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CETP EXPRESSION REVERSES THE RECONSTITUTED HDL-INDUCED INCREASE IN VLDL

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Journal of Lipid Research 2011; 52: 1533-41

ABSTRACT

Human data suggest that reconstituted HDL (rHDL) infusion can induce atherosclerosis regression. Studies in mice indicated that rHDL infusion adversely affects VLDL levels, but this effect is less apparent in humans. This discrepancy may be explained by the fact that humans, in contrast to mice, express cholesteryl ester transfer protein (CETP). The aim of this study was to investigate the role of CETP in the effects of rHDL on VLDL metabolism by using *APOE*3-Leiden* (*E3L*) mice, a well-established model for human-like lipoprotein metabolism. At 1 h after injection, rHDL increased plasma VLDL-C and TG in *E3L* mice, but not in *E3L* mice cross-bred onto a human CETP background (*E3L.CETP* mice). This initial raise in VLDL was caused by competition between rHDL and VLDL for LPL-mediated TG hydrolysis, and was thus prevented by CETP. At 24 h after injection, rHDL caused a second increase in VLDL-C and TG in *E3L* mice, whereas rHDL even decreased VLDL in *E3L.CETP* mice. This secondary raise in VLDL was due to increased hepatic VLDL-TG production. Collectively, we conclude that CETP protects against the rHDL-induced increase in VLDL. We anticipate that studies evaluating the anti-atherosclerotic efficacy of rHDL in mice that are naturally deficient for CETP should be interpreted with caution, and that treatment of atherogenic dyslipidemia by rHDL should not be combined with agents that aggressively reduce CETP activity.

INTRODUCTION

Dyslipidemia is an important risk factor for cardiovascular disease (CVD). Current treatment mainly focuses on lowering of LDL-cholesterol (C), e.g. by statins. LDL-C lowering treatment results in a significant reduction in the morbidity and mortality of CVD, but can not prevent the majority of cardiovascular events [1, 2]. Prospective epidemiological studies have demonstrated a strong inverse correlation between HDL-C and CVD [3], and recent studies revealed that high HDL-C levels are indeed protective against plaque progression [4]. Although the exact mechanisms by which HDL protects are unclear, HDL has been shown to have antioxidant, antithrombotic and anti-inflammatory properties, and to mediate reverse cholesterol transport (RCT) via the hepatobiliary route [5]. Therefore, new strategies to raise HDL-C are currently being developed to prevent and treat CVD.

Various therapeutic strategies are currently under development to raise HDL levels, including cholesteryl ester transfer protein (CETP) inhibition, niacin, upregulation of apoAI expression and infusion of apoAI mimetics or reconstituted HDL (rHDL) [6]. Although still in early stage of development, infusion of (r)HDL seems to be a promising strategy for the treatment of CVD. Recent reviews have demonstrated that infusion of rHDL improves atherosclerotic plaque characteristics both in animal models and in humans [7-9]. For example, rHDL, composed of recombinant human apoAI$_{Milano}$ and phosphatidylcholine, rapidly mobilized tissue cholesterol and reduced the lipid and macrophage content of atherosclerotic plaques after a single injection into apoE-deficient mice [10]. Moreover, it prevented the progression of aortic atherosclerosis as well as promoted the stabilization of plaques after 6 weeks of administration [11]. Recent clinical trials assessed the effect of rHDL consisting of human apoAI and phosphatidylcholine (CSL-111) as a potential HDL-raising therapeutic strategy. Short-term infusion of CSL-111 significantly improved the plaque characterization index and coronary score on quantitative coronary angiography [12]. In addition, a single dose of rHDL led to acute changes in plaque characteristics with a reduction in lipid content, macrophage size and inflammatory mediators [13].

Albeit that rHDL thus seems to beneficially modulate atherosclerosis in mice and humans, differences have been observed with respect to modulation of VLDL levels. Infusion of rHDL into apoE-deficient mice increased (V)LDL-C in both acute and chronic studies [10, 11], whereas rHDL did not adversely affect (V)LDL-C in clinical studies [12, 13]. This discrepancy may be explained by the fact that, in contrast to mice [14], humans express CETP [15], a crucial factor involved in the metabolism of both (V)LDL and HDL by mediating the transfer of triglycerides (TG) and cholesteryl esters (CE) between these

lipoproteins. Therefore, the aim of this study was to elucidate the role of CETP in the effects of rHDL on VLDL metabolism. We used *APOE*3-Leiden* (*E3L*) transgenic mice, a unique model for human-like lipoprotein metabolism, which have been crossbred with mice expressing human CETP under control of its natural flanking regions [16], resulting in *E3L.CETP* mice. This allows distinguishing between the effect of rHDL administration on VLDL metabolism in the absence and presence of CETP-mediated lipid transfer.

MATERIALS AND METHODS

Animals

Hemizygous human CETP transgenic (*CETP*) mice, expressing human CETP under the control of its natural flanking regions [16], were purchased from the Jackson Laboratory (Bar Harbor, ME) and crossbred with hemizygous E3L mice [17] at our Institutional Animal Facility to obtain *E3L.CETP* mice [18]. In this study, female mice were used, housed under standard conditions in conventional cages with free access to food and water. At the age of 12 weeks, mice were fed a semi-synthetic Western-type diet, containing 1% (w/w) corn oil and 15% (w/w) cacao butter (Hope Farms, Woerden, The Netherlands) with 0.25% (w/w) cholesterol (*E3L* mice) or 0.1% (w/w) cholesterol (*E3L.CETP* mice) for three weeks, aimed at yielding comparable VLDL levels between both mouse genotypes. Upon randomization according to total plasma cholesterol (TC) and TG levels, mice received a single intravenous injection of rHDL (CSL-111; CSL Behring AG, Bern, Switzerland) (250 mg/kg in 250 µL PBS) or vehicle. Experiments were performed after 4 h of fasting at 12:00 pm with food withdrawn at 8:00 am. The institutional Ethical Committee on Animal Care and Experimentation has approved all experiments.

Reconstituted HDL

rHDL (CSL-111) consists of apoAI isolated from human plasma and phosphatidylcholine from soybean with a molar ratio of 1:150. Before infusion, rHDL was reconstituted with 50 mL of sterile water, yielding 62.5 mL of clear, pale yellow solution, pH 7.5, and 10% (w/v) sucrose as a stabilizing agent. The final apoAI and PL concentrations were 20 and 86 mg/mL, respectively.

Plasma lipid and lipoprotein analysis

Plasma was obtained via tail vein bleeding and assayed for TC, TG and phospholipids (PL) using the commercially available enzymatic kits 236691, 11488872 (Roche Molecular Biochemicals, Indianapolis, IN, USA) and phospholipids B (Wako Chemicals,

Neuss, Germany), respectively. The distribution of lipids over plasma lipoproteins was determined using fast protein liquid chromatography (FPLC). Plasma was pooled per group, and 50 µL of each pool was injected onto a Superose 6 PC 3.2/30 column (Äkta System, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and eluted at a constant flow rate of 50 µL/min in PBS, 1 mM EDTA, pH 7.4. Fractions of 50 µL were collected and assayed for TC, TG and PL as described above.

Plasma human apoAI concentration

Plasma human apoAI concentrations were determined using a sandwich ELISA. Goat anti-human apoAI antibody (Academy Biomedical Co., Inc., Houston, TX; 11A-G2b) was coated overnight on to Costar medium binding plate (Costar, Inc., New York, NY) (3 µg/mL) at 4°C and incubated with diluted mouse plasma (dilution, 1:100,000) for 2 h at 37°C. Subsequently, horseradish peroxidase-conjugated goat antihuman apoAI (Academy Biomedical; 11H-G1b) was added and incubated for 2 h at 37°C. Horseradish peroxidase was detected by incubation with tetramethylbenzidine (Organon Teknika, Boxtel, The Netherlands) for 15 min at room temperature. Human apoAI (Academy Biomedical; 11P-101) was used as a standard.

In vivo clearance of VLDL-like emulsions

Glycerol tri[^{3}H]oleate- and [1a,2a(n)-^{14}C]cholesteryl oleate-double labeled VLDL-like emulsion particles (80 nm) were prepared as described by Rensen *et al*. [19]. In short, radiolabeled emulsions were obtained by adding 200 µCi of glycerol tri[^{3}H]oleate and 20 µCi of [^{14}C]cholesteryl oleate to 100 mg of emulsion lipids before sonication (isotopes obtained from GE Healthcare, Little Chalfont, U.K.). Mice were fasted for 4 h, sedated with 6.25 mg/kg acepromazine (Alfasan), 6.25 mg/kg midazolam (Roche), and 0.3125 mg/kg fentanyl (Janssen-Cilag) and injected with the radiolabeled emulsion particles (0.15 mg TG in 200 µL PBS) via the tail vein. At indicated time points after injection, blood was taken from the tail vein to determine the serum decay of glycerol tri[^{3}H]oleate and 20 µCi of [^{14}C]cholesteryl oleate.

In vitro LPL activity assay

The effect of rHDL on LPL activity was determined essentially as described [20]. First, glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles (200 µg of TG, corresponding to a final concentration of 0.5 mg/mL), prepared as described above, were added to the indicated amounts of rHDL (or vehicle containing sucrose or sodium cholate only) and heat-inactivated human serum (20 µL, corresponding to a final concentration of

5% v/v) in a total volume of 75 µL of phosphate-buffered saline. Subsequently, 0.1 M Tris.HCl (pH 8.5) was added to a total volume of 200 µL and incubation mixtures were equilibrated at 37°C. At t=0, bovine LPL (final concentration 3.5 U/mL, Sigma) in 200 µL of 120 mg/mL free fatty acid-free BSA (Sigma), corresponding with a final concentration of 60 mg/mL, was added (37°C). At t=15, 30, 60, 90 and 120 min, [^{3}H] oleate generated during lipolysis by LPL was extracted. Hereto, 50 µL samples were added to 1.5 mL extraction liquid (CH_3OH: $CHCl_3$: heptane: oleic acid (1410: 1250: 1000: 1, v/v/v/v). Samples were mixed and 0.5 mL of 0.2 M NaOH was added. Following vigorous mixing and centrifugation (10 min at 1000 g), ^{3}H radioactivity in 0.5 mL of the aqueous phase was counted. After taking the last samples, 50 µL of the incubations were also directly counted, representing the total amount of radioactivity in the assay. Lipolysis rate (i.e. LPL activity) was calculated by linear regression between incubation time and percentage of [^{3}H]oleate released.

Hepatic VLDL-TG and VLDL-apoB production

Mice were fasted for 4 h, with food withdrawn at 8:00 a.m., prior to the start of the experiment. During the experiment, mice were sedated as described above. At t=0 min blood was taken via tail bleeding and mice were i.v. injected with 100 µL PBS containing 100 µCi Trans^{35}S label to measure de novo total apoB synthesis. After 30 min, the animals received 500 mg of tyloxapol (Triton WR-1339, Sigma-Aldrich) per kg body weight as a 10% (w/w) solution in sterile saline, to prevent systemic lipolysis of newly secreted hepatic VLDL-TG [21]. Additional blood samples were taken at 15, 30, 60, and 90 min after tyloxapol injection and used for determination of plasma TG concentration. At 120 min, the animals were sacrificed and blood was collected by orbital puncture for isolation of VLDL by density gradient ultracentrifugation. ^{35}S-apoB was measured in the VLDL fraction and VLDL-apoB production rate was shown as dpm.h^{-1} [22].

Statistical analysis

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

RESULTS

Infusion of rHDL transiently increases plasma apoAI and phospholipid levels in both *E3L* and *E3L.CETP* mice

To investigate the role of CETP in the effects of rHDL infusion on VLDL metabolism, female *E3L* mice with or without human CETP expression received a single intravenous injection of rHDL. To assess the kinetics of rHDL that consists of humans apoAI and PL, plasma levels of human apoAI and phospholipid was determined over time (Fig. 1) rHDL caused a transient increase in plasma human apoAI and PL levels in both *E3L* mice (Fig. 1A, C) and *E3L.CETP* mice (Fig. 1B, D). Human apoAI and PL were cleared at a similar rate, and were disappeared from plasma after approximately 24 h. At 1 h after injection, lipoproteins in plasma were separated and the distribution of human apoAI and PL were determined (Fig. 2). rHDL appeared to integrate into the endogenous HDL pool in both *E3L* and *E3L.CETP* mice, since both human apoAI (Fig. 2A, B) and PL (Fig. 2C, D) eluted in fractions representing HDL. In addition, PL derived from rHDL selectively integrated into (V)LDL fraction (Fig. 2C, D). The presence of rHDL-PL in (V)LDL is not due the presence of large rHDL aggregates that would elute in the void volume, since apoAI is not detected in the void volume (Fig. 3A), but is explained by a time-dependent transfer of PL to endogenous VLDL as evident from incubation of rHDL with plasma from *E3L* mice (Fig. 3B) and *E3L.CETP* mice (Fig. 3C) in vitro.

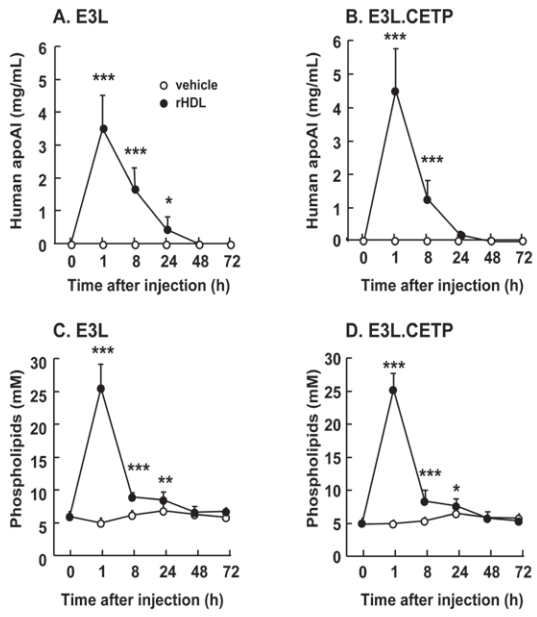

Figure 1. Effect of rHDL on plasma human apoAI and phospholipid in E3L and E3L.CETP mice. *E3L* (A, C) and *E3L.CETP* (B, D) mice were fed a Western-type diet for 3 weeks. Subsequently, they received a single intravenous injection of rHDL (250 mg/kg in 250 µL PBS) or vehicle. Blood was drawn at the indicated time points and plasma was assayed for human apoAI (A, B) and phospholipid (C, D). Values are means ± SD (n=8-10); **P*<0.05, ***P*<0.01, ****P*<0.001 as compared to the control group.

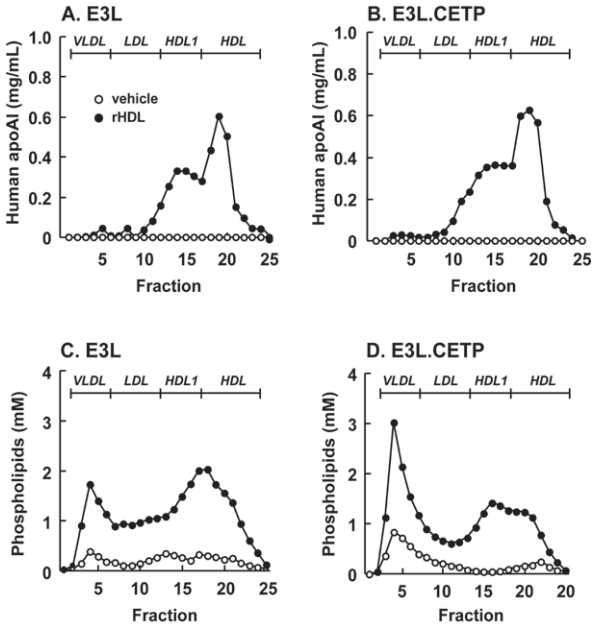

Figure 2. Effect of rHDL on the lipoprotein distribution of human apoAI and phospholipid at 1 h after injection in *E3L* and *E3L.CETP* mice. *E3L* (A, C) and *E3L.CETP* (B, D) mice were fed a Western-type diet for 3 weeks. Subsequently, they received a single intravenous injection of rHDL (250 mg/kg in 250 µL PBS) or vehicle. After 1 h, blood was drawn and plasma was pooled per group (n=8-10). Pooled plasma was fractionated using FPLC on a Superose 6 column and the individual fractions were assayed for human apoAI (A, B) and phospholipid (C, D).

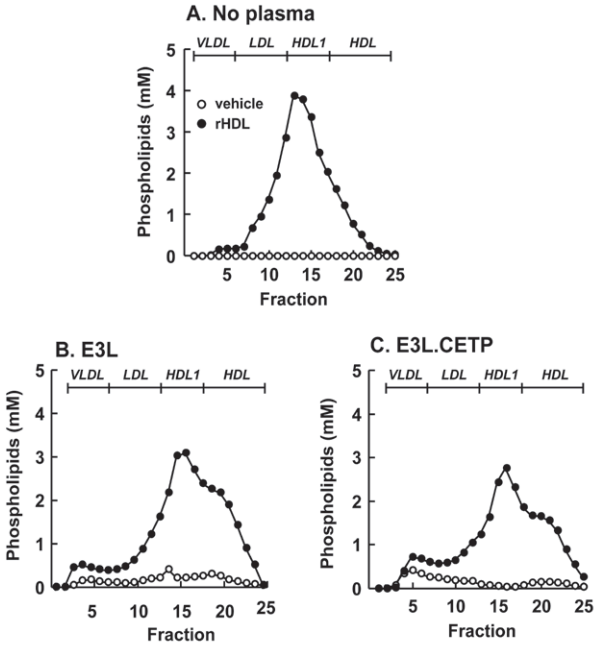

Figure 3. Effect of in vitro incubation of rHDL with *E3L* and *E3L.CETP* mouse plasma on phospholipid distribution. *E3L* and *E3L.CETP* mice were fed a Western-type diet for 3 weeks, and fresh plasma was collected. rHDL was incubated (1 h at 37°C) without mouse plasma (A) or with plasma of *E3L* mice (B) or *E3L.CETP* mice (C). Samples were pooled per group (n=8-10) and fractionated using FPLC on a Superose 6 column, and the individual fractions were assayed for phospholipid.

Infusion of rHDL affects plasma levels of endogenous lipids differentially in *E3L* and *E3L.CETP* mice

Albeit rHDL was cleared at a similar rate in *E3L* and *E3L.CETP* mice, its effects on endogenous plasma levels of cholesterol and TG were clearly different in both mouse types (Fig. 4). At 1 h after injection, rHDL significantly increased plasma cholesterol (C) in both *E3L* mice (+63%; P<0.001) (Fig. 4A) and *E3L.CETP* mice (+28%; P<0.01) (Fig. 4B). However, at 24 h after injection, rHDL still significantly increased plasma cholesterol in *E3L* mice (+26%, P<0.01) (Fig. 4A) but actually decreased plasma cholesterol in *E3L.CETP* mice (-22%, P<0.01) (Fig. 4B). In addition, whereas rHDL caused a significant increase in plasma TG levels in *E3L* mice at both 1 h (+89%; P<0.01) and 24 h after injection (+67%; P<0.01) (Fig. 4C), rHDL did not significantly increase plasma TG at any time point in *E3L.CETP* mice (Fig. 4D).

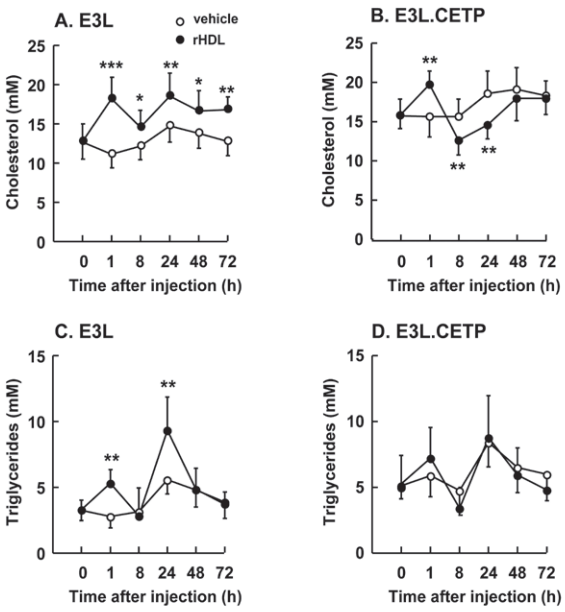

Figure 4. Effect of rHDL on plasma cholesterol and triglycerides in *E3L* and *E3L.CETP* mice. *E3L* (A, C) and *E3L.CETP* (B, D) mice were fed a Western-type diet for 3 weeks. Subsequently, they received a single intravenous injection of rHDL (250 mg/kg in 250 µL PBS) or vehicle. Blood was drawn at the indicated time points and plasma was assayed for total cholesterol (A, B) and triglycerides (C, D). Values are means ± SD (n=8-10); **P*<0.05, ***P*<0.01, ****P*<0.001 as compared to the control group.

At short term, rHDL raises HDL-C in *E3L* and *E3L.CETP* mice, and increases VLDL mainly in *E3L* mice

To investigate the mechanism underlying the early effects of rHDL infusion on plasma lipids, plasma was obtained at 1 h after injection and lipoproteins were fractionated by FPLC (Fig. 5). rHDL increased HDL-C in both *E3L* mice (Fig. 5A) and *E3L.CETP* mice (Fig. 5B), indicating that rHDL induces a rapid cholesterol efflux from peripheral tissues into

plasma. In addition, rHDL markedly increased VLDL-C (Fig. 5A) and VLDL-TG (Fig. 5C) in *E3L* mice, while its VLDL-increasing effect was only modest in *E3L.CETP* mice (Fig. 5B, D). To investigate whether the raise in VLDL was due to competition between rHDL and VLDL for binding and subsequent TG hydrolysis by LPL, we assessed the effect of rHDL on the plasma kinetics of intravenously injected glycerol tri[^{3}H]oleate [^{14}C]cholesteryl oleate double-labeled VLDL-like emulsion particles (Fig. 6). Indeed, rHDL decreased the plasma clearance of the VLDL-like emulsion particles, including glycerol tri[^{3}H]oleate and [^{14}C]cholesteryl oleate, in both *E3L* mice (Fig. 6A, C) and *E3L.CETP* mice (Fig. 6B, D). An in vitro LPL activity assay confirmed that rHDL dose-dependently decreases LPL-mediated lipolysis of VLDL-like emulsion particles (Fig. 7), whereas sucrose and sodium cholate at amounts present at the various rHDL concentrations did not (not shown). These data thus indicate that rHDL competes for the binding of VLDL-like emulsion particles with LPL in both *E3L* and *E3L.CETP* mice, resulting in delayed clearance of TG-derived fatty acids (i.e. ^{3}H-activity) as well as the resulting core remnants (i.e. ^{14}C-activity). The fact that rHDL does not substantially raise VLDL levels in *E3L.CETP* mice is thus probably related to rapid remodeling of VLDL by CETP.

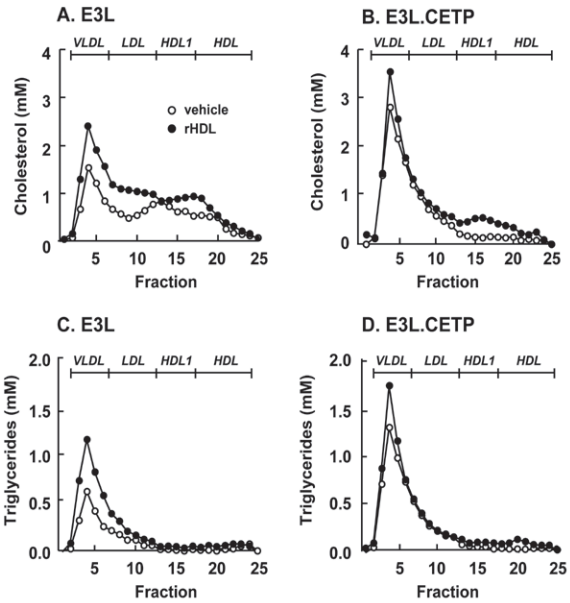

Figure 5. Effect of rHDL on lipoprotein distribution of cholesterol and triglycerides at 1 h after injection in *E3L* and *E3L.CETP* mice. *E3L* (A, C) and *E3L.CETP* (B, D) mice were fed a Western-type diet for 3 weeks. Subsequently, they received a single intravenous injection of rHDL (250 mg/kg in 250 µL PBS) or vehicle. Blood was drawn and plasma was pooled per group (n=8-10). Pooled plasma was fractionated using FPLC on a Superose 6 column and the individual fractions were assayed for total cholesterol (A, B) and triglycerides (C, D).

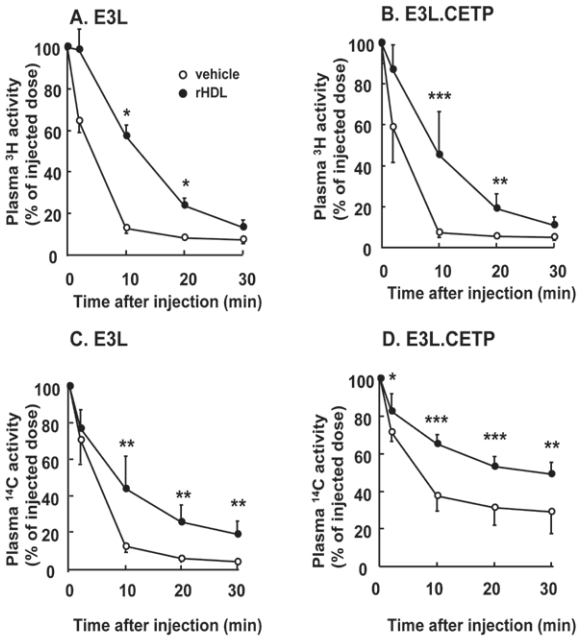

Figure 6. Effect of rHDL on the plasma clearance of VLDL-like emulsion particles in *E3L* and *E3L.CETP* mice. *E3L* (A, C) and *E3L.CETP* (B, D) mice were fed a Western-type diet for 3 weeks, and they received a single intravenous injection of rHDL (250 mg/kg in 200 µL PBS) or vehicle. After 1 min, mice were intravenously injected with glycerol tri[^{3}H]oleate- and [^{14}C]cholesteryl oleate-double labeled VLDL-like emulsion particles (0.15 mg TG in 200 µL PBS). Blood was drawn at the indicated time points and ^{3}H and ^{14}C-activity was determined. Values are means ± SD (n=8); **P*<0.05, ***P*<0.01, ****P*<0.001 as compared to the control group.

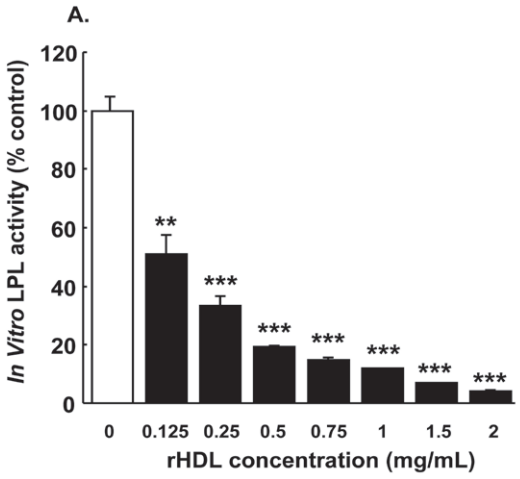

Figure 7. Effect of rHDL on in vitro LPL activity Glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles were incubated at 37 °C with bovine LPL (3.5 U/mL) in 0.1 M Tris.HCl (pH 8.5) in the presence of heat-inactivated human serum (5%, v/v) and free fatty acid-free BSA (60 mg/mL). [^{3}H]oleate generated during lipolysis was extracted after 15, 30, 60, 90 and 120 min of incubation. The lipolysis rate (i.e. LPL activity) was calculated by the linear regression between incubation time and percentage of [^{3}H]oleate generated. Values are means ± SD (n=3); ***P*<0.01, ****P*<0.001 as compared to control incubations containing vehicle.

At long term, rHDL raises VLDL in *E3L* mice and decreases VLDL in *E3L.CETP* mice

To determine the mechanism underlying the divergent long-term effects of rHDL infusion on plasma was also obtained at 24 h after administration and lipoproteins

were fractionated by FPLC (Fig. 8). In both *E3L* and *E3L.CETP* mice, the effect of rHDL on increasing HDL-C levels had disappeared (Fig. 8A, B). However, whereas rHDL still significantly raised VLDL-C (+60%) (Fig. 8A) and VLDL-TG (+86%) (Fig. 8C) in *E3L* mice, rHDL actually decreased VLDL-C (-25%) in *E3L.CETP* mice (Fig. 8B). Since it has been shown that increasing the flux of HDL to the liver can increase the availability of substrate for hepatic VLDL synthesis and subsequently VLDL-TG secretion [23], we speculated that rHDL may have increased the VLDL production. Therefore, the effect of rHDL on VLDL production was evaluated after injection of Triton WR1339 (tyloxapol) to block LPL-mediated lipolysis (Fig. 9). Indeed, at 24 h after administration of rHDL, the VLDL-TG production rate was increased in *E3L* mice (+36%; P<0.01) (Fig. 9A). ApoB production was not affected (Fig. 9C), indicating that rHDL increases lipidation of VLDL particles rather than increasing the VLDL particle secretion rate. Likewise, rHDL tended to increase the VLDL-TG production rate (Fig. 9B) without affecting the apoB production rate (Fig. 9D) in *E3L.CETP* mice.

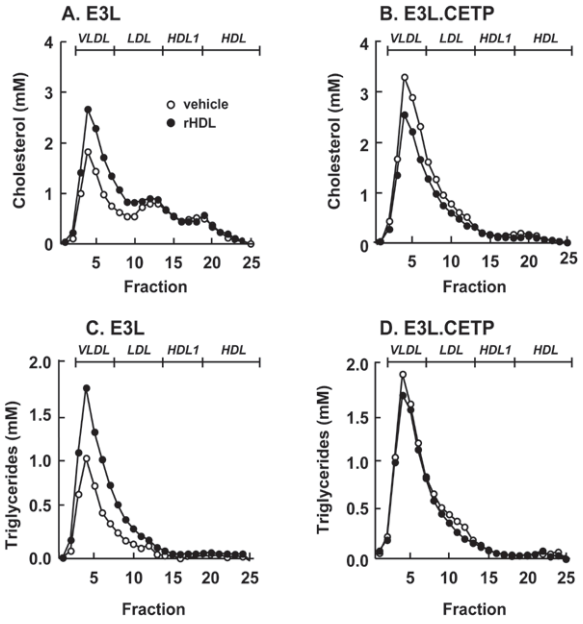

Figure 8. Effect of rHDL on lipoprotein distribution of cholesterol and triglycerides at 24 h after injection in *E3L* and *E3L.CETP* mice. *E3L* (A, C) and *E3L.CETP* (B, D) mice were fed a Western-type diet for 3 weeks. Subsequently, they received a single intravenous injection of rHDL (250 mg/kg in 250 µL PBS) or vehicle. Blood was drawn and plasma was pooled per group (n=8-10). Pooled plasma was fractionated using FPLC on a Superose 6 column and the individual fractions were assayed for total cholesterol (A, B) and triglycerides (C, D).

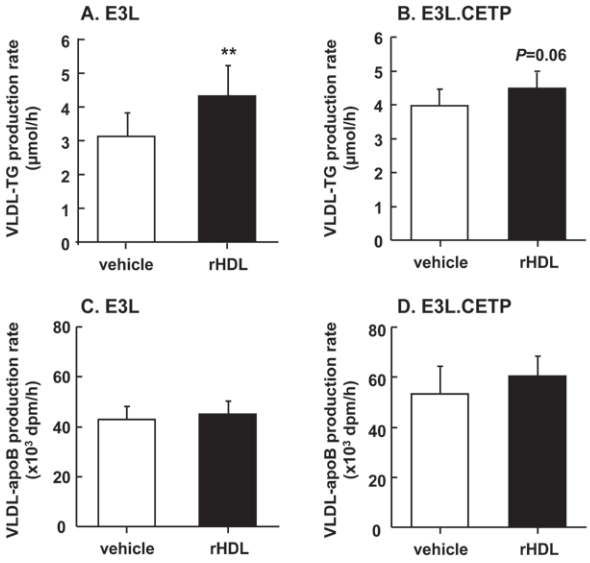

Figure 9. Effect of rHDL on the hepatic VLDL-TG production at 24 h after injection in *E3L* and *E3L.CETP* mice. *E3L* (A, C) and *E3L.CETP* (B, D) mice were fed a Western-type diet for 3 weeks, and they received a single intravenous injection of rHDL (250 mg/kg in 250 µL PBS) or vehicle. At 24 h after rHDL or vehicle injection, mice were injected with Trans^{35}S label and tyloxapol to block VLDL-TG clearance. Blood was drawn at the indicated time points and plasma TG concentrations were determined. VLDL-TG production rate was calculated from the slopes of the TG-time curves from the individual mice (A, B). At 120 after tyloxapol injection, mice were exsanguinated and VLDL was isolated by ultracentrifugation. ^{35}S-activity was determined and VLDL-apoB production rate was calculated as dpm.h^{-1} (C, D). Values are means ± SD (n=7-11); ***P*<0.01 as compared to the control group.

DISCUSSION

In this study, we investigated the role of CETP in the effects of rHDL on VLDL metabolism by using *E3L* mice with or without human CETP expression. In both *E3L* and *E3L.CETP* mice, rHDL caused a similar transient increase in plasma human apoAI and PL levels and induced a transient increase in the endogenous HDL-C pool. However, rHDL caused an increase in VLDL in *E3L* mice, at both 1 h and 24 h after injection, which was prevented by CETP expression in *E3L.CETP* mice.

We observed that rHDL caused a rapid increase in VLDL-TC and VLDL-TG in *E3L* mice within 1 h after administration. This is in line with previous observations showing that rHDL, composed of human apoAI$_{Milano}$ and PL, also increased the VLDL-TC pool in apoE-deficient mice at 1 h after injection [10]. These effects can not be simply explained by transfer of lipid compounds from rHDL to VLDL, since rHDL does not contain cholesterol or triglycerides. Rather, we speculated that infusion of a substantial amount of rHDL may interfere with endogenous VLDL catabolism. Indeed, rHDL decreased the plasma clearance of VLDL-like emulsion particles, including the clearance of both glycerol

tri[^{3}H]oleate and [^{14}C]cholesteryl oleate. Apparently, rHDL competes with endogenous VLDL for binding to triacylglycerol hydrolases, resulting in impaired hydrolysis of TG within VLDL. Indeed, rHDL dose-dependently inhibited LPL activity in an in vitro assay. At an rHDL concentration of 0.625 mg/mL, resulting in an rHDL: TG ratio similar to the in vivo situation, rHDL inhibited LPL activity by as much as 80%. Sucrose and sodium cholate, both present in rHDL, did not inhibit LPL activity in vitro and are thus unlikely to inhibit LPL in vivo. As a consequence of LPL inhibition by rHDL in vivo, the clearance of core remnants is attenuated and plasma VLDL levels are increased. Our finding that normalization of elevated PL levels at 8 h after injection also normalized plasma TG levels in *E3L* mice is consistent with such a mechanism.

In addition to increasing VLDL at 1 h after injection, rHDL caused a second raise in VLDL-TC and VLDL-TG in *E3L* mice at 24 h after administration. Since competition of rHDL for VLDL clearance mechanisms can be excluded at this time point, as rHDL has been cleared from the circulation, we hypothesized that rHDL may have caused an increase in hepatic VLDL production. Indeed, we observed that rHDL increased the production rate of VLDL-TG without affecting the production rate of VLDL-apoB. Since each VLDL particle contains a single molecule of apoB, this indicates that rHDL infusion increases the lipidation of hepatic apoB rather than increasing the number of VLDL particles produced. Interestingly, it has previously been observed that increasing the flux of HDL to the liver by hepatic overexpression of SR-BI also increases the VLDL-TG production rate, and that HDL-derived cholesterol can be re-secreted from the liver within VLDL particles [23]. Therefore, we postulate that, in a similar manner, infusion of rHDL causes an increased net flux of lipids to the liver. We did show that rHDL transiently enhanced HDL-C, which may at least partly be attributed by efflux of cholesterol from peripheral tissues. This increased HDL-C may subsequently be taken up by the liver via SR-BI, which could then be re-secreted within VLDL. However, it is even more likely that a high flux of rHDL-associated PL to the liver enhances the amount of hepatic TG available for secretion as VLDL-TG. Indeed, it has been demonstrated that HDL-associated phosphatidylcholine that has been taken up by hepatocytes is converted to TG after phosphatidylcholine-phospholipase C-mediated hydrolysis of phosphatidylcholine resulting in diacylglycerol that is subsequently converted into TG by DGAT2 [24].

Although rHDL infusion caused a clear hyperlipidemic side-effect in *E3L* mice, there was no increase in VLDL in *E3L.CETP* mice at any time point, albeit rHDL induced a significant decrease of VLDL clearance at short term and tended to increase VLDL production at long term in *E3L.CETP* mice similarly to *E3L* mice. Expression of CETP thus clearly prevents the adverse effects of rHDL on VLDL levels. These data are in line with various human studies in which infusion of rHDL was used as an experimental

treatment of CVD [12], diabetes [25] and inflammation [26]. In these clinical studies, no specific adverse VLDL increasing effects have been reported, which is in line with our data that human CETP may protect against rHDL-induced elevation of VLDL.

It is interesting to speculate on the mechanism(s) underlying the protective effect of CETP on the rHDL-induced raise in VLDL. It is well-known that CETP mediates the transfer of CE from HDL particles to LDL and VLDL particles in exchange for TG, and that this reciprocal neutral lipid transfer approaches equilibrium under physiological conditions. However, under conditions of increased VLDL levels as observed at 1 and 24 h after administration of rHDL to *E3L* mice, the increase in VLDL results in elevated acceptor activity for CETP, which would result in an increased net rate of TG transfer from VLDL particles to both HDL and LDL particles [27]. Both TG-enriched HDL and LDL particles are avidly bound to hepatic lipase (HL) that effectively hydrolyzes TG (as well as PL) to form small dense LDL and HDL, respectively [28], thereby effectively eliminating TG from plasma.

Based on our data and literature studies, we propose the following mechanism by which CETP has a protective effect on rHDL-induced increase in VLDL. Infusion of rHDL initially decreases VLDL clearance via blocking of LPL-mediated lipolysis and, at a later stage, increases VLDL production via HDL-mediated delivery of lipids to the liver, both of which processes increase plasma VLDL levels. The transient accumulation of VLDL particles leads to an increase of the CETP activity, resulting in an accelerated transfer of TG from VLDL to HDL and LDL, in which TG is hydrolyzed quickly through the action of HL.

Since our primary research question was to examine the effect of CETP on the rHDL-induced increase on VLDL metabolism, we used saline as a control for rHDL treatment similarly as applied in clinical trials. It should be noted that such a study set-up does not allow evaluating the individual contributions of apoAI versus lipids. As the cholesterol-efflux properties of rHDL could largely depend on the apoAI moiety, it would be interesting to investigate the effect of rHDL as compared to apoAI-free lipid micelles on cholesterol mobilization into plasma as well as VLDL metabolism in future studies.

In addition to rHDL infusion therapy, other strategies to raise HDL-C are currently being developed to prevent and treat CVD, alone or combined with LDL-lowering drugs, among which CETP inhibitors. The first CETP inhibitor torcetrapib increased HDL-C levels by approximately 60% [29], but failed to demonstrate any effect on the primary atherosclerotic burden as assessed by carotid intima-media thickness and coronary intravascular ultrasound imaging [30, 31] and even increased cardiovascular events and mortality, accompanied by off-target effects [32]. However, other CETP inhibitors such as dalcetrapib [33] and anacetrapib [34] have now progressed into phase III clinical trails

without showing any off-target toxicity. Additionally, niacin effectively increases HDL by 25-30% [35] and improves carotid intima-media thickness [36]. We recently showed that the HDL-raising effect of niacin is caused by reducing the hepatic CETP expression and plasma CETP protein [37]. Since the HDL-raising effects of CETP inhibitors and niacin thus both depend on reducing CETP activity in plasma, whereas CETP activity now appears crucial to prevent the raise in VLDL as induced by rHDL infusion, combining rHDL with these HDL-raising agents could reveal adverse VLDL effects, and as a consequence counteract the potentially protective effect of rHDL in CVD.

In conclusion, our results show that rHDL infusion induces an increase in VLDL levels, which is prevented by CETP expression. Therefore, we anticipate that studies evaluating the anti-atherosclerotic efficacy of rHDL in mouse models that are naturally deficient for CETP should be interpreted with caution, and that treatment of atherogenic dyslipidemia as a risk factor for CVD by rHDL should not be combined with agents that aggressively reduce CETP activity.

ACKNOWLEDGEMENTS

This work was performed in the framework of the Leiden Center for Cardiovascular Research LUMC-TNO, and supported by the Netherlands Heart Foundation (NHS grant 2007B81 to P.C.N.R.). P.C.N.R. is an Established Investigator of the Netherlands Heart Foundation (grant 2009T038).

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