Niacin Increases HDL by Reducing Hepatic Expression and Plasma Levels of Cholesteryl Ester Transfer Protein in APOE*3Leiden.CETP Mice

José W.A. van der Hoorn, Willeke de Haan, Jimmy F.P. Berbéé, Louis M. Havekes, J. Wouter Jukema, Patrick C.N. Rensen, Hans M.G. Princen

Objective—Niacin potently decreases plasma triglycerides and LDL-cholesterol. In addition, niacin is the most potent HDL-cholesterol–increasing drug used in the clinic. In the present study, we aimed at elucidation of the mechanism underlying its HDL-raising effect.

Methods and Results—In APOE*3Leiden transgenic mice expressing the human CETP transgene, niacin dose-dependently decreased plasma triglycerides (up to −77%, \( P<0.001 \)) and total cholesterol (up to −66%, \( P<0.001 \)). Concomitantly, niacin dose-dependently increased HDL-cholesterol (up to +87%, \( P<0.001 \)), plasma apoAI (up to +72%, \( P<0.001 \)), as well as the HDL particle size. In contrast, in APOE*3Leiden mice, not expressing CETP, niacin also decreased total cholesterol and triglycerides but did not increase HDL-cholesterol. In fact, in APOE*3Leiden.CETP mice, niacin dose-dependently decreased the hepatic expression of CETP (up to −88%; \( P<0.01 \)) as well as plasma CETP mass (up to −45%, \( P<0.001 \)) and CETP activity (up to −52%, \( P<0.001 \)). Additionally, niacin dose-dependently decreased the clearance of apoAI from plasma and reduced the uptake of apoAI by the kidneys (up to −90%, \( P<0.01 \)).

Conclusion—Niacin markedly increases HDL-cholesterol in APOE*3Leiden.CETP mice by reducing CETP activity, as related to lower hepatic CETP expression and a reduced plasma (V)LDL pool, and increases HDL-apoAI by decreasing the clearance of apoAI from plasma. (Arterioscler Thromb Vase Biol. 2008;28:2016-2022)

Key Words: APOE*3Leiden.CETP transgenic mice ■ CETP ■ HDL-cholesterol ■ hyperlipidemia ■ niacin

Dyslipidemia is an important risk factor for the development of cardiovascular disease (CVD). Although lowering of LDL-cholesterol (C) by eg, statins reduces CVD risk by approximately 30%, substantial residual cardiovascular risk remains, even with very aggressive reductions in levels of LDL-C.1-3 Because of clinical studies, which have shown that HDL-C, independently of LDL-C, is inversely correlated with the risk of CVD,4,5 attention has shifted toward strategies for targeting HDL composition as adjunctive therapy to prevent and treat CVD. Current strategies to mildly increase HDL-C levels include aggressive overall lifestyle modification (ie, exercise, diet, weight loss, and smoking cessation), and modest increases in HDL-C levels are achieved with statins6 and fibrates (5% to 10%).7

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Niacin (nicotinic acid, vitamin B3) has been described to exhibit lipid-modifying capacities already since the 1950s. Since then various (clinical) studies have shown the beneficial effects of niacin on plasma lipid levels. Treatment with niacin alone was associated with a 27% reduction in nonfatal myocardial infarction and it reduced all cause mortality by 11%.8,9 In combination with colestipol (FATS trial) or simvastatin (HATS trial), niacin reduced cardiac events by as much as 80% to 90%.10,11 These potent atherogenic properties of niacin are thought to be attributable to its marked HDL-raising effect (+20% to +30%), besides it potent effect on reducing plasma TG (−40% to −50%) and LDL-C (−20%).7,12 In fact, niacin is currently the most effective therapy for elevating HDL-C.

The mechanism underlying the ability of niacin to reduce the plasma (V)LDL level has been well studied. By selective binding to GPR109A on adipocytes, niacin suppresses hormone sensitive triglyceride lipase (HSL) activity, resulting in a decreased release of free fatty acids (FFA) from adipose tissue and decreased plasma FFA levels.13 The resulting reduced supply of FFA toward the liver is believed to bring about a decreased hepatic VLDL-TG production, resulting in reduced VLDL-TG and (V)LDL-C levels.13,14 In contrast, the mechanism underlying the HDL-C raising effect of niacin has not been elucidated as yet. This is probably related to the lack of suitable animal models that respond in a human-like manner.
manner to HDL-raising drug interventions. In wild-type mice and apoE-knockout mice (the classical animal model for hyperlipidemia and atherosclerosis), rats, and dogs, niacin only transiently reduced plasma levels of TG but failed to raise HDL-C.15,16 An HDL-C-elevating effect of niacin has been reported in rabbits, but with 30% ethanol as dosing vehicle and only after 12 weeks of treatment.17

Therefore, the aim of this study was to elucidate the mechanism underlying the HDL-C raising effect of niacin. To this end, we used our recently developed APOE*3Leiden (E3L).CETP transgenic mouse model. We have previously demonstrated that E3L mice have a human-like lipoprotein profile in which the elevated plasma cholesterol and TG levels are mainly confined to the (V)LDL-sized lipoprotein fractions.18,19 These mice develop atherosclerosis on dietary cholesterol feeding and respond in a human-like manner to drugs used in the treatment of CVD (eg, statins, fibrates, cholesteryl uptake inhibitors, calcium channel blockers, and angiotensin II receptor antagonists20–23), but they did not yet respond to HDL-modulating interventions. By cross-breeding E3L mice with mice expressing human CETP under control of its natural flanking regions, E3LCETP were obtained24 that respond to the HDL-raising effects of fenofibrate,25 atorvastatin,26 and torcetrapib.27 We now fed these mice a Western-type diet without or with increasing doses of niacin to reveal the mechanism underlying its HDL-C raising effect.

Methods

Animals

Hemizygous human CETP transgenic (CETP) mice28 were crossbred with E3L mice.18 At the age of 12 weeks, female E3L and E3LCETP mice were fed a semisynthetic cholesterol-rich diet for 3 weeks to obtain similar total cholesterol levels in both strains (about 12 to 14 mmol/L). After matching mice (n=8 per group) received a Western-type diet without or with 0.03% (~36 mg/kg/d), 0.1% (~118 mg/kg/d), 0.3% (~360 mg/kg/d), or 1% (~1180 mg/kg/d) niacin (Sigma) for at least 3 weeks. These doses correspond well to the doses used in humans, if the 10-times faster metabolism of mice as compared to humans is taken into account. The institutional Ethical Committee on Animal Care and Experimentation has approved all experiments. For the full descriptions of the used methods, please see the supplemental materials (available online at http://atvb.ahajournals.org).

Statistical Analysis

All data are presented as means±SD unless indicated otherwise. Data were analyzed parametrically by 1-way ANOVA followed by Dunnett to correct for multiple testing. Probability values less than 0.05 were considered statistically significant. SPSS 14.0 was used for statistical analysis.

Results

Niacin Decreases Plasma Lipids in Both E3L and E3LCETP Mice, but Increases HDL Only in E3LCETP Mice

No adverse clinical signs were observed with increasing dosages of niacin as indicated by absence of differences in weight gain and plasma ALT levels between treatment groups and the control. Treatment of E3L mice with niacin (118 mg/kg/d) caused a sustained reduction in plasma TG by −26% (1.4±0.6 mmol/L versus 1.9±0.6 mmol/L; P<0.05) and in plasma TC by −35% (9.2±3.4 mmol/L versus 14.2±4.5 mmol/L; P<0.05). Lipoprotein fractionation by fast protein liquid (FPLC) showed that the reduction in cholesterol was confined to the apoB-containing lipoproteins (V)LDL, whereas HDL-C was not affected (Figure 1A). An equal dose of niacin even more potently reduced plasma TG (−57%, P<0.05) and TC (−44%, P<0.01) in E3LCETP mice. As in E3L mice, the TC-decreasing effect of niacin in E3LCETP mice was caused by a reduction of (V)LDL-C. However, whereas niacin did not affect HDL levels in E3L mice, it increased HDL-C in E3LCETP mice (Figure 1B). In E3LCETP mice, the effects of niacin on plasma TG and TC levels were dose-dependent as shown in Figure 2. At the highest dose of 1180 mg/kg/d, niacin reduced TG levels by −77% (P<0.001; Figure 2A) and TC levels by −66% (P<0.001; Figure 2B).

The HDL-Increasing Effect of Niacin in E3LCETP Mice Is Dose-Dependent

To investigate whether the HDL-increasing effect of niacin in E3LCETP mice was also dose-dependent, we determined HDL-C concentrations in whole plasma after precipitation of apoB-containing lipoproteins by heparin/MnCl2. Indeed, niacin appeared to decrease (V)LDL-C levels up to −79% (P<0.001; Figure 3A), and to increase HDL-C up to +87% (P<0.001; Figure 3B), both in a dose-dependent fashion. We next evaluated whether niacin also affects apoAI, the main apolipoprotein constituent of HDL. Indeed, niacin dose-dependently increased apoAI up to +72% (P<0.001; Figure 3C). Whereas niacin thus increases both HDL-C and apoAI, the effects on HDL-C at the various doses are somewhat more pronounced than on apoAI, suggesting that niacin increases the lipidation of apoAI. This was
reflected by a modest increase of the HDL particle size as determined by native PAGE (supplemental Figure I). Further analyses of the pooled HDL fractions showed a decrease in triglycerides (~45%) and an increase in cholesteryl ester (+56%) and phospholipids (+66%; data not shown). Niacin did not seem to affect the hepatic synthesis or clearance of HDL, at least judged from unchanged hepatic mRNA expression of genes involved in HDL synthesis (apoAI, apoa1) or clearance (sr-b1; data not shown). Hepatic plp mRNA expression was slightly increased on niacin treatment (data not shown). In plasma niacin did decrease the HL activity, albeit that the effect was not dose-dependent (maximal reduction of ~47% at 118 mg/kg/d; P<0.05).

Niacin Increases the Residence Time of ApoAI in Plasma
To evaluate whether the dose-dependently increased plasma apoAI level as induced by niacin treatment was caused by decreased clearance of apoAI from plasma, we determined the effect of niacin on the plasma kinetics of intravenously injected 125I-apoAI-labeled HDL (Figure 4). Indeed, niacin dose-dependently increased the residence of 125I-apoAI in plasma (Figure 4A). From the mono-exponential decay curves it was calculated that the plasma half-life of 125I-apoAI (3.5±0.1 hour) was increased by niacin at 118 mg/kg/d (5.5±1.3 hour; P<0.01) and 1180 mg/kg/d (6.6±1.3 hour; P<0.01). This was accompanied by a dose-dependent reduction in the uptake of 125I-activity by the liver (up to ~50%; P<0.05) and the kidneys (up to ~90%; P<0.01; Figure 4B). For comparison, the uptake of [3H]cholesteryl oleoyl ether-labeled HDL by the liver was much larger (approx. 40% of dose/g wet weight), whereas the uptake by the kidneys was undetectable (data not shown).

Niacin Reduces the Hepatic Lipid Content
The effects of niacin on plasma lipid metabolism in E3L.CETP mice are consistent with a niacin-induced reduction in CETP activity. Because CETP expression is regulated by the hepatic cholesterol content, we first examined effects of niacin on liver lipids (Figure 5A). Niacin decreased the hepatic TG content (~38%, P<0.05). This is consistent with the inhibitory effects of niacin on HSL in adipose tissue, thereby reducing the trafficking of FFA to the liver for TG synthesis. Niacin also decreased the hepatic TC content (~21%, P<0.01), which was mainly attributed to a reduction in hepatic cholesteryl esters (~23%, P<0.05). This effect was in line with a compensatory increase in hepatic Hmg-coated mRNA expression (+232%, P<0.05; not shown).

Niacin Decreases Hepatic CETP mRNA Expression and Plasma CETP Levels
The decrease in hepatic cholesterol was indeed accompanied by a dose-dependent reduction in hepatic CETP mRNA up to ~88% (P<0.01) at 1180 mg/kg/d (Figure 5B). To evaluate whether the niacin-induced decreased hepatic CETP mRNA expression was reflected by reduced CETP levels in plasma, we determined both CETP mass (Figure 6A) and activity (Figure 6B). Indeed, niacin dose-dependently decreased plasma CETP mass and CETP activity to a similar extent (up to ~45% and ~52%; P<0.001).

Niacin Does Not Affect Biliary and Fecal Cholesterol Output
To evaluate the consequences of the niacin-induced alterations in lipid metabolism on lipid excretion into bile and feces, we determined bile flow, biliary lipids, and sterols in
Niacin did not affect bile flow or the bile composition (cholesterol, phospholipids, and bile acids). The highest dose of niaicin (1180 mg/kg/d) did affect the composition of the fecal sterols to some extent, as reflected by a slight nonsignificant increase in neutral sterols and a decrease in bile acids (~22%; P<0.05). However, like the dietary input, the total fecal sterol output was not affected by niaicin (supplemental Table I).

Discussion

In this study, we investigated the mechanism(s) underlying the HDL-raising effect of niaicin. We demonstrated that CETP plays a crucial role in the niaicin-induced increase in plasma HDL-C and apoAI levels in E3L.CETP mice. Niaicin reduced CETP dependent transfer of cholesterol from HDL to (V)LDL as related to lower hepatic CETP expression and a reduced plasma (V)LDL pool. This resulted in an increased lipidation of apoAI, as reflected by an increased HDL particle size, and a reduced uptake of apoAI by the kidneys.

We previously showed 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 treatment of CVD (eg, statins, fibrates, cholesterol uptake inhibitors, calcium channel blockers and angiotensin II receptor antagonists) with respect to alterations in the lipoprotein profile and/or atherosclerosis development. This is in sheer contrast with wild-type C57Bl/6 mice and conventional hyperlipidemic mice, such as apoE-deficient or LDL receptor–deficient mice, which show either an adverse response or no response to such interventions. In particular, administration of niaicin to wild-type mice or apoE-deficient mice did show a transient decrease in plasma TG and FFA levels, but failed to increase plasma HDL-C in these mice. Likewise, we now showed that niaicin lowered TG and cholesterol within apoB-containing lipoproteins in E3L mice but did not affect HDL-C levels.

Recently, we showed that introduction of the human CETP gene in E3L mice results in a mouse model which also shows a human-like response with regard to raising HDL-C after treatment with fenofibrate, atorvastatin, and torcetrapib. Because the introduction of CETP permits cross-talk between (V)LDL and HDL metabolism via the exchange of neutral lipids, we reasoned that the E3L.CETP mouse would also be an excellent mouse model to study the effects of niaicin on HDL metabolism.

First, we observed that niaicin dose-dependently reduced VLDL-TG and (V)LDL-C levels. The primary action of niaicin is inhibition of HSL activity in adipose tissue after binding to the GPR109A receptor that is selectively expressed by adipocytes. This results in a decreased liberation of FFA from adipose tissue, and a decreased flux of albumin-bound FA to the liver, which is required for substrate-driven hepatic TG synthesis and VLDL production. As a consequence we thus observed a concentration-dependent drop in VLDL-TG and (V)LDL-C levels. In addition, we observed that niaicin reduced the hepatic cholesterol content.
This may be caused by reduced input of cholesterol from plasma into the liver, because plasma (V)LDL-C concentrations are reduced and cholesterol-enriched HDL is formed from which cholesterol esters are presumably not being delivered efficiently to the liver. The decreased hepatic cholesterol content cannot be explained by differences in biliary sterol output, because the excretion of bile acids and cholesterol remained unchanged. Alternatively, niacin may reduce the endogenous hepatic synthesis of cholesterol.

Second, we showed that niacin dose-dependently raised HDL-C levels in E3L.CETP mice, but not in E3L mice, as paralleled by a less pronounced raise in apoAI. The presence of CETP thus plays a crucial role in the HDL-raising effect of niacin, and we reasoned that niacin may dose-dependently inhibit CETP activity. It is well-known that VLDL-TG is a driving force for CETP activity, and the relative proportions of VLDL and HDL have been shown to play a determinant role in CETP activity. It has been demonstrated that the capacity of apoB-containing lipoproteins to accept CE from HDL is closely correlated with the relative TG content of the lipoprotein acceptor particles.30–33 By decreasing VLDL levels, niacin may thus reduce CETP activity simply by decreasing the availability of VLDL-TG as substrate for CETP.

Our data corroborate recent observations from Hernandez et al.15,34 who showed that niacin increased HDL-C levels in CETP mice and APOB.CETP mice, but not their CETP-deficient wild-type littermates. In fact, they speculated the reduced VLDL levels to be the main mechanism underlying the HDL-raising effect of niacin. However, we observed that niacin not only reduced plasma CETP activity but also dose-dependently reduced plasma CETP mass to a similar extent, suggesting that niacin reduces the synthesis of CETP leading to less CETP protein being released in plasma as reflected by similar reductions in CETP mass and activity. Indeed, niacin dose-dependently reduced hepatic CETP mRNA expression. It has been reported that hepatic cholesterol determines the hepatic CETP mRNA expression in CETP transgenic mice,28 presumably via an LXR responsive element in the CETP promoter.35 Therefore, it is likely that niacin decreases the hepatic CETP mRNA expression as a result of the observed decreased cholesterol content of the liver on niacin treatment.

Besides increasing HDL-C, niacin also dose-dependently increased plasma apoAI levels. Niacin has been shown to inhibit the uptake of HDL-apoAI (but not HDL-CE) by cultured hepatocytes,36 which we now confirmed in vivo. This may partly contribute to the increased apoAI levels. Such a potential effect of niacin should be independent of GPR109A, because expression of this receptor has not been detected in hepatocytes.13,37,38 Together with our observations that hepatic mRNA expression of genes involved in HDL synthesis (apoai, acbal) and clearance (sr-b1) were not affected by niacin, and an increase of PLTP would rather lead to a decrease in HDL-C levels,39,40 it is most likely that the raise in apoAI is explained directly by the niacin-induced decreased CETP activity, which prevents cholesteryl ester transfer from HDL to (V)LDL. This leads to increased lipidation of apoAI, resulting in larger and cholesteryl ester-enriched HDL particles, and thus decreased glomerular filtration and excretion of lipid-poor apoAI via the cubulin/megalin receptor complex.40 Indeed, we demonstrated a clear dose-dependent reduction in the uptake of 125I-apoAI by the kidney.

Based on our collective data, we thus propose the following mechanism by which niacin reduces TG and (V)LDL-C and concomitantly raises HDL-C, as summarized in supplemental Figure II. By inhibiting HSL in adipose tissue on binding of the niacin receptor GPR109A, niacin decreases TG lipolysis and thereby the supply of FFA to the liver, required for lipid synthesis. The consequently reduced hepatic lipid content results in a lower VLDL production and thus lower (V)LDL levels. In addition, reduction in hepatic cholesterol results in reduced hepatic expression of CETP, as well as diminished release of CETP into the plasma. Additionally, HL activity is reduced which may contribute to reduced remodeling of HDL in plasma, resulting in decreased clearance of HDL. The HDL particles become CE enriched, and less lipid-poor apoAI is cleared by the kidney. Niacin thus increases HDL-C and apoAI levels by (1) reducing levels of (V)LDL, the acceptor of CETP-mediated HDL-CE transfer, (2) decreasing CETP expression, (3) decreasing HL activity, and (4) decreasing the clearance of apoAI.

As concluded from many clinical trials using statins, lowering LDL-C alone is no longer regarded to be sufficient to treat CVD. Therefore, comprehensive lipid management, in which raising HDL-C is an important target, is becoming a new standard.4,7 Niacin (at dosages of 2 to 4 g/d) is unsurpassed in raising HDL-C. We show that niacin (in a clinically relevant range if we take into account the 5- to 10-times faster metabolism of mice) significantly improves the plasma lipid levels in E3L.CETP mice, eg, reduces TG and (V)LDL-C and increases HDL-C, albeit that total fecal sterol output is
unaffected. Whether this will lead to improved HDL function and HDL-related reductions in CVD in the clinic still remains to be investigated.

Niacin has not been a very successful drug thus far because of its side-effect: severe flushing. Niacin is nowadays produced as an extended release (ER) compound, which enhances the tolerability. Clinical trials AIM-HIGH\(^1\) and ARBITER-6 (HALTS)\(^2\) evaluating the secondary prevention of CVD by ER niacin treatment are currently running. Posthoc analysis of a subgroup of ARBITER-2, a randomized placebo-controlled trial, showed increases in HDL-C on daily intake of ER niacin (+20%), which were related to reduced progression of carotid intima-media thickness in the setting of both normal glycemic status and diabetes mellitus.\(^3\)\(^,\)\(^4\) Because the flushing effects of niacin appeared to be prostaglandin D\(_2\) (PGD\(_2\)) receptor–mediated,\(^5\) a combination therapy is currently being evaluated combining ER niacin and PGD\(_2\) receptor antagonist laropiprant, which is better tolerated than ER niacin alone.\(^6\) Currently one trial evaluating effects of this combination drug on hard clinical end points such as myocardial infarction, stroke, or revascularization (HPS2-THRIVE) is underway.

In conclusion, our results show that niacin increases HDL-C by reducing the hepatic CETP expression and plasma CETP protein and 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 niacin, which should be subject of further investigation.

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Disclosures

None.

References


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METHODS

Animals

Hemizygous human CETP transgenic (CETP) mice, expressing a human CETP minigene under the control of its natural flanking sequences\(^1\) were purchased from the Jackson Laboratory (Bar Harbor, ME) and crossbred with hemizygous E3L mice\(^2\) at our Institutional Animal Facility to obtain E3L and E3L.CETP littermates.\(^3\) 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, E3L and E3L.CETP mice were fed a semi-synthetic cholesterol-rich diet, containing 15% (w/w) fat and 0.25% (E3L) or 0.1% (E3L.CETP) (w/w) cholesterol (Western-type diet; Hope Farms, Woerden, The Netherlands) for three weeks to obtain similar total cholesterol levels in both strains (about 12-14 mmol/L). After matching based on total plasma cholesterol (TC), triglyceride (TG) levels, and age, mice (n=8 per group) received a Western-type diet without or with 0.03% (~36 mg/kg/day), 0.1% (~118 mg/kg/day), 0.3% (~360 mg/kg/day) or 1% (~1180 mg/kg/day) niacin (Sigma, St. Louis, MO, USA) for at least 3 weeks. These doses correspond well to the doses used in humans, if the 10 times faster metabolism of mice as compared to humans is taken into account. 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\(^3\) and assayed for TC, TG and phospholipids (PL), using the commercially available enzymatic kits 236691 and
11488872 (Roche Molecular Biochemicals, Indianapolis, IN, USA) and ‘Phospholipids B’ (Instruchemie, The Netherlands), respectively. The distribution of lipids over plasma lipoproteins was determined by fast-performance liquid chromatography (FPLC) using a Superose 6 column as described previously.\(^3\) HDL-C was isolated by precipitating the apoB-containing lipoproteins from 20 µL EDTA plasma by adding 10 µL heparin (LEO Pharma, The Netherlands; 500 U/mL) and 10 µL 0.2 M MnCl\(_2\). Mixtures were incubated for 20 min at room temperature and centrifuged for 15 min at 13,000 rpm at 4°C. In the supernatant HDL-C was measured using enzymatic kit 236691 (Roche Molecular Biochemicals, Indianapolis, IN, USA).

**Plasma apoAl concentration**

Plasma apoAl concentrations were determined using a sandwich ELISA. Here to, rabbit anti-mouse apoAl polyclonal antibody (ab20453; Abcam plc, Cambridge, UK) was coated overnight onto Costar strips (Costar, Inc., New York, NY) (at 3 µg/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. 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.

**HDL size by native PAGE**

The HDL size was determined essentially as described.\(^4\) Total lipoproteins were isolated from plasma by ultracentrifugation (5 h at 541,000 g) as the d < 1.21 g/mL
plasma fraction in a TLA 100.3 rotor (Beckman). Lipoproteins (7.5 µg protein) were loaded onto a 4-20% polyacrylamide Tris.HCl gel (BioRad, Hercules, CA, USA) and electrophoresis was performed according to the manufacturer’s protocol. Gels were stained with Coomassie Brilliant Blue (Merck) and HDL size was compared with globular protein standards (HMW native marker kit, GE Healthcare).

**Plasma lipolysis**

Post-heparin plasma from overnight fasted mice was collected from the tail vein at 20 minutes after intraperitoneal injection of heparin (1.0 U/g body weight). Post-heparin plasma triacylglycerol hydrolase activity was determined in the presence or absence of 1 mol/L NaCl to estimate the hepatic lipase (HL) activity, which was calculated as the portion of total triacylglycerol hydrolase activity not inhibited by 1 mol/L NaCl.5

**Preparation of ^125^I-apoAl-labeled autologous HDL**

ApoAl was radiolabeled at pH 10 with carrier-free ^125^I according to the ICl method6, and separated from unbound ^125^I by Sephadex G50 gel filtration. ^125^I-apoAl (~75 µg) was incubated with 1.4 mL of plasma from E3L.CETP mice (3 h at 37°C), and ^125^I-apoAl-HDL was isolated after density gradient ultracentrifugation. The specific activity was ~15 cpm/ng HDL protein.

**In vivo kinetics of ^125^I-apoAl-labeled HDL**

E3L.CETP mice were injected via the tail vein with ^125^I-apoAl-HDL (40 µg protein) in a total volume of 200 µL PBS. At the indicated time points after injection, blood was collected from the tail vein to determine the plasma decay of ^125^I-apoAl. The total plasma volumes of the mice were calculated from the equation V (mL) = 0.04706 x body weight (g), as determined from previous ^125^I-BSA clearance studies.7 At 6 h after injection, the mice were sacrificed and organs were taken and counted for ^125^I-activity. Values were corrected for serum radioactivity present in the liver (84.7 µL/g
wet weight), kidneys (135.2 µL/g wet weight), skeletal muscle (13.7 µL/g wet weight) and white adipose tissue (16.1 µL/g wet weight).\(^8\)

**Hepatic lipid analysis**

Liver tissue samples were homogenized in phosphate-buffered saline (approx. 10% wet w/v), and the protein content was measured according to the method of Lowry et al. Lipids were extracted, separated by high-performance thin-layer chromatography on silica gel plates and analyzed with TINA2.09 software (Raytest Isotopen Messgeräte, Straubenhardt, Germany), as described before.\(^9\)

**Hepatic mRNA expression**

Total RNA extraction from liver tissue samples was performed using RNA-Bee (Amsbio, Oxon, UK) according to the manufacturer’s instructions. RNA was converted to single-stranded cDNA by a reverse transcription procedure (Promega) according to the manufacturer’s protocol using random primers. cDNA levels were measured by real-time polymerase chain reaction (PCR) using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions. PCR master mix from Eurogentec was used. Primers and probes were obtained from Biosource (Nivelles, Belgium). The probes were labelled with 3-BHQ1 and 5-FAM or 5-TET. The mRNA levels were normalized to mRNA levels of three housekeeping genes (i.e., cyclophilin, HPRT and GAPDH). Primers and probes used for this study were described previously.\(^10\) The level of mRNA expression for each gene of interest was calculated according to the manufacturer’s instructions (Applied Biosystems) as described previously.\(^11\)
CETP mass and activity in plasma

Plasma CETP mass was analyzed by ELISA using kit 'CETP ELISA Daiichi' (Daiichi Pure Chemicals Co, Ltd, Tokyo, Japan). Plasma CETP activity was measured as the transfer of \([^{3}H]\)cholesteryl oleate ([\(^{3}\)H]CO) from exogenous LDL to HDL as described.\(^{12}\) CETP activity was calculated as µmol CE transfer per mL plasma per hour.

Biliary lipid secretion

The common bile duct of anesthetized mice was ligated, the gall bladder was cannulated, and bile was collected during 90 minutes.\(^{30}\) Cholesterol, PL and total bile acids in bile were determined using kits ‘236691’ (Roche Molecular Biochemicals, Indianapolis, IN, USA), ‘Phospholipids B’ (Instruchemie, The Netherlands) and ‘Total bile acids assay’ (Bio-Stat, UK), respectively.

Fecal excretion of bile acids and neutral sterols

The mice were housed at 3 mice per cage. Feces produced during 2 subsequent periods (48 h each) were separated from the wood shavings by sieving. Aliquots of lyophilized feces were used for determination of neutral and acidic sterol content by gas-liquid-chromatography procedures as described.\(^{5}\)
References


11. Post SM, Groenendijk M, Solaas K, Rensen PC, Princen HM. Cholesterol 7alpha-hydroxylase deficiency in mice on an APOE*3-Leiden background

Supplementary table

**Table I.** Effect of niacin on biliary and fecal lipid output. *E3L.CETP* mice received a Western-type diet without or supplemented with niacin for 3 weeks. The bile bladder was cannulated, and bile flow and composition were measured during 90 minutes (n=6-7). Feces were collected per cage (3 mice per cage) in two subsequent periods of 48 h each (n=8). Fecal composition was measured by gas-liquid-chromatography and fecal sterol output was calculated. Data are presented as mean ± SD, *P*<0.05.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Niacin 118 mg/kg/d</th>
<th>Niacin 1180 mg/kg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bile</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile flow (µL/min/100g bw)</td>
<td>2.0 ± 0.5</td>
<td>2.0 ± 0.4</td>
<td>2.3 ± 0.7</td>
</tr>
<tr>
<td>Bile acid output (nmol/min/100g bw)</td>
<td>50 ± 14</td>
<td>67 ± 18</td>
<td>65 ± 25</td>
</tr>
<tr>
<td>Cholesterol output (nmol/min/100g bw)</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Phospholipid output (nmol/min/100g bw)</td>
<td>14 ± 3</td>
<td>14 ± 3</td>
<td>16 ± 5</td>
</tr>
<tr>
<td><strong>Feces</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutral sterols (µmol/100g bw/d)</td>
<td>32.7 ± 2.2</td>
<td>34.8 ± 4.6</td>
<td>36.1 ± 4.5</td>
</tr>
<tr>
<td>Bile acids (µmol/100g bw/d)</td>
<td>8.6 ± 1.5</td>
<td>8.4 ± 1.6</td>
<td>6.7 ± 0.9*</td>
</tr>
<tr>
<td>Total sterols (µmol/100g bw/d)</td>
<td>41.3 ± 3.1</td>
<td>43.2 ± 5.1</td>
<td>42.7 ± 4.7</td>
</tr>
</tbody>
</table>
Supplementary figures

**Figure I.** Dose-dependent effect of niacin on the HDL particle size. *E3L.CETP* mice received a Western-type diet without or supplemented with incremental doses of niacin for 3 weeks. Total lipoproteins from pooled plasma (n=15 per group) were subjected to native 4-20% PAGE, the gel was stained with Coomassie Brilliant Blue.

**Figure II.** Proposed mechanism underlying the HDL-raising effect of niacin. For explanation see text. CE, cholesteryl ester; FA, fatty acids; HSL, hormone sensitive lipase; TC, total cholesterol; TG, triglycerides.
Figure I

Niacin (mg/kg/day)
Figure II

A. control

B. niacin