ApoE*2-Associated Hyperlipidemia is Ameliorated by Increased Levels of ApoAV, but Unaffected by ApoCIII-Deficiency

Gery Gerritsen¹, Caroline C. van der Hoogt²-⁴, Frank G. Schaap³, Peter J. Voshol²-⁴, Kyriakos E. Kypreos¹,⁶, Nobuyo Maeda⁷, Albert K. Groen⁵, Louis M. Havekes²,³,⁴, Patrick C.N. Rensen²,⁴, Ko Willems van Dijk¹,⁴

Departments of ¹Human Genetics, ²General Internal Medicine, and ³Cardiology, Leiden University Medical Center, Leiden, The Netherlands; ⁴TNO Quality of Life, Gaubius Laboratory, Leiden, The Netherlands; ⁵AMC Liver Center, Amsterdam, The Netherlands; ⁶Boston University School of Medicine, Boston, MA, USA; ⁷Department of Pathology, University of North Carolina, Chapel Hill, USA

Submitted
Chapter 5

Objective - ApoE*2-associated hyperlipidemia is characterized by a disturbed clearance of apoE*2-enriched VLDL-remnants. Since excess apoE*2 inhibits lipoprotein lipase (LPL)-mediated triglyceride (TG)-hydrolysis in vitro, we investigated whether direct or indirect stimulation of LPL activity in vivo reduces the apoE*2-associated hyperlipidemia.

Methods and Results - Hereto, we studied the role of LPL and two potent modifiers, the LPL-inhibitor apoCIII and the LPL-activator apoAV in APOE*2-knockin (APOE*2) mice. Injection of heparin in APOE*2 mice reduced plasma TG by 55% and plasma total cholesterol (TC) by 28%. Similarly, adenovirus-mediated overexpression of LPL reduced plasma TG by 85% and TC by 40%, indicating that apoE*2-enriched particles can serve as substrate for LPL. Indirect activation of LPL activity via deletion of apoCIII in APOE*2 mice did neither affect plasma TG nor TC levels, whereas overexpression of Apoa5 did reduce plasma TG by 81% and plasma TC by 41%.

Conclusion - In conclusion, the combined hyperlipidemia in APOE*2 mice can be ameliorated by direct activation of LPL activity. Indirect activation of LPL via overexpression of apoAV does, whereas deletion of apoCIII does not affect the lipid phenotype of APOE*2 mice. These data indicate that changes in apoAV levels have a dominant effect over changes in apoCIII levels in the improvement of APOE*2-associated hyperlipidemia.
ApoE*2-associated hyperlipidemia is characterized by increased plasma levels of chylomicron and VLDL remnants and is associated with xanthomatosis and premature atherosclerosis. ApoE*2 has a single amino acid substitution (Arg158 to Cys) as compared with the common apoE3 variant, resulting in a low binding affinity for the LDLR. In vivo, this is associated with impaired hepatic clearance of VLDL and chylomicron remnant particles, resulting in increased plasma TG and TC levels. Simultaneously, apoE*2 accumulates in plasma leading to an increase in apoE-mediated inhibition of LPL-mediated TG hydrolysis. It has been postulated that both impaired remnant clearance and impaired remnant generation via lipolysis contribute to the hyperlipidemia associated with apoE*2.

We and others have found that VLDL obtained from hyperlipidemic patients homozygous for APOE*2 is a relatively poor substrate for LPL-mediated lipolysis. Two potent modifiers of LPL activity have been described, apoAV and apoCIII, that are encoded in same gene cluster on chromosome 11. In vitro and in vivo mouse studies indicate that apoAV stimulates LPL-mediated TG hydrolysis and that apoCIII inhibits this process. Overexpression of apoAV in mice reduces plasma TG levels via stimulation of LPL activity and overexpression of apoCIII results in increased plasma TG levels via inhibition of LPL. Studies in Apoc3-knockout mice show accelerated LPL-mediated TG hydrolysis. Deficiency in apoAV in both mice and humans is associated with hypertriglyceridemia.

In the present study, we have investigated the role of LPL-mediated TG-hydrolysis in apoE*2 associated hyperlipidemia in vivo. Direct stimulation of LPL activity in APOE*2 knockin (APOE*2) mice via heparin injection and via adenovirus mediated gene transfer of LPL both reduced the TG and TC levels. Indirect stimulation of the LPL activity via deletion of endogenous Apoc3 did not affect the lipid levels, whereas indirect stimulation via adenovirus mediated overexpression of apoAV did result in decreased plasma TG and TC levels. Thus, stimulation of LPL activity via apoAV overexpression or deficiency of apoCIII occur via different mechanisms. Moreover, these data indicate that apoAV represents a potential target for the improvement of APOE*2 associated hyperlipidemia.

Methods

Adenoviral Constructs
The adenoviral vector expressing active LPL (AdLPL) was kindly provided by Dr. Santamarina-Fojo. The generation of the adenoviral vectors expressing apoAV (AdApoa5), the control empty vector (AdEmpty) and β-galactosidase (AdLacZ) have been described. Expansion, purification and titration of the adenoviral vectors were performed as described previously. Before in vivo administration, the adenoviral vectors were diluted to a dose of 5x10⁸ pfu in 200 µl sterile PBS.

Mouse Models
APOE*2 knockin mice, carrying the human APOE*2 gene in place of the mouse Apoe gene have been described previously. These mice were backcrossed 8 times with
Chapter 5

C57Bl/6 mice to achieve a more homogenous genetic background and subsequently intercrossed to obtain homozygous APOE*2 mice. Apoc3/- mice were obtained from The Jackson Laboratories (Bar Harbor, ME, USA) and intercrossed with APOE*2 mice to obtain APOE*2, APOE*2.ApoC3/- and APOE*2.ApoC3/- mice. The mice were fed a regular mouse diet (SRM-A: Hope Farms, Woerden, The Netherlands) and given free access to food and water. At least five days before adenovirus injection, mice were transferred to filter-top cages in designated rooms. All animal experimentation protocols were approved by the Committee on Animal Experimentation of the Leiden University Medical Center.

Adenovirus-Mediated Gene Transfer in Mice
Male APOE*2 mice at the age of 13-18 weeks were selected for injection with AdLPL. A dose of 5x10⁸ pfu adenovirus was injected into the tail vein. Prior to and 5 days after administration of AdLPL, mice were fasted for 4 h and a blood sample for lipid determination was collected by tail bleeding, using diethyl-p-nitro phenyl phosphate (paraoxon, Sigma) coated heparinised capillary tubes (Hawksley, Sussex, England).

Female APOE*2 mice between the age of 13 and 18 weeks were injected with a dose of 5x10⁸ pfu of AdApoa5 or 5x10⁸ of empty vector (AdEmpty). Three hours prior to this virus injection, the mice were injected with 5x10⁸ pfu AdLacZ to saturate the uptake of viral particles by hepatic Kupffer cells. Prior to injection and 4 days after virus injection, mice were fasted for 4 h and a blood sample for lipid determinations was collected in paraoxon-coated capillaries by tail bleeding.

Lipid Determinations
Plasma was isolated from blood samples obtained from the mice by centrifugation. TG and TC levels were measured enzymatically (Sigma). Human apoE levels were measured by sandwich ELISA as described previously. The circulating human apoE level in homozygous APOE*2 carrying mice was 3.1±0.9 mg/dl.

Lipoprotein fractions were separated using fast protein liquid chromatography (FPLC). Hereto, a plasma pool obtained from the groups of mice were diluted 5 times using PBS. A volume of 50 µl was injected onto a Superose 6 column (3.2 x 30 mm, Äkta System, Pharmacia, Uppsala, Sweden) to separate lipoprotein fractions. Elution fractions of 50 µl were collected and assayed enzymatically for TG and TC levels as described above.

Heparin Treatment
Heparin was administered to APOE*2 mice after a period of 4 hours fasting and via i.v. injection of a dose of 0.5 U/g body weight. Blood samples of approx. 30 µl were drawn via the tail vein at t = 0, 10, 30, 60 and 120 minutes after heparin injection, using paraoxon coated capillaries. Plasma TG levels were measured enzymatically, as described above.
Fat-Load
The fat-load response was determined in male APOE*2, APOE*2.Apoc3+/- and APOE*2. Apoc3-/- mice aged 13 to 20 weeks. The mice were fasted over night and given an intragastric olive oil load (Carbonell, Cordoba, Spain) of 400 µl. Prior to the olive oil load and 3 and 6 h after the load, a blood sample was drawn via the tail vein for TG determination. The circulating levels were corrected for the TG level prior to the fat-load. The Area Under the Curve (AUC) was determined over the period of 6 h.

Statistical Analysis
Data were analyzed using the non-parametric Mann-Whitney U test. P-values less than 0.05 were regarded as statistically significant.

Results

Effect of Increased LPL Activity on Lipid Levels in APOE*2 Mice
I.v. injection of heparin results in activation of LPL and its release from the endothelial surfaces. Stimulation of LPL activity in APOE*2 mice via injection of heparin reduced the hyperlipidemia (Fig. 1). The maximum reduction was observed at 60 minutes after injection of 0.5 U heparin/g body weight. The plasma TG levels decreased 55% (P<0.005, n=4). The TC levels in APOE*2 mice decreased 28% (P<0.05, n=4).

APOE*2 mice were injected with adenovirus expressing LPL to determine the effect on hyperlipidemia (Fig. 2). At day 5 after injection of 5x10⁸ pfu AdLPL, APOE*2

Figure 1. Plasma lipid levels of APOE*2 mice after heparin treatment. Fasted APOE*2 mice were injected with heparin. Before (open bars) and 1 hour after injection (black bars), plasma samples were obtained and assayed for triglyceride (A) and cholesterol (B). The values are represented as means ± SD for n=4 mice per group. *P<0.05, **P<0.005.
mice exhibited a 85% decrease in plasma TG levels (n=3). The TC levels decreased 40% (n=3). The lipoprotein distribution as determined by FPLC showed a decrease in VLDL-TG and VLDL-TC to wild type levels after injection of AdLPL, indicating an accelerated conversion of APOE*2-containing VLDL particles by overexpression of LPL (data not shown).

**Figure 2. Plasma lipid levels of APOE*2 mice injected with AdLPL.** APOE*2 mice were injected with 5x10⁸ pfu AdLPL. Before (open bars) and at day 5 after adenovirus injection (black bars), fasted plasma samples were assayed for triglyceride (A) and cholesterol (B). Values are represented as means ± SD for n=3 mice per group.

**Effect of ApoCIII-Deficiency on Lipid Levels in APOE*2 Mice**

The main endogenous inhibitor of LPL, apoCIII, was deleted from the genetic background of APOE*2 mice by crossbreeding with Apoc3 knockout mice. The effect of Apoc3-deficiency on APOE*2-associated hyperlipidemia was investigated in APOE*2 mice heterozygous or homozygous deficient for the endogenous Apoc3 gene (Fig. 3). Surprisingly, the plasma TG levels were not different between APOE*2, APOE*2.Apoc3+/− and APOE*2.Apoc3−/− mice. Also, the TC levels were not affected by Apoc3-deficiency in the presence of APOE*2. No differences in plasma lipid levels were found between male and female mice (data not shown). The distribution of TG and TC over the lipoprotein fractions was measured after separation via FPLC. No differences were observed between APOE*2, APOE*2.Apoc3+/− and APOE*2.Apoc3−/− mice (data not shown).

To further analyse the effect of apoCIII-deficiency in APOE*2 mice on TG metabolism, mice were given an intragastric olive oil load. The increase in plasma TG levels were measured over a period of 6 h and the AUC was determined. The response in APOE*2 carrying mice was not different (APOE*2 AUC 5.8; APOE*2.Apoc3+/− AUC 5.5 and APOE*2.Apoc3−/− mice AUC 4.3 mM/6 h, n.s. for n=5 mice per group, data not shown).
Effect of Adenovirus-Mediated Expression of Apoa5 on Lipid Levels in APOE*2 Mice.

The activator of LPL, apoAV, was expressed in APOE*2 mice via a recombinant adenoviral vector. Injection of a moderate dose of AdApoa5 (5x10^8 pfu) reduced plasma TG by 81% (P<0.05) and TC by 41% (P<0.05) as compared to AdEmpty (Fig. 4). Analysis of lipoprotein fractions separated by FPLC revealed that the apoAV-mediated reduction of plasma TG was associated with a 4-fold reduction in VLDL-TG, whereas the TG level in the IDL/LDL fraction was affected to a minor degree. The reduction in plasma TC level was associated with a 2-fold reduced VLDL-TC level (data not shown).
Discussion

In the current study, we have addressed the hypothesis that alleviating the apoE*2-mediated inhibition of lipolysis can reduce the apoE*2-associated hyperlipidemia. Using the APOE*2 mouse model, we first stimulated LPL activity directly via heparin injection, which releases and activates endogenous LPL. This resulted in a reduction of the TG and TC levels in APOE*2 mice (Fig. 1). Likewise, injection of adenovirus expressing LPL in APOE*2 mice reduced the plasma TG and TC levels (Fig. 2). The reduction in TG and TC was mainly confined to the VLDL-sized fractions (data not shown). Subsequently, LPL was stimulated indirectly via its oppositely acting modulators apoCIII and apoAV. APOE*2 overexpression did reduce the APOE*2-associated hyperlipidemia in APOE*2 knock-in mice (Fig. 4). In contrast, the APOE*2-associated hyperlipidemia was not affected by Apoc3-deficiency (Fig. 3). Our data indicate that a direct increase of LPL activity by increasing circulating LPL levels reduces APOE*2 associated hyperlipidemia. The indirect stimulation of LPL activity via apoAV overexpression but not apoCIII-deficiency ameliorates the APOE*2-associated hyperlipidemia. We conclude that apoAV is apparently dominant over apoCIII in the improvement of APOE*2-associated hyperlipidemia. Moreover, apoAV and apoCIII modulate LPL activity via distinct mechanisms.

Addition of apoE to lipoproteins results in a decrease in the LPL-mediated TG hydrolysis.25-27 This can at least partially explain the hypertriglyceridemia that is found in APOE*2-associated familial dysbetalipoproteinemia (FD), which is characterized by plasma accumulation of apoE-enriched lipoproteins. It has been proposed that inhibition of LPL activity is caused by displacement of the LPL-coactivator apoCII from the apoE*2-rich lipoprotein particles.5 However, this is difficult to reconcile with the observation that indirect stimulation of LPL activity via apoAV overexpression ameliorates the APOE*2-associated hyperlipidemia. Especially, since it has been demonstrated that the LPL-activating effect of apoAV is dependent on the presence of apoCII.8 Thus other mechanisms might underlie the inhibitory effect of apoE*2 on LPL activity.

Under normal conditions, LPL-mediated TG hydrolysis takes place mainly at the endothelial cell surface and may thus be affected by the interaction between the TG-containing particle and the cell surface where LPL is localized. This interaction involves the association of TG-rich particles and endothelial surface bound heparan sulfate proteoglycans (HSPG) via apoE.28 It has been shown that apoE*2 is partly defective in the association with HSPG29 and this could also explain part of the apoE*2-associated hypertriglyceridemia. In agreement with this hypothesis, it has been found in vitro that VLDL obtained from APOE*2 homozygous FD patients is effectively lipolysed by LPL in solution, but poorly lipolysed by HSPG-bound LPL.8 Thus, apoE*2-containing VLDL may be defective in the physical association with the endothelial surfaces where LPL-mediated TG hydrolysis takes place in vivo. This would explain why additional LPL via adenovirus mediated gene transfer and endothelial release and activation of endogenous LPL by heparin do rescue the apoE*2-associated hyperlipidemia. Intriguingly, this explanation is also in line with the observation that additional apoAV rescues the apoE*2-associated hyperlipidemia. It has recently been found that the LPL-activating
Role of ApoAV and ApoCIII in APOE*2-Associated Hyperlipidemia

effect of apoAV involves enhanced binding to HSPG. Thus additional apoAV on the TG-rich particle apparently overcomes the apoE*2-mediated inhibition of HSPG binding. It is interesting to note that apoCIII-deficiency cannot overcome this binding defect, despite postulated inhibition of HSPG-binding by apoCIII. However, the in vivo contribution of HSPG in the lipolysis of TG-rich lipoprotein particles still remains to be determined.

The AdLPL and heparin-induced decrease in plasma TG levels was accompanied by a decrease in TC levels. This is likely due to increased clearance of TC and can be explained by two mechanisms. First, stimulation of LPL-mediated processing of VLDL and chylomicrons will lead to accelerated generation of remnant particles that are more easily cleared by the liver. Second, the AdLPL and heparin induced increase in the pool of LPL may result in enhanced binding of apoE*2-containing lipoproteins to the liver via an LPL-mediated bridging effect. This would result in enhanced hepatic clearance of whole particles and thus a reduction in both plasma TG and TC. Whether one or both of these mechanisms play a dominant role in mediating the hypocholesterolemic effect of AdLPL and heparin remains to be determined.

Previously, we have shown that Apoc3-deficiency is a potent tool to accelerate LPL-mediated TG-hydrolysis and to reduce the severe combined hyperlipidemia induced by adenovirus-mediated overexpression of APOE4. This hyperlipidemia is caused by an apoE4-induced increase in VLDL-production and simultaneous apoE4-mediated inhibition of VLDL-TG lipolysis. Despite a 10-fold increase in VLDL-TG production rate in AdAPOE4 treated mice, Apoc3-deficiency did result in a normalization of circulating lipid levels. To our surprise, Apoc3-deficiency did not affect the hyperlipidemia or lipoprotein lipid distribution (data not shown) in APOE*2 mice. Moreover, stressing the TG metabolism by an intragastric bolus injection of olive oil also did not induce a different post prandial TG response in APOE*2 mice on Apoc3 deficient or wild-type backgrounds. The absence of a hypolipidemic effect of Apoc3-deficiency in APOE*2 mice indicates that the defect in APOE*2-associated hyperlipidemia is upstream from the positive effect associated with apoCIII deficiency.

Apart from a stimulatory effect on LPL, the decrease in plasma TG of APOE*2 mice after expression of apoAV may have resulted from a decrease in the VLDL-TG secretion rate by the liver. We have previously shown a 30% decreased VLDL-TG secretion rate after adenovirus-mediated overexpression of Apoa5 in wild-type C57Bl/6 mice, whereas others have found no effects of apoAV on VLDL production in neither APOA5 transgenic mice nor in Apoa5/6 mice. Intriguingly, in the APOE*2 mice, we did not observe differences in the VLDL-TG secretion rate between AdApoa5- or AdEmpty-treated mice (data not shown). At present, we have no explanation for these apparent discrepancies, but cannot exclude that apoAV has additional yet unrecognized functions.

Polymorphisms in both the APOA5 and APOC3 genes have been associated with hypertriglyceridemia. Since both genes are expressed in the same gene cluster and have opposing effects on TG levels, it has been hypothesized that these genes act synergistically. Our current data clearly indicate that apoAV and apoCIII affect different steps in the conversion of TG rich lipoproteins to remnants. Moreover, within the con-
text of APOE*2-associated hyperlipidemia, it seems likely that variation in apoAV level and activity will have a more pronounced effect on the expression of hyperlipidemia as compared to variation in apoCIII level and activity.

Acknowledgements

This research was conducted in the framework of the “Leiden Center for Cardiovascular Research LUMC-TNO” and supported by the Netherlands Heart Foundation (project 2000.099), by the Leiden University Medical Center (Gisela Thier Fellowship to P.C.N. Rensen), the Netherlands Organization for Scientific Research (NWO VIDI grant 917.36.351 to P.C.N. Rensen, and NWO program grant 903-39-291 to L.M. Havekes), the American Heart Association (grant SDG 0535443T to K.E. Kypreos), and National Institutes of Health (grant HL42630 to N. Maeda).

References

Role of ApoAV and ApoCIII in APOE*2-Associated Hyperlipidemia


28. Mahley RW and Ji ZS. Remnant lipoprotein metabolism: key pathways involving cell-surface heparan sulfate


