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

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Severe Hypertriglyceridemia in Human *APOC1* Transgenic Mice is Caused by ApoCI-Induced Inhibition of LPL

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Objective - Studies in humans and mice have shown that increased expression of apoCI results in combined hyperlipidemia with a more pronounced effect on triglycerides (TG) as compared to total cholesterol (TC). The aim of this study was to elucidate the main reason for this effect using human apoCI-expressing (*APOC1*) mice.

Methods and Results - Moderate plasma human apoCI levels (*i.e.* 4-fold higher than human levels), caused a 12-fold increase in TG, along with a 2-fold increase in TC, mainly confined to VLDL. Cross-breeding of *APOC1* mice on an apoE-deficient background resulted in a marked 55-fold increase in TG, confirming that the apoCI-induced hyperlipidemia cannot merely be attributed to blockade of apoE-recognizing hepatic lipoprotein receptors. The plasma half-life of [³H]TG-VLDL-mimicking particles was 2-fold increased in *APOC1* mice, suggesting that apoCI reduces the lipolytic conversion of VLDL. While total post-heparin plasma LPL activity was not lower in *APOC1* mice as compared to controls, apoCI was able to dose-dependently inhibit the LPL-mediated lipolysis of [³H]TG-VLDL-mimicking particles *in vitro*, with a 60% efficiency as compared to the main endogenous LPL inhibitor apoCIII. Finally, purified apoCI impaired the clearance of [³H]TG-VLDL-mimicking particles independent of apoE-mediated hepatic uptake, in lactoferrin-treated mice.

Conclusion - Therefore, we conclude that apoCI is a potent inhibitor of LPL-mediated TG lipolysis.

The human apolipoprotein CI (apoCI)-encoding gene *APOC1* is part of the *APOE/APOC1/APOC2* gene cluster.¹ *APOC1* is primarily expressed in the liver, but also in the lung, skin, spleen, adipose tissue, and brain.² ApoCI is secreted as a 6.6-kD protein into the plasma, where it is present at a relatively high concentration of approximately 10 mg/dl,³ and is mainly bound to chylomicrons, VLDL and HDL.⁴ Although human studies have not revealed any polymorphism in the *APOC1* gene leading to functional apoCI variants thus far, an *HpaI* polymorphism in the promoter region has been described that leads to 57% increased expression of the *APOC1* gene.⁵ Interestingly, *HpaI* carriers display increased plasma triglyceride (TG) levels, which are independent of total cholesterol (TC) levels.⁶ To get more insight into the function of apoCI in lipoprotein metabolism, we and others have generated mice that either lack endogenous apoCI^{7,8} or express the human *APOC1* gene.^{9,10} Although apoCI-deficient mice did not show a phenotype with respect to plasma lipid levels,⁷ *APOC1* transgenic mice indeed showed an *APOC1* gene dose-dependent increase in plasma levels of TG, TC, and free fatty acids (FFA). The most prominent increasing effect of *APOC1* was observed on TG levels, and could be attributed to severely increased levels of VLDL.^{9,11}

Early reports have postulated that apoCI may function by both modulation of the activity of plasma enzymes involved in lipid metabolism and by modulation of TG-rich lipoprotein (remnant) binding and uptake by hepatic receptors. *In vitro* studies have shown that apoCI may interfere with VLDL metabolism by partial activation of lecithin:cholesterol acyl transferase (LCAT),¹² inhibition of lipoprotein lipase (LPL)¹³ and inhibition of hepatic lipase (HL).¹⁴ Recently, Conde-Knape *et al.*¹⁵ have confirmed such an HL-modulating function of apoCI *in vitro*, and have suggested that HL modulation may contribute to the hypertriglyceridemic phenotype of *APOC1* transgenic mice. Strikingly, HL-deficient mice do not show any sign of disturbed TG metabolism.¹⁶⁻¹⁸ In addition, LCAT transgenic mice do not show elevated VLDL,¹⁹ suggesting that potential LCAT-activating properties of apoCI do not contribute to the phenotype of *APOC1* mice.

Besides modulation of plasma enzymes, apoCI has also been reported to interfere with the apoE-dependent hepatic uptake of lipoprotein remnants by the LDL receptor (LDLr) and LDLr-related protein (LRP). In the isolated rat liver perfusion model, it was demonstrated that addition of human apoCI inhibits the catabolism of chylomicrons and TG-rich emulsions.^{20,21} Subsequently, it was shown that apoCI can interfere with the apoE-mediated uptake of VLDL by the LDLr²² and LRP,²³ possibly related to apoCI-induced masking of the receptor binding domain of apoE²² or displacement of apoE from the lipoprotein particle.²³ More recent studies from our group^{9,24} have suggested that the inhibiting properties of apoCI towards the LRP may exceed those towards the LDLr, since the apoCI-associated hyperlipidemia was severely aggravated on an LDLr-deficient background.⁹ In addition, it was shown that transfection of *ldlr*^{-/-}*APOC1* mice with a recombinant adenovirus encoding the 39-kDa receptor associated protein (RAP) further increased plasma TG levels 4-fold. However, although these studies certainly point at a modulating role of apoCI with respect to hepatic lipoprotein recognition, it remains remarkable that complete blockade of the apoE-mediated lipoprotein clearance in apoE-deficient mice hardly affects plasma TG levels^{18,25,26} whereas TG levels

are severely increased by *APOC1* expression, indicating that apoCI has a profound additional effect.

Since the proposed functions of apoCI cannot explain the severe hypertriglyceridemic phenotype of *APOC1* mice, the aim of the present study was to elucidate the main mechanism underlying the apoCI-induced combined hyperlipidemia in *APOC1* mice. We demonstrate that apoCI is a potent inhibitor of LPL, which can explain the combined hyperlipidemia observed in *APOC1* transgenic mice both in the presence and absence of endogenous apoE.

Materials and Methods

Transgenic Animals

Transgenic *APOC1* mice with hemizygous expression of the human *APOC1* gene have been generated previously as described,^{7,9} and back-crossed at least 10 times to C57Bl/6 background. *APOC1* mice were intercrossed with apoE-deficient (*apoe*^{-/-}) mice (C57Bl/6 background) that have originally been obtained from the Jackson Laboratories (Bar Harbor, ME) to generate mice hemizygous for the *APOC1* gene on an apoE-deficient background (*apoe*^{-/-}*APOC1*). After initial characterization of both male and female mice, 10-12 weeks-old male *APOC1* and *apoe*^{-/-}*APOC1* mice were used for subsequent experiments, with wild-type and *apoe*^{-/-} littermates as controls. Mice were housed under standard conditions in conventional cages and were fed ad libitum with regular chow (Ssniff, Soest, Germany). Experiments were performed after 4 h of fasting at 1:00 pm with food withdrawn at 9:00 am, unless stated otherwise.

Plasma Lipid and Lipoprotein Analysis

In all experiments blood was collected from the tail vein into chilled paraoxon (Sigma, St. Louis, MO)-coated capillary tubes to prevent ongoing *in vitro* lipolysis,²⁷ unless indicated otherwise. These tubes were placed on ice, centrifuged at 4°C, and the obtained plasma was assayed for TC, TG (without free glycerol), and FFA using the commercially available enzymatic kits 236691 (Roche Molecular Biochemicals, Indianapolis, IN, USA), 337-B (GPO-Trinder kit; Sigma), and NEFA-C (Wako Chemicals, Neuss, Germany), respectively. For determination of the plasma lipoprotein distribution by fast performance liquid chromatography (FPLC), 50 µl of pooled plasma from 10 mice per group was injected onto a Superose 6 column (Äkta System; Amersham Pharmacia Biotech, Piscataway, NJ, USA), and eluted at a constant flow rate of 50 µl/min with PBS, 1 mM EDTA (Sigma), pH 7.4. Fractions of 50 µl were collected and assayed for TC and TG as described above. Human apoCI was quantified by ELISA as described below.

VLDL-Isolation and Characterization

Fasted mice were sacrificed by cervical dislocation and blood was drawn from the retro-orbital vein into Microvette[®] CB 1000 Z capillaries (Sarstedt, Nümbrecht, Germany). Sera were collected after centrifugation at 4°C, and pooled from 10 mice. VLDL was isolated by flotation (*d* < 1.006 g/ml) after ultracentrifugation in a Beckman SW 40 Ti rotor (Beckman Instruments, Geneva, Switzerland) at 40,000 rpm during 18 h at 4°C.

The VLDL fractions were assayed for TG and TC as described above, and for free cholesterol (FC) and phospholipids (PL) using the commercially available analytical kits 274-47109 and 990-54009 (Wako Chemicals), respectively. Cholesteryl esters (CE) were calculated by subtracting the molar concentration of FC from the molar concentration of TC, and corrected for the presence of the fatty acid. Protein was determined by the method of Lowry *et al.*²⁸ with BSA as a standard. VLDL-particle size was determined by photon correlation spectroscopy using a Zetasizer 3000 HSA (Malvern Instruments, Malvern, UK) at 25°C with a 90° angle between laser and detector.

Human ApoCI ELISA

Plasma human apoCI concentrations were determined using a human apoCI-specific sandwich ELISA. Hereto, a polyclonal goat anti-human apoCI antibody (Academy Biomedical Company, Houston, TX, USA) was coated overnight onto Costar medium binding plates (Corning Incorporated, New York, NY) (dilution 1:10⁴) at 4°C and incubated with diluted mouse plasma (dilution 1:10⁶-10⁷) or FPLC fractions (1:10⁴), for 2 h at 4°C. Subsequently, horse radish peroxidase (HRP)-conjugated polyclonal goat anti-human apoCI antibody (dilution 1:500; Academy Biomedical Company) was added, incubated for 2 h at room temperature, and HRP was detected by incubation with tetramethylbenzidine (Organon Teknika, Boxtel, The Netherlands) for 20 min at room temperature. Plasma from wild-type mice spiked with human apoCI (Labconsult, Brussels, Belgium) was used as a standard.

Intestinal Triglyceride Absorption

APOC1 mice and wild-type littermates were fasted overnight and injected intravenously with 500 mg of Triton WR 1339 (Sigma) per kg body weight as a 10% (v/v) solution in sterile saline, to block LPL-mediated TG-hydrolysis.²⁹ Subsequently, mice were given an intragastric load of glycerol tri[9,10(n)-³H]oleate (10 µCi; Amersham, Buckinghamshire, UK) ([³H]TO) in 200 µl of olive oil. Blood samples were drawn before and at the indicated times after olive oil administration by tail bleeding. Lipids were extracted from plasma according to the method of Bligh and Dyer,³⁰ and TG was separated from the other lipid components by thin layer chromatography on Kieselgel 60 F254 plates (Merck, Darmstadt, Germany) by using hexane: diethyl ether: acetic acid (83: 16: 1, v/v/v) as eluents. The radioactivity in the TG fraction was determined by scintillation counting (Packard Instruments, Dowers Grove, IL) according to Voshol *et al.*³¹

Hepatic VLDL-Triglyceride Production

Mice were fasted, anesthetized by intraperitoneal injection of domitor (0.5 mg/kg; Pfizer, New York, NY, USA), dormicum (5 mg/kg; Roche Netherlands, Mijdrecht, The Netherlands), and fentanyl (0.05 mg/kg; Janssen-Cilag B.V., Tilburg, The Netherlands), and injected via the tail vein with 500 mg of Triton WR1339 per kg body weight.³² Blood samples were drawn at 1, 30, 60, 90, and 120 min after administration and plasma TG levels were measured as described above.

Preparation and In Vivo Clearance of VLDL-Like Triglyceride-Rich Emulsion Particles

The preparation and characterization of 80 nm-sized protein-free VLDL-like emulsion particles have previously been described.³³ Briefly, emulsion particles were prepared by sonication from 100 mg of total lipid at an egg yolk phosphatidylcholine (Lipoid, Ludwigshafen, Germany): triolein: lysophosphatidylcholine: cholesteryl oleate: cholesterol (all from Sigma) weight ratio of 22.7: 70: 2.3: 3.0: 2.0, in the presence of either 75 μ Ci of [³H]TO or 150 μ Ci of [$1\alpha,2\alpha(n)$ -³H]cholesteryl oleate ([³H]CO; Amersham) using a Soniprep 150 (MSE Scientific Instruments, Crawley, UK) at 10 μ m output. The lipid composition of the emulsions was determined as described above. Emulsions were stored at 4°C under argon and were used within 7 days. To study the *in vivo* serum clearance of the radiolabeled emulsions, mice were anesthetized as described above and the abdomens were opened. The emulsion (100 μ g of TG), preincubated (30 min at 37°C) with or without human apoC1 (50 μ g), was injected intravenously via the vena cava inferior. When indicated, mice received a preinjection of bovine lactoferrin (70 mg/kg; Serva, Heidelberg, Germany) at 1 min before injection of the radiolabeled emulsion. Blood samples (< 50 μ l) were taken via the vena cava inferior at the indicated times, and the radioactivity in serum was counted as described above. The total plasma volumes of the mice were calculated from the equation: V (ml) = 0.04706 x body weight (g), as determined from ¹²⁵I-BSA clearance studies as previously described.³⁴

Plasma LPL Level Assay

Fasted *APOC1* mice and wild-type littermates were injected via the tail vein with heparin (0.1 U/g; Leo Pharmaceutical Products B.V., Weesp, The Netherlands) and blood was collected after 10 min. The plasma thereof was snap-frozen and stored at -80°C until analysis of the total LPL activity as modified from Zechner.³⁵ In short, a TG substrate mixture containing triolein (4.6 mg/ml), [³H]TO (2.5 μ Ci/ml), essentially fatty acid-free BSA (20 mg/ml; Sigma), Triton X-100 (0.1%; Sigma) and heat-inactivated (30 min 56°C) human serum (20%) in 0.1 M Tris-HCl, pH 8.6, was generated by 6 sonication periods of 1 min using a Soniprep 150 at 7 μ m output, with 1 min intervals in between on ice. 10 μ l of post-heparin plasma was added to 0.2 ml of substrate mixture and incubated for 30 min at 37°C in the presence or absence of 1 M NaCl, which completely inhibits LPL activity, to estimate both the LPL and HL levels. The reaction was stopped by the addition of 3.25 ml of heptane: methanol: chloroform (1: 1.28: 1.37, v/v/v), and 1 ml of 0.1 M K₂CO₃ in saturated H₃BO₃ (pH 10.5) was added. To quantify the generated [³H]oleate, 0.5 ml of the aqueous phase obtained after vigorous mixing (15 sec) and centrifugation (15 min at 3600 rpm) was counted in 4.5 ml of Ultima Gold (Packard Bioscience, Meriden, CT, USA). The LPL activity was calculated as the fraction of total triacylglycerol hydrolase activity inhibited by 1 M NaCl and expressed as the amount of FFA released per hour per ml of plasma.

In Vitro LPL Activity Assay

The effect of apolipoproteins on the TG hydrolysis of VLDL-like emulsion particles was determined as described.³⁶ Hereto, [³H]TO-labeled emulsion particles (0.5 mg of TG/ml) were preincubated with apoCI, apoCIII (Labconsult), apoAI (Calbiochem, San Diego, CA, USA), or recombinant apoAV³⁷ at the indicated TG: protein weight ratios (30 min at 37°C). Subsequently, the protein-enriched particles were incubated with LPL in the presence of essentially fatty acid-free BSA (60 mg/ml) and heat-inactivated human serum (5%) in 0.1 M Tris-HCl, pH 8.5. At the indicated times, 50 µl samples from a 400 µl total incubation volume were added to 1.5 ml methanol: chloroform: heptane: oleic acid (1410: 1250: 1000: 1, v/v/v/v) and 0.5 ml of 0.2 N NaOH to terminate lipolysis. Generated [³H]oleate was counted as described above and expressed as percentage of the total [³H]activity added.

Statistical Analysis

Statistical differences with respect to *in vivo* serum half-lives were determined using a two-way main effects analysis of variance (ANOVA). All other data were analyzed using nonparametric Mann-Whitney U tests. *P* values less than 0.05 were regarded as significant.

Results

Effect of APOC1 on Plasma ApoCI and Lipid Levels

Table 1 summarizes the plasma human apoCI and lipid levels in fasted male *APOC1* mice and wild-type littermates on chow diet. *APOC1* mice had approximately 4-fold higher plasma levels of human apoCI as compared to humans,³ which was accompanied by severe combined hyperlipidemia. The enhancing effect of *APOC1* expression on TG (12-fold) was much more pronounced than that on TC (2.1-fold) and FFA (1.5-fold), which is in agreement with our previous reports.^{9,11} Similar effects of human apoCI expression were observed in females as compared to males (data not shown). Lipoprotein fractionation by FPLC showed that the plasma human apoCI was primarily distributed towards HDL and VLDL (Fig. 1A). The increase in both plasma TG and TC as a result of *APOC1* expression could be mainly attributed to highly elevated levels observed in VLDL and mildly increased levels in IDL/LDL, whereas the neutral lipid levels of the HDL-fraction were hardly affected (Figs. 1B and C).

Effect of APOC1 in ApoE-Deficient Mice

Although apoCI has been postulated to inhibit the apoE-dependent hepatic uptake of TG-rich lipoprotein remnants, apoE-deficient (*apoe*^{-/-}) mice show only minor elevation of plasma TG. To investigate the effects of *APOC1* in the absence of endogenous apoE, *APOC1* mice were intercrossed with *apoe*^{-/-} mice, to generate *apoe*^{-/-}*APOC1* mice. Plasma human apoCI levels in *apoe*^{-/-}*APOC1* mice were approximately 4-fold higher as compared to *APOC1* mice, and severely further aggravated the hyperlipidemia as observed in *apoe*^{-/-} littermates (Table 1). Similar as on a wild-type background, *APOC1* expression on an *apoe*^{-/-} background had a much more pronounced effect on TG (55-

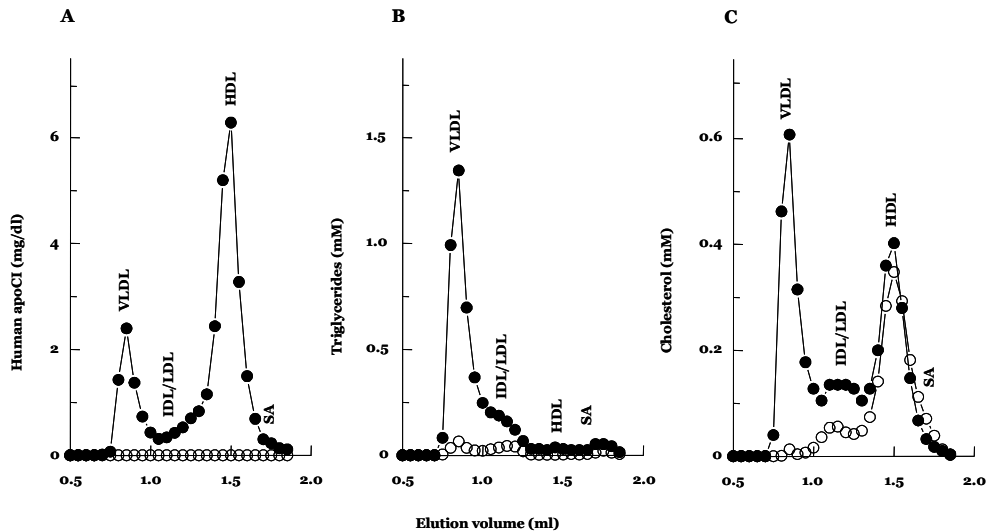


Figure 1. Effect of *APOC1* on FPLC profiles of human apoCI and lipids. Plasmas of male *APOC1* (closed circle) and wild-type (open circle) mice (n=10) were pooled and size-fractionated by FPLC on a Superose 6 column. The individual fractions were analyzed for human apoCI (A), TG (B) and TC (C).

Table 1. Effect of *APOC1* on plasma lipid levels in wild-type and *apoe*^{-/-} mice.

Genotype	Human apoCI (mg/dl)	TG (mmol/l)	TC (mmol/l)	FFA (mmol/l)
Wild-type background				
Wild-type	n.d.	0.32±0.06	2.06±0.17	0.79±0.15
<i>APOC1</i>	39.7±9.4	3.86±0.75***	4.28±0.57***	1.18±0.20***
<i>Apoe</i> ^{-/-} background				
<i>Apoe</i> ^{-/-}	n.d.	0.59±0.20	11.0±5.2	0.78±0.13
<i>Apoe</i> ^{-/-} <i>APOC1</i>	160±60	32.6±8.8***	35.7±7.1**	2.52±0.77***

4 h fasted plasma was obtained from 10-12 weeks-old male *APOC1* (n=23), wild-type (n=10), *apoe*^{-/-}*APOC1* (n=10) and *apoe*^{-/-} (n=6) mice. Plasma human apoCI, TG, TC and FFA levels were measured and values are represented as means ± S.D. Statistical differences were assessed between *APOC1* and wild-type mice, and between *apoe*^{-/-}*APOC1* and *apoe*^{-/-} mice. ***P*<0.01, ****P*<0.001; n.d., not detectable.

fold), than on TC (3.2-fold) and FFA (3.2-fold). Again, similar data were observed in females as compared to males (data not shown).

Effect of APOC1 on VLDL Composition

To investigate whether the effect of *APOC1* expression on plasma lipid levels was accompanied by a change in VLDL composition and/or size, VLDL was isolated from *apoe^{-/-}APOC1* mice and their *apoe^{-/-}* littermates, and their relative lipid compositions were determined (Table 2). The composition of VLDL from wild-type mice could not be determined accurately, because of low circulating levels (see Fig. 1). VLDL of *apoe^{-/-}APOC1* mice was predominantly enriched in TG, as compared to VLDL from *apoe^{-/-}* littermates, and had a higher core lipid (TG + CE) to surface lipid (FC + PL) ratio (2.7 vs. 2.4, respectively), indicative for larger VLDL-particles. Indeed, VLDL particle size analysis confirmed that *APOC1* expression markedly increased VLDL-size compared to control littermates, both on wild-type background (average size 72.9 vs. 44.4 nm, respectively) and *apoe^{-/-}* background (average size 64.5 vs. 50.6 nm, respectively).

Table 2. Effect of *APOC1* on VLDL lipid composition.

Genotype	TG	CE	FC	PL
	<i>mg/mg VLDL protein</i>			
<i>Apoe^{-/-}</i>	1.3	2.0	0.55	0.82
<i>Apoe^{-/-}APOC1</i>	3.0	0.94	0.56	0.89

VLDL (d<1.006 g/ml) was isolated from pooled plasma of fasted mice by ultracentrifugation and the TG, CE, FC, PL and protein content was measured.

Effect of APOC1 on Intestinal Triglyceride Absorption and Hepatic VLDL-Triglyceride Production

To further address the mechanism(s) underlying the hypertriglyceridemia in *APOC1* mice, we determined whether the intestinal TG absorption and/or the hepatic VLDL-TG production rate were enhanced in these mice. First, the intestinal TG absorption was studied by injecting intravenously Triton WR1339, to block LPL-mediated TG-hydrolysis,²⁹ after which an intragastric load of olive oil containing [³H]TO was given. As shown in Fig. 2, no differences were observed between *APOC1* and wild-type mice with respect to appearance of radioactivity in plasma TG, indicating that apoC1 expression does not enhance triglyceride absorption from the intestinal lumen.

The hepatic VLDL-TG production rate was measured by determining plasma TG levels at the indicated times after intravenous Triton WR1339 injection (Fig. 3). Whereas the TG levels in the *APOC1* mice were higher as compared to the wild-type littermates at the start of the experiment (4.9 ± 2.1 vs. 0.42 ± 0.08 mM, respectively; Fig. 3A), the relative increase in TG was similar for both types of mice (7.4 ± 0.9 vs. 6.6 ± 0.8 mM/h,

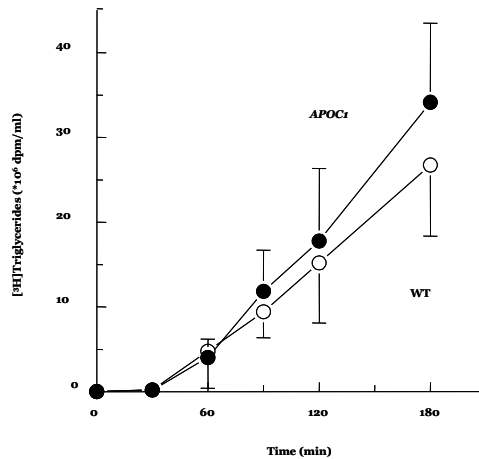


Figure 2. Effect of *APOC1* on intestinal lipid absorption. Overnight fasted *APOC1* (closed circle) and wild-type (open circle) mice were injected intravenously with Triton WR1339 (500 mg/kg), and subsequently given an intragastric load of [³H]TO in 200 μ l of olive oil. Blood samples were drawn at the indicated times and lipids were extracted from plasma. Lipids were separated by thin layer chromatography and the radioactivity in the TG fraction was determined by scintillation counting. Values are means \pm S.D. (n=7).

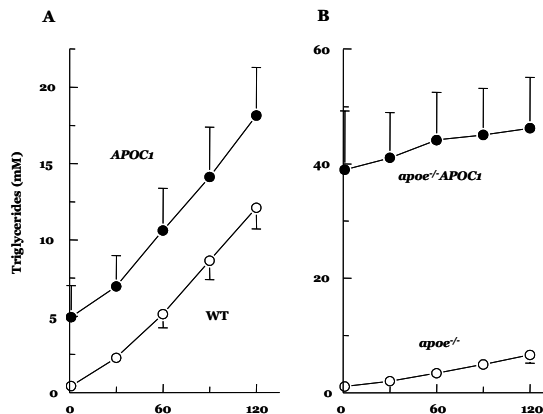


Figure 3. Effect of *APOC1* on hepatic VLDL-triglyceride production. Triton WR1339 (500 mg/kg) was injected (t=0) into fasted *APOC1* (closed circle) and wild-type (open circle) mice (A) and in *apoe*^{-/-}*APOC1* and *apoe*^{-/-} mice (B). Plasma TG levels were determined at 1, 30, 60, 90 and 120 min after injection. Values represent means \pm S.D. (n=6).

respectively). Likewise, we found no difference in the relative increase in TG levels in *apoe*^{-/-}*APOC1* as compared to *apoe*^{-/-} mice (3.3 ± 1.4 vs 3.1 ± 0.7 mM/h, respectively; Fig. 3B), indicating that apoCI expression does not affect the hepatic VLDL-TG production rate either.

Effect of APOC1 on In Vivo Clearance of VLDL-Like Emulsion Particles

To investigate whether an impaired lipolytic processing of TG-rich lipoproteins may contribute to the hypertriglyceridemia observed in *APOC1* mice, mice were injected with [^3H]TO-labeled protein-free VLDL-like emulsion particles, which have previously been shown to mimic the metabolic behaviour of TG-rich lipoproteins.^{33,38} As shown in Fig. 4, the clearance of [^3H]TO was markedly decreased in *APOC1* mice compared to their wild-type littermates, as evident from a 2-fold increased serum half-life of [^3H]TO (7.9 ± 2.1 vs. 4.0 ± 0.3 min, respectively; $P < 0.05$). This observation indicates that *APOC1* expression impairs TG clearance, which may result from inhibition of the LPL-mediated VLDL-TG hydrolysis.

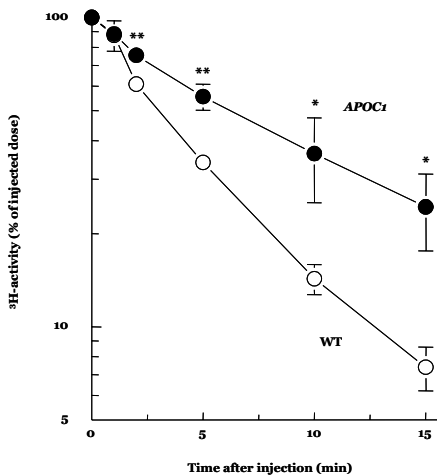


Figure 4. Effect of *APOC1* on serum clearance of VLDL-like emulsion particles *in vivo*. [^3H]TO-labeled emulsion particles (100 μg of TG) were injected via the vena cava inferior into anesthetized *APOC1* (closed circle) and wild-type (open circle) mice. Blood samples were taken at the indicated times, and ^3H -activity was determined in serum. Values are means \pm S.D. ($n=3$). * $P < 0.05$, ** $P < 0.01$.

Effect of APOC1 on Plasma LPL Levels

An impaired clearance of VLDL-TG in *APOC1* mice can be due to either a decreased expression of LPL and/or apoC1-induced inhibition of the activity of LPL. Therefore, we first determined plasma lipase levels in post-heparin plasma by incubation with a [^3H]TO-containing substrate mixture (Fig. 5). Whereas the post-heparin HL level was only slightly increased in *APOC1* mice as compared to wild-type littermates (12.8 ± 1.2 vs. 11.3 ± 1.0 μmol FFA/h/ml, respectively; $P < 0.05$), the post-heparin LPL level was even 1.8-fold increased in *APOC1* mice as compared to wild-type mice (40.7 ± 6.1 vs. 22.5 ± 2.2 μmol FFA/h/ml, respectively; $P < 0.01$). Therefore, the impaired lipolytic conversion of VLDL in *APOC1* mice cannot be due to decreased levels of LPL.

Effect of ApoC1 on LPL Activity

To investigate whether the apoC1-related impaired lipolytic conversion of VLDL can be due to a direct inhibiting effect of apoC1 on LPL activity, protein-free VLDL-like emul-

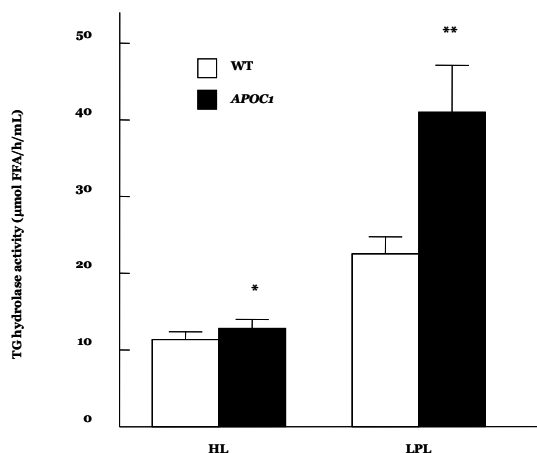


Figure 5. Effect of APOC1 on plasma HL and LPL levels *in vivo*. Fasted APOC1 (closed bars) and wild-type (open bars) mice were injected intravenously with heparin (0.1 U/g). Plasma, collected at 10 min after injection, was incubated (30 min at 37°C) with a [³H]TO containing substrate mixture in the absence or presence of 1 M NaCl, to estimate both the LPL and HL activity. Generated [³H]oleate was extracted and determined as described. Values represent means ± S.D. (n=8). **P*<0.05, ***P*<0.01.

sion particles were enriched with increasing concentrations of purified human apoCI and subsequently incubated with LPL. The well-established endogenous LPL inhibitor apoCIII^{39,40} was used as a control. ApoCI and apoCIII were compared on a mass basis,

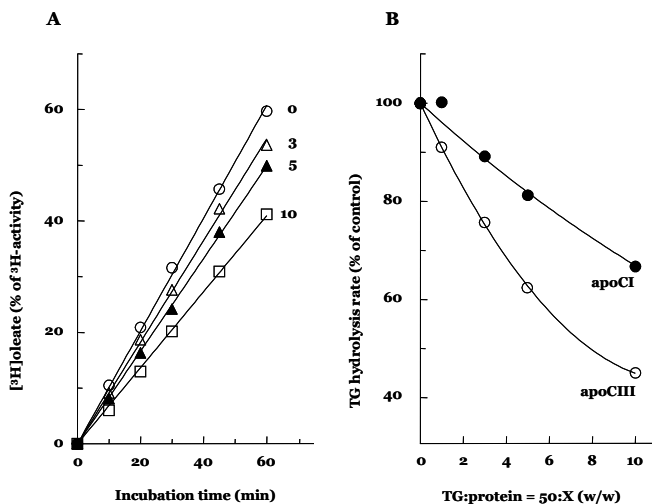


Figure 6. Effect of apoCI on LPL-mediated hydrolysis of VLDL-like emulsion triglycerides. (A) [³H]TO-labeled protein-free emulsion particles were preincubated (30 min at 37°C) in the absence (open circle) and presence of apoCI at TG: apoCI = 50: 3 (open triangle), 50:5 (closed triangle), and 50:10 (open square) weight ratios. At the indicated times after addition of LPL, generated [³H]oleate was extracted and quantified. (B) The effect of apoCI (closed circle) on LPL-mediated TG hydrolysis was compared with that of apoCIII (open circle), and depicted as percentage of the TG hydrolysis rate in the absence of protein.

as they are also present in human plasma at similar mass concentrations (*i.e.* 10 and 13 mg/dl).³ ApoCI appeared to dose-dependently inhibit the TG hydrolysis rate (Fig. 6A). At a TG: protein = 50: 10 weight ratio, apoCI and apoCIII inhibited the triacylglycerol hydrolase activity of LPL by 33% and 55%, respectively (Fig. 6B). In contrast, apoAI did not affect lipolysis, and apoAV even dose-dependently increased the lipolysis rate up to 1.5-fold at a TG: apoAV = 50: 3 weight ratio (data not shown), which is in agreement with our previous observations.^{36,37}

Effect of ApoCI-Enrichment of VLDL-Like Emulsion Particles on In Vivo Clearance

To assess whether apoCI can directly inhibit lipolysis *in vivo*, the effect of apoCI protein was determined on the plasma decay of [³H]TO-labeled protein-free VLDL-like emulsion particles in wild-type mice. To focus on the effects of apoCI on peripheral lipolysis rather than on liver uptake, mice were preinjected with lactoferrin. Lactoferrin has previously been shown to block the interaction of chylomicrons and emulsion particles with the liver *in vivo*,^{33,41} which we confirmed using [³H]CO-labeled protein-free VLDL-like emulsion particles (results not shown). As depicted in Figure 7, preincubation of emulsion particles with apoCI markedly delayed the clearance of [³H]TO as evident from a 1.9-fold increased serum half-life (17.6 ± 5.7 vs. 9.2 ± 3.7 min, respectively; $P < 0.05$), indicating that apoCI can indeed inhibit the lipolytic TG conversion *in vivo*.

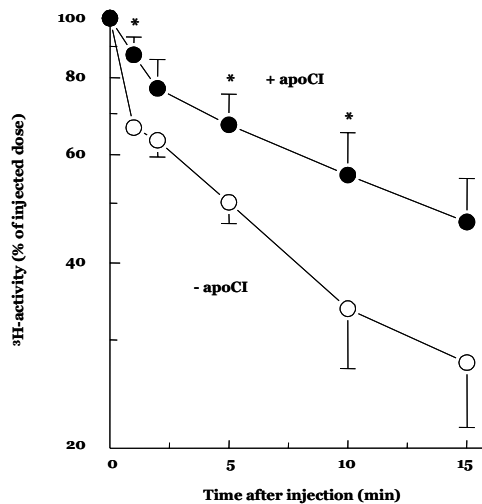


Figure 7. Effect of human apoCI-enrichment of VLDL-like emulsion particles on their serum clearance in lactoferrin-treated mice. [³H]TO-labeled emulsion particles (100 μ g of TG) were preincubated without (open circle) or with (closed circle) human apoCI (50 μ g) for 30 min at 37°C, and injected via the vena cava inferior into anesthetized lactoferrin-treated wild-type mice. Blood samples were taken at the indicated times, and ³H-activity was determined in serum. Values are means \pm S.E.M. (n=3). * $P < 0.05$.

Discussion

Studies in both humans⁶ and mice^{9,10} have shown that increased expression of apoCI results in combined hyperlipidemia, with a more pronounced enhancing effect on TG as compared to TC. Since a variety of effects on lipid metabolism has been attributed to apoCI, including activatory effects (*e.g.* LCAT) and inhibitory effects (*e.g.* HL, CETP, intestinal absorption, and apoE-dependent recognition by LRP, LDLr and VLDLr), the aim of the present study was to elucidate the main mechanism underlying the apoCI-related hypertriglyceridemia using *APOC1* transgenic mice. We demonstrated that at moderate plasma human apoCI levels (*i.e.* 4-fold higher than those found in humans³), the 12-fold increase in plasma TG levels was mainly due to inhibition of the lipolytic processing of VLDL.

The effects of apoCI on lipid metabolism were mainly confined to VLDL metabolism, leaving HDL metabolism (which crucially involves both CETP and LCAT) unaffected. Analysis of the HDL-protein constituents for CETP-modulating properties showed that apoCI is a very potent and highly selective inhibitor of CETP.⁴² In addition, Gautier *et al.*⁴³ have shown that cross-breeding of human *CETP* transgenic mice with apoCI-deficient mice resulted in a higher CETP activity *in vivo*. Although apoCI thus appears to be a physiologically relevant inhibitor of CETP, this function of apoCI cannot contribute to the phenotype of *APOC1* mice, since mice do not express CETP.⁴⁴ Activation of LCAT should be expected to lead to increased HDL size and HDL lipids as was observed in mice and rabbits that overexpress LCAT.^{19,45} Since both the cholesterol level and size of HDL are not affected by apoCI expression in *APOC1* mice, potential LCAT-activating properties of apoCI¹² do not appear to be relevant for determining HDL levels in mice.

ApoCI expression thus predominantly affects VLDL-TG metabolism, which can result from either i) increased intestinal TG absorption, ii) increased VLDL-TG production, and/or iii) disturbed lipolytic conversion and/or hepatic clearance of VLDL. Previously, we have reported that mice deficient for apoCI showed a significantly lower intestinal lipid absorption as compared to wild-type mice.³⁴ However, no changes in intestinal lipid absorption were observed in *APOC1* mice as compared to wild-type littermates, which can be related to a relatively low expression of human apoCI in the intestine. Previously, we have shown that apoE-deficient mice show a decreased VLDL-TG secretion rate,^{18,25,26} and we confirm this observation in our present study. Although human apoCI is highly expressed in the liver, we did not detect any effect of human apoCI expression on hepatic VLDL-TG production rate on both a wild-type and apoE-deficient background, which is in line with our previous studies showing that apoCI-deficiency did not alter the hepatic VLDL production rate.⁷ Apparently, expression of human apoCI can not compensate for the decreased VLDL-TG production in apoE-deficient mice. Collectively, the hypertriglyceridemia in *APOC1* mice is not caused by either an effect on intestinal TG absorption or hepatic TG production.

Next, we evaluated the effect of apoCI expression on apoE-dependent VLDL uptake by the liver. ApoCI has been shown to inhibit the apoE-mediated binding of TG-rich lipoprotein remnants by hepatic lipoprotein receptors (*i.e.* LDLr and LRP),^{22,23,46} although Quarfordt *et al.*²⁰ reported that the apoCI-mediated inhibition of the uptake of TG-rich

emulsions by cultured hepatocytes was (at least partly) independent on the presence of apoE. Indeed, we have shown that *APOC1* expression in mice can interfere with hepatic interaction of VLDL primarily via LRP.^{9,24} However, the contribution of this effect to the *APOC1*-induced severe hypertriglyceridemia can be questioned since complete blockade of the apoE-dependent hepatic lipoprotein clearance in *apoE*^{-/-} mice only mildly affects plasma TG levels,²⁶ whereas *APOC1* expression increases plasma TG as much as 12-fold. In addition, we now show that *APOC1* expression on an *apoE*^{-/-} background further dramatically increased TG levels, showing that the hypertriglyceridemic effects of apoCI can be independent of the presence of apoE. Taken together, these data indicate that the hypertriglyceridemia observed in *APOC1* mice can also not be explained by inhibition of apoE-mediated hepatic remnant uptake.

Finally, we evaluated the possibility that the lipolytic conversion of VLDL may be impaired in *APOC1* mice, since such a mechanism may explain the dramatic accumulation of plasma TG in primarily VLDL. In addition, a decreased plasma TG hydrolysis may also explain the increased VLDL particle size observed in *APOC1* expressing mice on both wild-type and *apoE*^{-/-} background, and the observed impaired clearance of VLDL-like emulsion particles upon intravenous administration.

Recently, Conde-Knape *et al.*¹⁵ described the cross-breeding of their human apoCI-expressing mouse strain with *apoE*^{-/-} mice, which resulted in a comparable, albeit more modest, hypertriglyceridemic phenotype as our *apoE*^{-/-}*APOC1* mice, and they suggested that the hypertriglyceridemia in these mice was due to inhibition of HL-mediated TG-hydrolysis. However, HL-deficiency or overexpression in mice and rabbits predominantly affects plasma HDL-TC levels with only mild effects (if any) on TG levels on both wild-type and *apoE*^{-/-} backgrounds.^{16-18,47} Furthermore, HL has a much lower preference for TG as compared to LPL⁴⁸ and HL is known to primarily mediate the conversion of IDL to LDL, and of HDL₂ to HDL₃,⁴⁹ while both our studies and those of Conde-Knape *et al.*¹⁵ indicate that *APOC1* expressing mice merely have a disturbed VLDL metabolism. Therefore, although a potential inhibiting effect of apoCI on the activity of HL *in vivo* cannot be ruled out, and it may add to the observed hypercholesterolemia, it does not contribute to the severe hypertriglyceridemia observed in *APOC1* mice.

Thus, impairment of LPL remains as the most likely mechanism explaining the hypertriglyceridemic phenotype of *APOC1* mice. Although the *APOC1* mice showed elevated LPL levels in postheparin plasma, we indeed found that apoCI is very effective in attenuating the LPL activity *in vitro*, with a 60% efficiency on mass basis as compared to the well-known endogenous LPL-inhibitor apoCIII.^{34,50} Our observations confirm previous *in vitro* studies by Havel *et al.*¹³ who showed that apoCI and apoCIII were equally effective on a mass basis with respect to inhibition of the apoCII-stimulated LPL-mediated TG hydrolysis. In fact, the LPL-inhibitory properties of apoCI and apoCIII are specific for these apolipoproteins, since addition of the negative control apoAI³⁶ had no effect on the LPL activity, and the recently identified LPL-stimulator apoAV³⁷ enhanced the LPL activity in this assay. Importantly, the TG: apoCI ratios applied in the *in vitro* assay at which apoCI inhibited LPL (50: 3-10, w/w) were similar as found in both *APOC1* mice (50: 6) and *apoE*^{-/-}*APOC1* mice (50: 3), indicating that the LPL-inhibitory properties observed *in vitro* are relevant for the *in vivo* situa-

tion. Indeed, preincubation of VLDL-like emulsion particles with apoCI inhibited the liver-independent serum clearance of emulsion-TG, as was demonstrated in lactoferrin-treated mice. Concomitantly, the uptake of TG-derived fatty acids by white adipose tissue was 1.8-fold decreased (not shown). Given the fact that apoCI readily exchanges between lipoproteins, a part of the injected emulsion-associated apoCI will presumably rapidly redistribute towards endogenous lipoproteins, which will even lead to underestimation of the inhibiting effect of apoCI on emulsion-TG clearance. In a previous study from our group in which VLDL clearance was assessed in functionally hepatectomized *APOC1* mice on a LFC diet, we also found a tendency towards a decreased VLDL-TG lipolysis rate in *APOC1* mice (*i.e.* 32%), albeit that a statistically significant difference was not reached under the applied experimental conditions.⁹

The phenotype of *APOC1* mice closely resembles that of human apoCIII-expressing *APOC3* mice with respect to predominant elevation of VLDL-TG levels.⁵¹ In addition, both *APOC1* mice and *APOC3* mice⁵² show a modest increase in plasma cholesterol levels. In fact, the relative increase in TG as compared to cholesterol as induced by apoCI-expression (*i.e.* 5.8) is similar to that as induced by apoCIII expression (*i.e.* 5.5).⁵² Indeed, it has been established that LPL activity strongly determines plasma TG levels. Overexpression of LPL in mice markedly reduces plasma VLDL-TG levels,^{53,54} whereas heterozygous deficiency of LPL results in accumulation of plasma VLDL-TG.⁵⁵ Similar to *APOC1* and *APOC3* mice, the effects of LPL modulation on plasma TG exceeded those on TC.

Inhibition of the lipolytic conversion of TG-rich lipoproteins in *APOC1* mice can fully account for our previous observation that *APOC1* protects against the development of obesity on a genetically obese leptin-deficient (*ob/ob*) background,⁵⁶ by impeding the disposition of LPL-liberated fatty acids into adipose tissue. Likewise, we have recently observed that deletion of the main endogenous LPL-inhibitor apoCIII in *apoc3*^{-/-} mice markedly aggravates diet-induced obesity as related to increased adipose tissue stores (unpublished observations). Interestingly, we have reported that VLDLr-deficient (*vldlr*^{-/-}) mice are protected from diet-induced obesity on both a wild-type and *ob/ob* background.⁵⁷ Subsequently, Yagyu *et al.*⁵⁸ have shown that *vldlr*^{-/-} mice have reduced LPL activity as related to the LPL-chaperone function of the VLDLr,⁵⁹ which may partially explain their resistance to obesity. In addition, the VLDLr may also be involved in LPL-mediated lipolysis by bridging of lipoproteins to the endothelial surface, thereby facilitating the LPL-particle interaction. Since we have firmly established that apoCI strongly inhibits the interaction of VLDL with the VLDLr,²⁴ a concurring VLDLr-inhibiting effect of apoCI may certainly add to further hampering of the LPL-mediated VLDL-TG hydrolysis *in vivo* as observed in *APOC1* mice.

In conclusion, we have demonstrated that the hypertriglyceridemic effect of moderate human apoCI expression in mice is the consequence of an impaired lipolytic conversion of VLDL-TG. This effect probably results from a direct inhibiting effect of apoCI on LPL activity, although a concomitant inhibiting effect of apoCI on VLDL binding to the VLDLr, which facilitates lipolysis, cannot be excluded. The mechanism by which apoCI inhibits LPL activity is currently under investigation.

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