in exchange for TG.<sup>7</sup> Treatment of patients with combined hyperlipidemia with atorvastatin resulted in increased levels of relatively CE-rich large HDL2a with a concomitant decrease in CE-poor small HDL3c,<sup>8</sup> as associated with a reduction in CETP mass.<sup>8</sup> Likewise, in type 2 diabetic subjects carrying the CETP *TaqIB* polymorphism, the increase in HDL-cholesterol (+7.2%) after atorvastatin treatment was correlated with a reduction in CETP mass (-32%).<sup>9</sup> These data suggest that the effects of statin treatment on HDL-cholesterol levels may actually be caused by a reduction in the CETP-mediated transfer of CE.

Therefore, the aim of this study was to evaluate whether the statin-induced increase in HDL-cholesterol would depend on CETP expression. Previously, we demonstrated that *APOE\*3-Leiden (E3L)* mice, with a human-like lipoprotein profile<sup>10</sup> show a human-like response to atorvastatin with reduced (V)LDL-cholesterol levels accompanied by reduced VLDL production.<sup>11</sup> In the current study, these mice were crossbred with mice expressing human CETP under control of the natural flanking regions, resulting in *E3L.CETP* mice.<sup>12</sup> We treated *E3L* and *E3L.CETP* mice with atorvastatin to investigate whether CETP expression contributes to the HDL-raising effect of atorvastatin.

### Methods

#### *Animals*

Hemizygous human CETP transgenic (*CETP*) mice, expressing a human CETP minigene under the control of natural flanking sequences were crossbred with hemizygous *E3L* mice<sup>10</sup> at our Institutional Animal Facility to obtain *E3L* and *E3L.CETP* littermates (C57Bl/6J background).<sup>12</sup> In this study, mice were housed under standard conditions in conventional cages with free access to food and water. Male mice were fed a semi-synthetic diet containing 15% (w/w) fat (Hope Farms, Woerden, The Netherlands), supplemented with 0.25% (w/w) cholesterol (Sigma, St. Louis, MO) for two weeks. Subsequently, the mice received the same diet without or with 0.01% (w/w) atorvastatin (Lipitor® 20, Pfizer B.V., Capelle a/d IJssel, The Netherlands) for 6 weeks (*i.e.* approx. 10 mg/kg/day, which corresponds to a dose of 70 mg/day for an average 70 kg person, assuming a 10-fold higher metabolic rate in mice as compared to humans). To study whether atorvastatin sorts similar effects in female mice, and to evaluate the dose-response relationship, female *E3L.CETP* mice were fed a diet containing 15% (w/w) fat, supplemented with 0.1% (w/w) cholesterol and 0.001% or 0.01% of atorvastatin for two weeks successively. 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 and assayed for total cholesterol (TC) using the enzymatic kit 236691 (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.). The distribution of lipids over plasma lipoproteins was determined by fast-performance liquid chromatography (FPLC) as described previously.<sup>12</sup>

#### *Hepatic liver lipid levels*

Livers were isolated from control-treated and atorvastatin-treated mice after cervical dislocation. A small piece of liver was homogenated in 400 µL PBS and 1.5 mL CH<sub>3</sub>OH:CHCl<sub>3</sub> (2:1, v/v) was added. After centrifugation, lipids were extracted from the supernatant with CHCl<sub>3</sub> and H<sub>2</sub>O (1:1, v/v) and the CHCl<sub>3</sub> phase was dried. Lipids were dissolved in H<sub>2</sub>O with 2% Triton-X100. TC levels were assayed as described above. Free cholesterol (FC) was analyzed with the Free Cholesterol C kit (WAKO, Neuss, Germany), and cholesteryl esters (CE) were determined as the difference between TC and FC. Phospholipids (PL) and TG were analyzed with the, phospholipids B kits (Wako, Neuss Germany) and the enzymatic kit 1488872 (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.), respectively.

### Plasma CETP level

The total CETP level in plasma was measured as the transfer of [ $^3\text{H}$ ]cholesteryl oleate from exogenous human LDL to HDL as described.<sup>12</sup>

### Plasma cholesteryl ester transfer activity

The transfer of newly synthesized CE in plasma was assayed by a radioisotope method as previously described.<sup>13</sup> In short, [ $^3\text{H}$ ]cholesterol was complexed with BSA and incubated overnight at 4°C with mouse plasma to equilibrate with plasma free cholesterol. Subsequently, the plasma samples were incubated for 3 h at 37°C. VLDL and LDL were then precipitated by addition of sodium phosphotungstate/ $\text{MgCl}_2$ . Lipids were extracted from the precipitate by methanol: hexane (1:2, v/v) and [ $^3\text{H}$ ]CE was separated from [ $^3\text{H}$ ]cholesterol on silica columns, followed by counting of radioactivity.

### 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) (3  $\mu\text{g}/\text{ml}$ ) 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; 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,

**Table 1.** Primers for quantitative real-time PCR analysis.

| Gene               | Forward primer (5'-3') | Reverse primer (5'-3')  |
|--------------------|------------------------|-------------------------|
| <i>Hmgcoa red.</i> | CCGCAACAACAAGATCTGTG   | ATGTACAGGATGGCGATGCA    |
| <i>Pltp</i>        | TCAGTCTGCGCTGGAGTCTCT  | AAGGCATCACTCCGATTTGC    |
| <i>Abca1</i>       | CCCAGAGCAAAAAGCGACTC   | GGTCATCATCACTTTGGTCCTTG |
| <i>Sr-b1</i>       | GTTGGTCACCATGGGCCA     | CGTAGCCCCACAGGATCTCA    |
| <i>Apoa1</i>       | GGAGCTGCAAGGGAGACTGT   | TGCGCAGAGAGTCTACGTGTGT  |
| <i>CETP</i>        | CAGATCAGCCACTTGTCCAT   | CAGCTGTGTGTTGATCTGGA    |
| <i>Abcg5</i>       | TGTCCTACAGCGTCAGCAACC  | GGCCACTCTCGATGTACAAGG   |
| <i>Abcg8</i>       | GACAGCTTCACAGCCCACAA   | GCCTGAAGATGTGAGAGCGA    |
| <i>Lpl</i>         | GTGGCCGAGAGCGAGAAC     | TCCACCTCCGTGTAAATCAAGA  |
| <i>Srebp-1c</i>    | GGAGCCATGGATTGCACATT   | CCTGTCTCACCCCCAGCATA    |
| <i>Ldlr</i>        | GCATCAGCTTGGACAAGGTGT  | GGAACAGCCACCATTGTTG     |

*Abca1*, ATP-binding cassette transporter A1; *Abcg5/8*, ATP-binding cassette transporter G5/G8, *Apoa1*, apolipoprotein A1; *CETP*, cholesteryl ester transfer protein; *Hmgcoa reductase*, 3-hydroxy-3-methylglutaryl coenzyme A reductase; *Ldlr*, low density lipoprotein receptor; *Lpl*, lipoprotein lipase; *Pltp*, phospholipid transfer protein; *Sr-b1*, scavenger receptor class B type I; *Srebp-1c*, sterol regulatory element-binding protein-1c.

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 and SR-BI protein analysis*

Livers were isolated after cervical dislocation. Total RNA was isolated using the NucleoSpin<sup>®</sup> 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 are listed in online Table 1. Expression levels were normalized, using hypoxanthine-guanine phosphoribosyl transferase (HPRT) and cyclophilin as housekeeping genes.<sup>14</sup> Hepatic SR-BI protein was determined by immunoblot analysis exactly as described previously.<sup>15</sup>

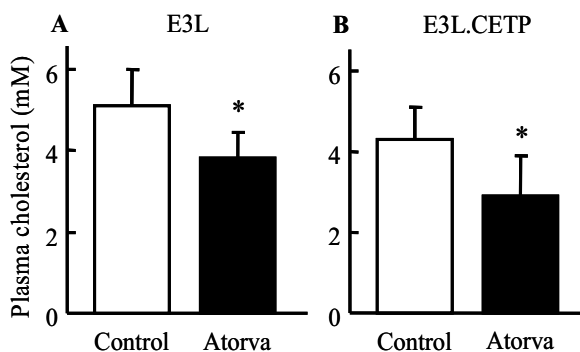
#### *Statistical analysis*

All data are presented as means  $\pm$  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. SPSS 12.0.01 was used for statistical analysis.

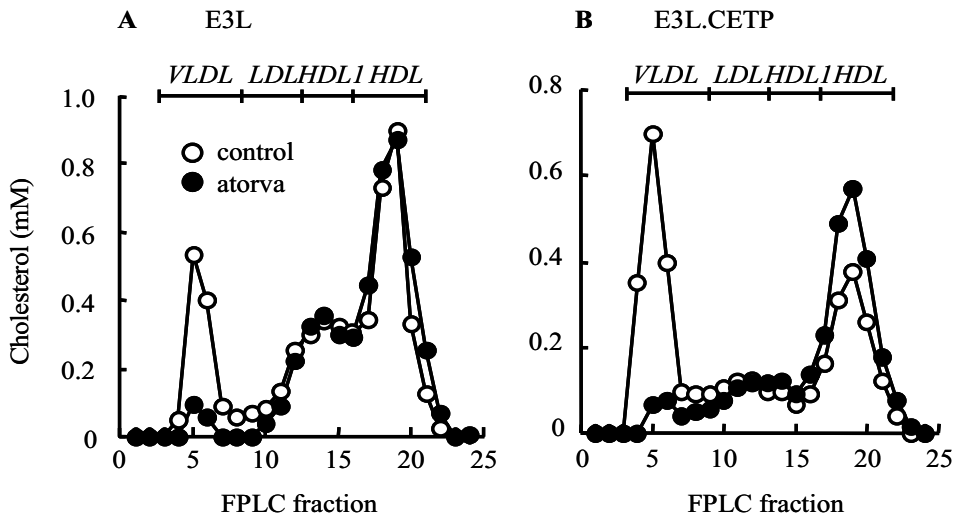
## Results

#### *Atorvastatin increases HDL-cholesterol in E3L.CETP mice*

On a diet containing 0.25% (w/w) cholesterol, atorvastatin (0.01%, w/w) reduced plasma total cholesterol in both *E3L* mice from  $5.1 \pm 0.9$  mM to  $3.8 \pm 1.2$  mM (-26%;  $P < 0.05$ ) and *E3L.CETP* mice from  $4.3 \pm 0.8$  mM to  $2.9 \pm 1.0$  mM (-33%;  $P < 0.05$ ) (Fig. 1), without substantially affecting TG levels (not shown). These effects were reflected by a strong decrease in (V)LDL-cholesterol in *E3L* mice (-86%) and *E3L.CETP* mice (-88%) (Fig. 2). However, whereas atorvastatin did not affect HDL-cholesterol *E3L* mice (3.2 mM vs 2.9 mM) (Fig. 2A), it did raise HDL-cholesterol (+52%) in *E3L.CETP* mice (2.1 mM vs 1.4 mM) (Fig. 2B).



**Figure 1.** Effect of atorvastatin on plasma total cholesterol levels. *E3L* (A) and *E3L.CETP* (B) mice received a diet containing 0.25% (w/w) cholesterol without (white bars) or with (black bars) 0.01% (w/w) atorvastatin for 6 weeks. Plasma was obtained, and assayed for total cholesterol. Values are means  $\pm$  SD ( $n=6$  per group). \* $P < 0.05$  compared to control.



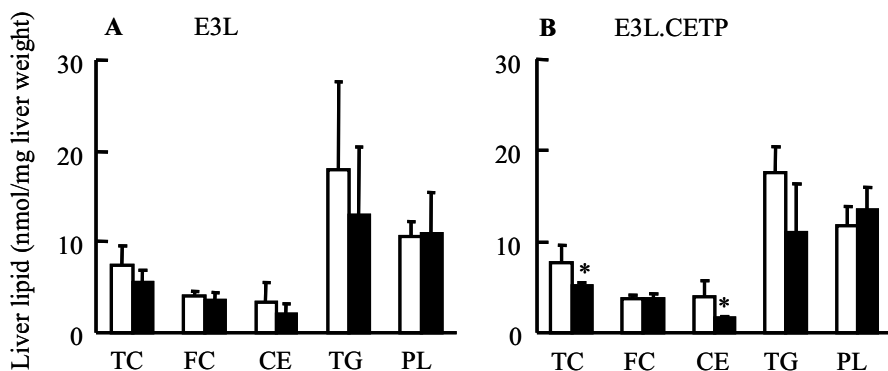
**Figure 2.** Effect of atorvastatin on the distribution of cholesterol over lipoproteins. *E3L* (A) and *E3L.CETP* (B) mice received a cholesterol-containing diet without (white circles) or with (black circles) atorvastatin for 6 weeks. Plasmas of the various mouse groups were pooled ( $n=6$  per group). Lipoproteins were separated by FPLC, and fractions were analyzed for cholesterol.

Atorvastatin also reduced cholesterol levels in the liver of *E3L* mice (-24%) and *E3L.CETP* mice (-32%) ( $P<0.05$ ). This decrease in hepatic cholesterol was mainly confined to the cholesteryl ester content in *E3L* mice (-38%) and *E3L.CETP* mice (-60%) ( $P<0.05$ )(Fig. 3).

#### *Atorvastatin does not differentially affect hepatic mRNA expression of genes involved in HDL metabolism in E3L versus E3L.CETP mice*

Atorvastatin increased the hepatic expression of *Hmgcoa reductase* both in *E3L* mice (2.5-fold;  $P<0.05$ ) and in *E3L.CETP* mice (2.8-fold;  $P<0.05$ ) (Fig. 4). Concomitantly, *Ldlr* expression was increased in *E3L* mice (+22%) and *E3L.CETP* mice (+24%) (not shown). These effects are in line with previous observations in *E3L* mice,<sup>16</sup> and likely reflect an attempt of the liver to maintain its cholesterol balance.

Since atorvastatin may affect PLTP, ABCA1, SR-BI, and apoAI, which are crucially involved in HDL metabolism, and may account for the increase in HDL-cholesterol in *E3L.CETP* mice upon atorvastatin treatment, we examined the effect of atorvastatin on their hepatic mRNA expression (Fig. 4). The expression of these genes was not substantially different in *E3L.CETP* mice as compared to *E3L* mice ( $<16\%$ , not significant). Atorvastatin tended to increase the expression of *Pltp*, involved in remodeling of HDL by mediating transfer of phospholipids between lipoproteins, in *E3L* mice (+34%) and *E3L.CETP* mice (+69%), which did not reach statistical significance. The expression of *AbcA1*, which is an important determinant for HDL formation, was decreased by



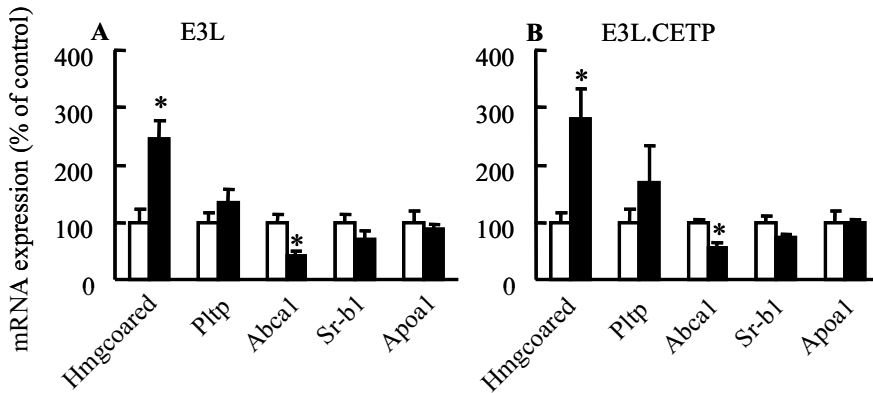
**Figure 3.** Effect of atorvastatin on hepatic lipid levels. *E3L* (A) and *E3L.CETP* (B) mice were fed a cholesterol-containing diet without (white bars) or with (black bars) atorvastatin. After 6 weeks, livers were collected and lipids were extracted. Total cholesterol (TC), free cholesterol (FC), cholesteryl esters (CE), triglycerides (TG) and phospholipids (PL) were quantified. Values are means  $\pm$  SD ( $n=3-5$  per group). \* $P<0.05$  compared to control.

atorvastatin in *E3L* mice (-59%;  $P<0.05$ ) and *E3L.CETP* mice (-45%;  $P<0.05$ ) to a similar extent. The expression of *Sr-b1*, which is largely involved in the selective uptake of HDL-CE in mice, tended to be decreased in *E3L* (-30%) and *E3L.CETP* (-27%) mice, but hepatic SR-BI protein levels were unaffected in both mouse groups (not shown). Also, in both types of mice, atorvastatin did not increase hepatic *Apoa1* expression or the plasma apoAI levels (not shown). Atorvastatin thus affects the mRNA expression of *Pltp*, *Abca1*, *Sr-b1*, and *Apoa1* to a similar extent in *E3L* and *E3L.CETP* mice, and is thus unlikely to explain the differentially raised HDL in *E3L.CETP* mice as compared to *E3L* mice.

In general, atorvastatin tended to decrease the expression of LXR target genes, including *Abcg5* (-2% and -38%), *Abcg8* (-26% and -46%), *Lpl* (-85% and -77%) and *Srebp-1c* (-31% and -32%) in *E3L* and *E3L.CETP* mice, respectively.

#### *Atorvastatin decreases hepatic CETP mRNA expression and cholesteryl ester transfer activity in plasma of E3L.CETP mice*

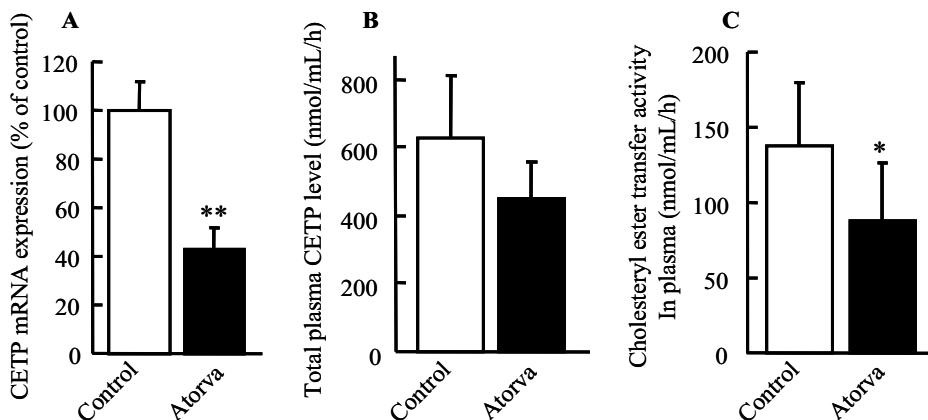
To investigate whether atorvastatin increases HDL-cholesterol in *E3L.CETP* mice by reduction of CETP activity, we determined the hepatic *CETP* mRNA expression, the total plasma CETP level, and the CE transfer activity in plasma (Fig. 5). Indeed, atorvastatin markedly decreased *CETP* expression in *E3L.CETP* mice (-57%;  $P<0.01$ ) (Fig. 5A). This effect was accompanied by a trend towards a reduction in the total plasma CETP level (-29%), which did not reach statistical significance, probably related to the relatively high variation in combination with the limited group size (Fig. 5B). Additionally, the CE transfer activity in plasma of *E3L.CETP* mice was reduced (-36%;  $P<0.05$ ) (Fig. 5C). Taken together, the HDL-raising effect of atorvastatin in *E3L.CETP* mice appears a direct consequence of reduced *CETP* expression.



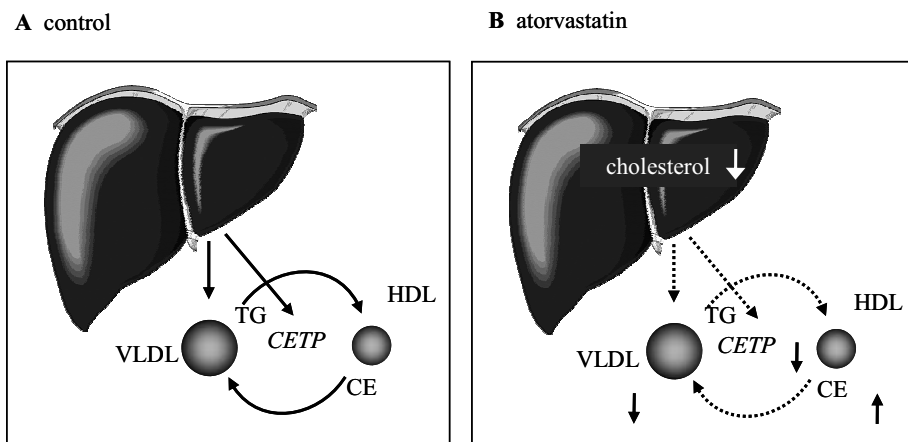
**Figure 4.** Effect of atorvastatin on hepatic mRNA expression of genes. *E3L* (A) and *E3L.CETP* (B) mice were fed a cholesterol-containing diet without (white bars) or with (black bars) atorvastatin. After 6 weeks, livers were collected to determine mRNA expression. Values are expressed as means  $\pm$  S.E. relative to control mice (n=4 per group). \* $P$ <0.05 compared to control.

#### *Atorvastatin dose-dependently decreases CETP and increases HDL*

To determine whether atorvastatin also reduces CETP and increases HDL-cholesterol in female mice, and to evaluate whether these effects would be dose-dependent, female *E3L.CETP* mice were fed a cholesterol-containing diet that successively contained 0.001% and 0.01% of atorvastatin (w/w) for two weeks each. Atorvastatin dose-dependently decreased plasma cholesterol (-34% and -71%,  $P$ <0.01). This was accompanied by a dose-dependent increase in HDL-cholesterol levels (+118% and +176%) and reductions in total plasma CETP activity (-31% and -61%;  $P$ <0.01) (not shown).



**Figure 5.** Effect of atorvastatin on hepatic CETP mRNA expression and cholesteryl ester transfer activity in plasma. *E3L.CETP* mice were fed a cholesterol-containing diet without (white bars) or with (black bars) atorvastatin. After 6 weeks, livers were collected to determine CETP mRNA expression (A), and plasma was assayed for total CETP level (B) and CE transfer activity (C). Values are means  $\pm$  SD (n=4-6 per group). \* $P$ <0.05; \*\* $P$ <0.01.



**Figure. 6.** Proposed mechanism underlying the HDL-raising effect of statins. See text for explanation. CE, cholesteryl ester; TG, triglyceride.

## Discussion

*E3L* mice respond to statin treatment with respect to lowering of apoB-containing lipoproteins and reduced atherosclerosis development similarly as humans,<sup>11,16,17</sup> whereas statins do not affect or even increase plasma cholesterol levels in apoE-deficient mice<sup>18,19</sup> and LDL receptor-deficient mice.<sup>20</sup> However, whereas statins increase HDL in humans, atorvastatin and rosuvastatin did not increase HDL levels in *E3L* mice.<sup>11,16,17</sup>

To investigate whether the statin-induced elevation of HDL-cholesterol in humans depends on CETP expression, we crossbred *E3L* mice with human CETP transgenic mice. We found that atorvastatin decreased (V)LDL in both *E3L* and *E3L.CETP* mice but increased the steady-state HDL-cholesterol level only in *E3L.CETP* mice, which was not observed in *E3L* littermates. We previously showed that atorvastatin reduces plasma cholesterol in *E3L* mice by reducing VLDL production.<sup>11</sup> Since atorvastatin similarly reduces (V)LDL cholesterol in *E3L.CETP* mice as compared to *E3L* mice, and CETP expression *per se* does not affect VLDL production,<sup>21</sup> it is likely that the mechanisms by which atorvastatin reduces (V)LDL-cholesterol are similar in *E3L.CETP* mice and *E3L* mice. In addition, the mild increase in LDLr expression in both *E3L* and *E3L.CETP* mice may contribute to lower plasma cholesterol levels. The increase in HDL was accompanied by decreased hepatic *CETP* mRNA expression levels with a concomitant reduction in plasma CE transfer activity. Apparently, the fact that mice naturally lack CETP expression prevents the atorvastatin-induced increase in HDL-cholesterol in mice.

Since several additional key players in HDL metabolism might have been affected differently by atorvastatin treatment in *E3L.CETP* as compared to *E3L*

mice, and thus participate in the HDL-cholesterol raising effect, we have also evaluated the effect of atorvastatin on the hepatic expression of *Apoa1*, *Abca1*, *Pltp*, and *Sr-b1*. ApoAI is involved in the generation formation nascent HDL particles,<sup>22</sup> which acquire cholesterol via ABCA1. In fact, the HDL-cholesterol level in mice is largely determined by the hepatic expression of ABCA1.<sup>23</sup> PLTP plays an important role in the remodeling of HDL, by facilitating phospholipid transfer to HDL during its maturation from discoidal HDL into spherical HDL.<sup>24</sup> Finally, at least in mice, hepatic SR-BI is crucially involved in the selective uptake of HDL-CE.<sup>25</sup> We found that atorvastatin did not affect the hepatic expression of *Pltp*, *Sr-b1*, and *Apoa1*. Atorvastatin did decrease *Abca1* expression in *E3L* and *E3L.CETP* mice. However, since a decreased *Abca1* expression would be expected to lower HDL levels, it also cannot be a causal factor for the selective elevation of HDL in *E3L.CETP* mice. Previous experiments in mice in which hepatic ABCA1 expression levels were modulated specifically, have shown a causal relationship between hepatic ABCA1 expression and plasma HDL-cholesterol. In our study, atorvastatin primarily decreases CETP expression as related to a reduced hepatic cholesterol content. We speculate that, as a consequence rather than as a cause, the liver attempts to maintain its cholesterol balance by an upregulation of LDL receptors to enhance cholesterol influx and a down-regulation of ABCA1 to decrease cholesterol efflux.

Taken together, the selective raise in HDL-cholesterol in *E3L.CETP* mice cannot be explained by atorvastatin-mediated effects on apoAI, ABCAI, PLTP, or SR-BI, but is primarily caused by the reduction in *CETP* expression. Both a decrease in plasma CETP activity and a reduction in (V)LDL (*i.e.* acceptor of HDL-CE) can account for a reduction in CE transfer activity, which in its turn causes the increase in HDL-cholesterol. In addition to its transfer activity, CETP has also been implicated in the direct<sup>26</sup> and in the SR-BI-mediated<sup>27</sup> HDL-CE uptake by hepatocytes. Inhibition of these uptake pathways by atorvastatin via reducing cellular CETP may thus potentially also contribute to the increase in HDL-cholesterol.

The atorvastatin-induced down-regulation of CETP expression is presumably caused by a reduction in plasma and hepatic cholesterol levels. Cholesterol feeding of *CETP* transgenic mice increases hepatic *CETP* mRNA expression via an LXR responsive element in the CETP promoter.<sup>28</sup> Conversely, atorvastatin may down-regulate CETP expression by reducing LXR signaling, as atorvastatin reduced plasma and hepatic cholesterol levels<sup>16</sup> and consequently probably also hepatic oxysterols, the natural ligands of LXR $\alpha$ . In line with this hypothesis, the expression of other LXR target genes such as ABCG5, ABCG8, LPL and SREBP-1c were also reduced upon atorvastatin treatment. In addition, the CETP promoter activity is affected by several other regulatory transcription factors,<sup>7</sup> which alone or in combination could also be responsible for decreased transcription. The fact that atorvastatin treatment of humans also decreases

plasma CETP<sup>8,9</sup> may well be explained by similar regulation of CETP expression.

Based on our collective data, we thus propose the following mechanism by which statins raise HDL-cholesterol, as summarized in Fig. 6. By inhibiting HMGCoA reductase activity, statins decrease the hepatic lipid content. This results in decreased (V)LDL levels by a lower VLDL production and a higher (V)LDL clearance. In addition, reduction in hepatic cholesterol results in reduced levels of hepatic oxysterols (*i.e.* the natural ligands of LXR $\alpha$ ) and, consequently, decreased LXR $\alpha$ -induced hepatic expression of CETP. Therefore, the HDL-cholesterol levels are raised by lower (V)LDL levels and lower CETP expression, resulting in decreased CE transfer activity from HDL to (V)LDL.

Clinical studies have established that statins improve the survival rate of patients with hypercholesterolemia and coronary artery disease by lowering LDL-cholesterol and by their pleiotropic anti-inflammatory effects.<sup>29</sup> However, a high residual cardiovascular risk still remains.<sup>3</sup> Even with aggressive atorvastatin treatment in the PROVE-IT study, the risk remained 60-70% despite greater protection against death or major cardiovascular events.<sup>30</sup> Therefore, concomitant raising of HDL-cholesterol is generally considered to enhance the anti-atherogenic potential of statins. Since our novel *E3L.CETP* mouse model is responsive to modulation of apoB-containing lipoproteins as well as HDL levels, we anticipate that our mouse model will be valuable to study the effect of such HDL-raising therapeutic strategies, alone or in combination with (V)LDL-lowering strategies, on plasma lipid metabolism and atherosclerosis development, and to study the underlying mechanisms.

In conclusion, our results show that atorvastatin increases HDL-cholesterol by reducing the hepatic CETP expression and plasma CE transfer activity in *E3L.CETP* mice. Therefore, we postulate that reduction of CETP expression contributes to the increase in HDL that is found in human subjects treated with statins.

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