The multifunctional role of apolipoprotein E in lipid metabolism

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Proefschrift

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wie ze liefheeft, onderzoekt ze.'
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General Introduction

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1. Introduction

Lipid disorders are a serious health problem in western society, because they are strongly associated with increased risk for cardiovascular diseases. In the Netherlands, cardiovascular diseases are a main cause of death (~34%, Hart- en vaatziekten in Nederland 2005, uitg. Nederlandse Hartstichting). The incidences of dyslipidemia and cardiovascular diseases correlate closely with the typical sedentary and hypercaloric Western lifestyle and are consequently increasingly significant. In addition to life style, hormonal status and gender clearly affect plasma lipid levels and cardiovascular risk. Detailed insight in lipid metabolism is important for our understanding of the link between the Western lifestyle and hyperlipidemia and to improve therapeutic strategies for lipid disorders.

Lipid disorders are classified according to their clinical features. Type III hyperlipoproteinemia (HLP III) is characterized by increased levels of lipoprotein remnant particles in the blood circulation(1) and predisposes subjects to features like xantomatosis and premature atherosclerosis(2,3,4). It is clear that environmental and genetic cofactors affect expression of the disease. In addition, secondary conditions such as hypothyroidism and systemic lupus erythematosus contribute to the severity of the hyperlipidemia(5,6,7).

Patients suffering from Familial Dysbetalipoproteinemia (FD) have a heritable defect in apolipoprotein E (apoE), causing HLP III and the associated clinical features(8,9,10,11,12,13,14). One of the consequences of defective apoE is a hampered clearance of lipoproteins resulting in hyperlipidemia. Since not all carriers of apoE mutations in a family display FD, it is again clear that additional factors affect expression of the disease(15,16).

2. Lipoprotein metabolism

The most common dietary lipids are cholesterol and triglycerides (TG). Cholesterol is necessary for the synthesis of steroid hormones, vitamins and bile acids. At the cellular level, cholesterol is an important component of the cell membrane. All the cholesterol the body requires can eventually be synthesized by

the body. TG are formed by esterification of glycerol with free fatty acids and supply cells with free fatty acids which are used as energy source for muscle or reconverted to TG and stored in adipose tissue. TG and cholesterol are insoluble in water and therefore transported through the circulation in lipoproteins. These lipoproteins have a hydrophobic core in which the cholesterol esters and TG are present. The outer layer consists of a monolayer of mainly polar phospholipids. Unesterified cholesterol and apolipoproteins are also present on the outer layer. Lipoproteins are classified according to their buoyant densities in ultracentrifugation gradients into 5 categories: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). These lipoproteins differ not only in density, but also in lipid content and apolipoprotein composition. The apolipoproteins on the surface of the lipoproteins determine the structure and metabolic fate of the particle(17,18).

The lipoproteins move through the body via three pathways:

- The exogenous pathway in which dietary lipids are taken up in the intestine and packaged in lipoproteins. These lipids pass through the periphery and are subsequently transported to the liver. Part of the exogenous pathway consists of the enterohepatic circulation; cholesterol and bile that are secreted by the liver as emulsifying agents are re-absorbed by the intestine.
- The endogenous pathway comprises the secretion of lipoproteins by the liver and the subsequent distribution of these lipids trough the periphery.
- The reverse cholesterol pathway transports excess of cholesterol from the periphery via lipoproteins back to the liver.

Exogenous pathway:

Dietary lipids enter the body via the intestine. They are packaged into chylomicrons that enter the blood circulation via the lymph. These chylomicrons have a very low density and have a diameter of 75-1200nm. ApoB48 serves as a backbone of chylomicrons and is a truncated variant of the apoB100 which is synthesized in the liver to produce VLDL particles(19,20). Chylomicrons contain apoAI, apoAII and apoAIV(21,22,23). In the circulation, apoAI and a part of AIV are exchanged for apoCI, apoCII , apoCIII and apoE from HDL particles(24,25). The

chylomicrons are very TG-rich and interact with Lipoprotein Lipase (LPL)(26). LPL uses apoCII as cofactor and hydrolyses TG to release FFA that is either stored as TG in adipose tissue or used as an energy source in muscle or other peripheral tissues(27). The chylomicron remnants that are formed are smaller due to loss of TG. Sheets of phospholipids and apolipoproteins (esp apoAI) separate from the particle and merge with pre-HDL particles. Also, the remnant particles loose affinity for apoC's that are transferred to HDL(28). The chylomicron remnants acquire apoE from other plasma lipoproteins and therefore gain affinity for rapid receptor mediated clearance by the liver(29). This hepatic uptake is a multi-step process. The remnant lipoproteins enter the space of Disse and become further enriched with surface bound apoE, derived from hepatocytes. Subsequently, the particles bind to hepatic lipoprotein receptors that mediate endocytosis(30).

After degradation of the particle, cholesterol is released to the liver cells(31,32). The cell regulates its cholesterol content by balancing the rates of cholesterol synthesis and degradation of the remnant particles(33,34). Cholesterol excretion from the body occurs almost exclusively via the bile. The bile maintains fatty components like cholesterol and phospholipids in solution due to its detergent effect. The level of secretion of biliary cholesterol is coupled with the level of secretion of bile acids(35). Most of the bile acids that enter the intestine is actively reabsorbed by the ileum. This is the so-called enterohepatic circulation.

Endogenous pathway:

Hepatocytes assemble VLDL particles from newly formed apoB100(36). In the endoplasmatic reticulum apoB is folded and lipidated. ApoE increases the lipidation of nascent VLDL particles, likely by stabilizing the particle(37,38). These nascent VLDL particles are very TG rich. In the blood circulation, the VLDL particles have a very similar metabolic fate as chylomicrons (fig 1). The VLDL particles gain apoE, apoCI, apoCII and apoCIII. Due to interaction with LPL on the endothelium, VLDL particles loose TG and release FFA to muscle and fat. The VLDL-remnant particles, also called IDL particles, that are formed can be taken up by the liver. ApoB100 and apoE both serve as a ligand for receptor mediated clearance by the liver(39). The VLDL remnants can also be further processed to LDL particles. This occurs via interaction with hepatic lipase (HL) and is accompanied by loss of phospholipids,

apoE and apoC(40). The LDL particle is very cholesterol rich and contains apoB100 as the sole protein on its surface. In humans most cholesterol is present in LDL particles. These LDL particles can be taken up again by the liver or by extra-hepatic receptors via apoB100(41).

Reverse cholesterol pathway:

Excess cholesterol in the peripheral cells can be transported back to the liver. This occurs via HDL particles that are synthesized as apoA1 containing phospholipid-rich particles by the liver and the intestine. HDL is further matured from interaction with chylomicrons and VLDL remnant particles(42,43).

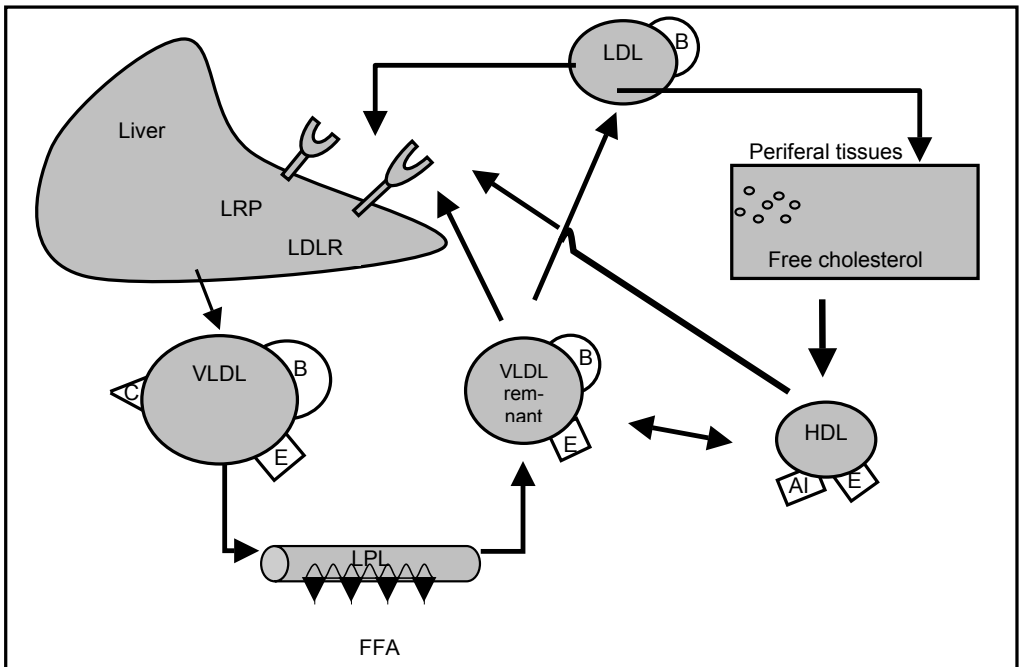

Figure 1. Schematic representation of the VLDL metabolism. AI, apoAI; B, apoB; C, apoC; E, apoE; FFA, free fatty acids; LPL, lipoprotein lipase.

The major apolipoproteins on HDL are apoAI and apoAII. Furthermore, mature HDL contains apoE and apoCI, CII and CIII(42). The HDL-cholesterol is converted to an esterified form by the enzyme lecithin:cholesterol acyl transferase (LCAT), with apoAI as cofactor. One pathway of delivery of HDL-cholesterol to the liver is via transfer of cholesterol to VLDL or LDL particles and subsequent hepatic clearance. This cholesterol transfer occurs via cholesterol ester transfer protein (CETP) that exchanges cholesterol with TG from HDL to VLDL or LDL(44). A second pathway of hepatic clearance of HDL-cholesterol is via enrichment of HDL with apoE and direct binding to hepatic receptors. Thirdly, cholesterol can be selectively taken up by the liver, without whole particle uptake of HDL. In this pathway, receptors like scavenger receptor B1 (SR-B1) and ABC-transporters on the liver are involved(45,46). The liver in turn excretes the cholesterol as bile acids into the intestinal tract.

3. Lipases

The lipase enzyme family is responsible for the hydrolysis of TG, phospholipids and cholesterolesters(47). The lipase enzyme family includes pancreatic lipase,(48), endothelial lipase(49,50), hormone-sensitive lipase(51), lipoprotein lipase (LPL) and hepatic lipase (HL)(52). The endothelial lipase is a secreted enzyme present on the endothelium and also in several other tissues, including placenta, lung, liver, testis, thyroid and ovary(53). Its main activity is hydrolysis of phospholipids. Hormone-sensitive lipase is an intracellular enzyme involved in the mobilization and hydrolysis of TG and diglycerides that are stored in adipose tissue. There is evidence of a second intracellular TG-hydrolyzing enzyme, adipose triglyceride lipase (ATGL)(51). LPL and HL both hydrolyse TG and PL and are key enzymes in the hydrolysis of TG-rich VLDL and chylomicron particles.

LPL is synthesized in several tissues, including skeletal and cardiac muscle, adipose tissue and mammary gland tissue. LPL delivers fatty acids to adjacent tissues by hydrolysing TG in circulating TG-rich chylomicrons and VLDL(26). The mature LPL protein is 56 kDa(54) and consists of two structural domains; the N-terminal domain (amino acids 1-312) and the C-terminal domain (amino acids 313-448). The N-terminal domain contains the catalytically active site(55,56,57). The C-

terminal domain is involved in the interaction of LPL with lipoproteins(58). Both C- and N-terminal domain can interact with heparin and HSPG and mediate binding to the extracellular matrix of hepatocytes and endothelial cells(57,59,60)(fig 2).

Active LPL is a homodimer that consists of two non-covalently linked glycoproteins(61,62). A dimeric state is necessary for the hydrolytic function, since access to the catalytic site is determined by the dimeric conformation(63,64). LPL is mainly located at the luminal surface of the vessels, where it is anchored to endothelial cells through electrostatic interation with HSPG(65,66) (fig 2). LPL can also bind lipoproteins(67,68,69,70) and exerts a bridging function between lipoprotein particles and the vessel wall. Heparin induces release of LPL from the vessel wall thereby increasing its accessibility to TG-rich lipoproteins. This results in a rapid clearance of plasma TG(71,72).

The hydrolytic activity of LPL is influenced by apolipoproteins that are mainly encoded for by two important gene clusters. In the cluster endocing apoE-apoCI-apoCII, apoCII is important as cofactor of LPL to hydrolyse TG(59,73,27,74). ApoE and ApoCI both have an inhibitory effect on LPL and both cause hypertriglyceridemia upon overexpression in mouse models(75,76,77,78,79,80). In the cluster of apoA1-apoCIII-apoAIV-apoAV, APOCIII inhibits LPL activity(81,82,83), whereas apoAV stimulates the activity of LPL(84,85). ApoCIII and apoAV seem to act in a synergistic way on the activity of LPL(86). A deficiency in the LPL enzyme due to genetic mutations also leads to severe hypertriglyceridemia(87,88,89,90).

Next to hydrolysis of TG that reside in the core of TG-rich VLDL and chylomicrons, LPL is also important for binding of lipoprotein particles to the liver(91,92)(fig 2). LPL bridges between lipoproteins and HSPG and thus increases initial binding to the liver(93,94,95,96). Subsequently, LPL increases receptor-mediated uptake(91,97,98,99,100). LPL mediates binding of lipoproteins to LRP and, with a lower affinity, to the LDLr via the C-terminal domain of LPL(101,102,103). This bridging of LPL between lipoproteins and hepatic receptors is independent of lipolytic activity(104,105). The bridging function of LPL not only leads to stimulated hepatic clearance of remnant particles, but also a higher selective cholesterol uptake by the liver from the lipoprotein particles(106).

HL is synthesized in hepatocytes. The glycoprotein of about 65 kDa resides on the endothelial cells lining the liver, adrenals and ovaries attached to

HSPG(107,108). TG present in chylomicron remnants, IDL and HDL and PL in HDL are hydrolysed by HL(99,109). The enzymatic activity is independent of a co-factor. Deficiency of HL is associated with hypertriglyceridemia and increased levels of IDL and HDL(110). Also, HL activity affects the risk on atherosclerosis(111). HL may serve as a ligand that mediates hepatic uptake of lipoproteins by concentrating lipoproteins on HSPG-sites and subsequent receptor binding and endocytosis(112). HL binds to LRP, indicating an LRP-dependent pathway of lipoprotein catabolism(113). The LDLr also contributes to HL-mediated binding and degradation of VLDL(114). This bridging occurs with active as well as inactive HL and is independent of apoE(105,115).

4. Hepatic lipoprotein uptake

The hepatic uptake of lipoproteins is mediated by lipoprotein receptors and HSPG. HSPG consists of a core protein and heparin sulphate chains. Three classes of HSPG are known, based upon their core protein: syndecan, glypican and perlecan(116).

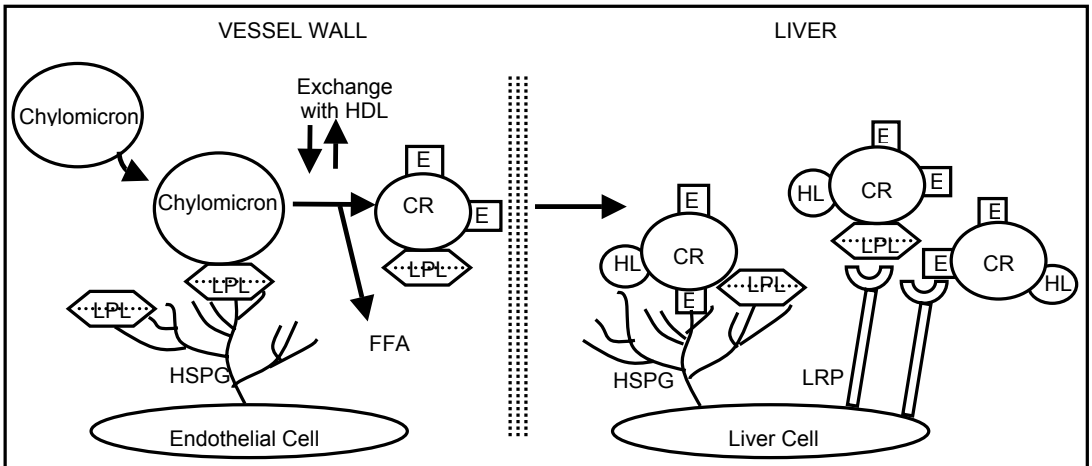

Figure 2. Role of LPL in metabolism of TG-rich lipoproteins. CR, Chylomicron remnant; E, apoE; FFA, free fatty acids; HL, hepatic lipase; LPL, lipoprotein lipase; HSPG, heparan sulphate proteoglycans.

Syndecans are a family of transmembrane HSPG that are expressed on the surface of vascular endothelial and smooth muscle cells. Glypicans are located at the extracellular space, anchored to the cell membrane of vascular endothelial and smooth muscle cells. Perlecans are also located in the extracellular matrix and expressed by various cell types.

HSPG bind a large number of extracellular ligands and participates in the cellular interaction with these ligands(116,117). The ligands mostly have a role in cell-matrix and cell-cell adhesion, cell migration and proliferation(118). HSPG have a crucial role in lipoprotein metabolism(30,119). In the blood vessels, HSPG bind VLDL via LPL(91,92). HSPG are abundantly present in the space of Disse in the liver. The lipoprotein remnants that are sequestered here are captured by the HSPGs with apoE and HL as important ligands(120). These lipoproteins can be further processed by LPL or HL and are enriched with apoE(97,121,122,123). ApoE contains heparin binding sites and mediates internalization of the particle(120,124,125,126). The endocytosis of lipoproteins by hepatocytes mainly occurs via LDLr(127) and LRP(128,129). HSPG can also mediate internalization of lipoproteins, although this occurs at a slower rate as compared with receptor mediated uptake(130,131). Still, HSPG are important in the hepatic uptake of remnant particles, since absence of HSPG disturb the binding of remnant particles and the uptake via receptors(130).

The LDLr is the main receptor for hepatic uptake of lipoproteins. The LDLr mediates clearance via binding to apoB100 and apoE. After endocytosis, the remnant lipoproteins are transported into the endosomes and lysosomes where the lipid components are hydrolyzed. A genetic defect in the LDLr causes familial hypercholesterolemia, characterized by accumulation of apoB100 containing lipoproteins. Overexpression of LDLr results in a clearly increased clearance of apoB and apoE containing lipoproteins(132,133). Studies in LDLr knockout mice indicate that alternative pathways for hepatic clearance exist(134,135). The LRP is the backup receptor in case the hepatic uptake via the LDLr is hampered(136,137). The majority of the remnant lipoproteins that enter the space of Disse will be taken up by the LDLr(29). When the remnant lipoproteins are not directly taken up, they undergo enrichment with surface-bound apoE that makes them competent to bind to the LRP(138).

LRP is a multifunctional receptor that is expressed in many tissues including the liver(139,140). The LRP has structural similarity to the LDLr(141). In addition to its function in lipoprotein metabolism, the LRP has a role in the homeostasis of proteinases and proteinase inhibitors, cellular entry of viruses and toxins, activation of lysosomal enzymes, cellular signal transduction and neurotransmission(142). LRP recognizes at least 30 ligands and is identical to the receptor for a2 macroglobulin(142,143). The function of LRP as hepatic receptor for proteinase complexes affects several blood factors and the development of atherosclerosis(144). LRP deficiency leads to early embryonic lethality(145). The importance of LRP in lipoprotein metabolism is proven by inducible tissue specific inactivation of LRP in the liver. In absence of the LDLr, inactivation of LRP leads to hyperlipidemia and accumulation of cholesterol-rich remnants(146). The hepatic uptake via LRP is a complex process that involves LPL, HL, HSPG and cell surface bound apoE(129,147,148,149). Although the hepatic uptake is less efficient via LRP as compared with the LDLr, it is suggested that presence of both LDLr and LRP is necessary for efficient hepatic clearance(146).

5. Apolipoprotein E

ApoE is a glycoprotein that is mainly synthesized in the liver where it is associated with VLDL. Several additional organs, including astrocytes in the brain and macrophages in various tissues produce apoE(1). The intestine does not produce apoE, although it is a major site of lipoprotein synthesis. These particles have to gain apoE in the blood circulation by exchange from other lipoproteins.

ApoE consists of two functional domains: a N-terminal domain (amino acids 1-191) and a C-terminal domain (amino acids 216-299). The N-terminal domain contains an antiparallel four-helix bundle (fig 3). The LDLr binding domain of apoE is located at helix 4 between aminoacid 136 and 150(1) and contains a heparin-binding site at amino acid 142-147(124).

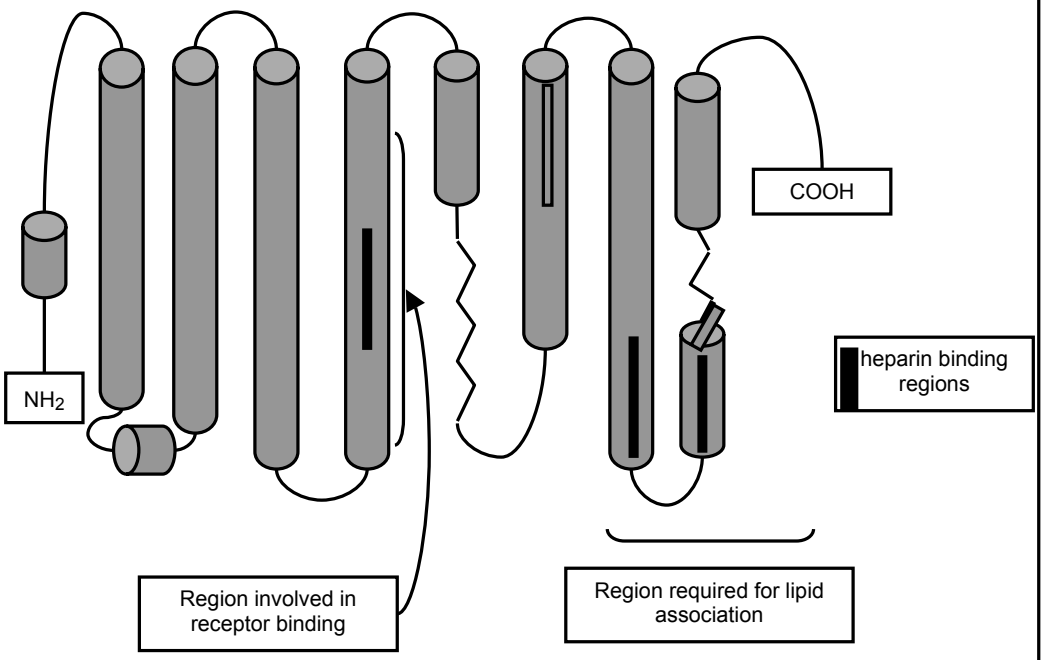

Figure 3. Schematic presentation of the structure of apoE.

The C-terminal domain has a strongly amphipatic character and consists of 3 α-helices. Helix 3 of apoE mediates association to lipoproteins(150). The C-terminal domain also contains a heparin binding site between amino acid 214-236 that may mediate binding of apoE with HSPG(124).

Mature apoE is a 299-amino acid polypeptide(151). The molecular weight of the apoE polypeptide is 34.2 kDa. The protein is polymorphic and can be distinguished by isoelectric focusing(152,153,154). About 30 variants of apoE have been characterized(9). The 3 major isoforms in human apoE are apoE2, apoE3 and apoE4. The ApoE2, apoE3 and apoE4 isoform differ in amino acid sequence at 2 sites.

ApoE3 is the most common variant that is considered as wildtype apoE. Compared to wildtype apoE3, the most common apoE2 variant has an aminoacid substitution at position 158 (Arg → Cys). Other ApoE2 variants have aminoacid substitution 146 Lys → Gln, 145 Arg → Cys and 136 Arg → Ser. ApoE4 differs from apoE3 by a change at aminoacid 112 (Cys→ Arg).

ApoE plays a central role in lipoprotein metabolism. It resides on VLDL, VLDL remnant and HDL particles(151) and affect many steps in VLDL metabolism(155,38). In addition to its role as ligand for receptor mediated hepatic clearance of lipoprotein remnants, apoE stimulates the production and secretion of TG-rich VLDL by the liver(37). Low levels of apoE are sufficient for normal production of VLDL-TG. Expression of high levels of apoE elevates the VLDL-TG production by the liver, which might result in hypertriglyceridemia(79,156,78). Likely, apoE participates in the assembly of TG into VLDL particles in the liver. Recycling of apoE in the liver that is obtained from internalized VLDL makes the apoE available for lipoprotein assembly(157,158). ApoE inhibits LPL-mediated VLDL-TG hydrolysis. This occurs in a dose dependent manner(76,77,79). Displacement of apoCII, the cofactor of LPL, from the particle has been postulated to contribute to this inhibitory effect of apoE(78).

6. ApoE-associated hyperlipidemia and transgenic mouse models

Different variants of ApoE are associated with hyperlipidemia. To study the contribution of apoE in lipoprotein metabolism, mouse models are frequently used(155,160). Mouse models give the opportunity to study biochemical and genetic variables in a controlled environment. Mice are easily genetically modified and have a short breeding time. The lipoprotein metabolism of mice shows many similarities with the metabolism in humans. Mostly the same genes control the lipoprotein metabolism(161). However, some important differences between humans and mice have to be taken into account. Mice have a different cholesterol distribution among lipoprotein classes as compared to humans. In mice, cholesterol is mainly present in HDL, whereas in humans cholesterol mainly resides in LDL(162,163). Secondly, the liver of mice edits part of apoB100 to apoB48. In humans the liver produces only

apoB100, whereas apoB48 is exclusively generated in the intestine. ApoB48 lacks the LDLr binding domain and is therefore not a ligand for hepatic clearance(164,165,166). Thus, part of the VLDL particles that are generated by the liver of mice depend on apoE for hepatic clearance. Thirdly, mice lack cholesteryl ester transfer protein (CETP). CETP transfers cholesteryl esters from HDL particles to VLDL and chylomicrons in exchange for TG. The presence of CETP contributes to the difference in distribution of cholesterol over the lipoprotein particles between humans and mice(167,168,169). Several mouse strains have been developed to investigate the role of apoE in lipoprotein metabolism.

ApoE-deficient mice

Mice deficient for apoE revealed that apoE is required to maintain plasma lipid levels within a normal range. ApoE-deficiency leads to severe hypercholesterolemia characterized by accumulation of VLDL particles in the circulation that are cholesterol-rich and depleted from TG. Also, these mice are susceptible to develop atherosclerosis(170,171,172). *Apoe-/-* mice have a decreased production of VLDL-TG by the liver, although the number of VLDL particles produced is not altered(38,173,174). ApoE has an inhibitory effect on the lipolysis rate of the TG in circulating VLDL particles. Absence of apoE stimulates the hydrolysis of VLDL-TG which results in TG-poor and relatively cholesterol rich VLDL remnant particles. Due to the absence of apoE, the VLDL particles depend on apoB for hepatic clearance. In mice, VLDL particles contain apoB100 or apoB48. The latter has no receptor binding domain and will not be cleared by the liver at all, thereby aggravating the hyperlipidemia(175,176). Particles also circulate longer, exposing them longer to LPL and thus reducing the TG levels.

APOE2 expressing mice

The common apoE2 isoform binds poorly to the LDLr(177,178). The substitution in apoE2 at position 158 is located outside the receptor binding domain of apoE with the LDLr. Nevertheless, the binding capacity to the LDLr is reduced to less than 1%. This is likely due to a conformational change induced by the single amino acid substitution(178,179). The LDLr binding defect results in accumulation of apoE2-enriched remnant particles. The majority of patients with FD are homozygous

for the the APOE2 allele. However, in human only some 4% of homozygous APOE2 carriers develop FD(152,180). Thus, additional genetic and/or environmental factors are required for the development of hyperlipidemia. Strikingly, healthy APOE2 carriers are characterized by hypolipidemia. They have decreased plasma cholesterol and LDL levels(181). Mice have been generated that carry APOE2(158) as a transgene(177,182) or as a knock-in gene(183). Comparison between APOE2 knockin and APOE3 knockin mice indicates that, at equal expression of the human APOE gene, APOE2 expressing mice develop hyperlipidemia and are defective in clearing remnant particles(183). *In-vitro* studies show that, although apoE2 is binding defective, it can bind LRP normally(184). Also, the high cholesterol levels in APOE2 transgenic mice can be reduced by overexpression of the LDLr(38,185). Thus, the receptor mediated clearance is disturbed, but not abolished. The VLDL-TG lipolysis is inhibited by apoE2 in a dose dependent manner, as was also found for wt apoE3(186). Importantly, the lipolysis of VLDL-TG by HSPG bound LPL is less efficient with apoE2 as compared with apoE3(187). Thus, in subjects with elevated levels of APOE2, both inefficient receptor binding and disturbed interaction with LPL might contribute to the hyperlipidemia.

ApoE*3-L expressing mice

A minor fraction of FD patients carry APOE*3-Leiden, associated with a dominant mode of inheritance of FD(188,189). The APOE*3-L allele contains a 7-amino acid tandem repeat of residues 120-126 or 121-127(190). APOE*3-L has an impaired ability to bind the LDLr(189,191). Studies in APOE*3-L transgenic mice also indicated impaired lipolysis and decreased hepatic uptake of VLDL(177,187,192,193). Also, the apoE*3-L variant is less efficient in hepatic secretion of VLDL-TG as compared to wt apoE3(194). Mice expressing APOE*3-L as a transgene accumulate cholesterol and TG rich remnant lipoproteins in circulation, very similar to FD patients(192).

APOE3 and APOE4 expressing mice

The differences between human apoE and mouse apoE are investigated via knockin mouse models in which the mouse Apoe gene is replaced by human APOE. Characterization of APOE3 knockin mice showed unaffected fasting plasma lipid levels as compared to wildtype mice. However, expression of APOE3 caused differences in lipoprotein distribution; the LDL and apoB100 levels are decreased, whereas the VLDL fraction is increased. Furthermore, the apoE distribution is shifted from HDL to VLDL particles. On a high fat diet, APOE3 knockin mice develop more atherosclerosis(195). APOE4 knockin mice show elevated plasma lipid levels as compared to wildtype mice. The serum cholesterol levels are increased and VLDL and LDL particles accumulate in the blood circulation(196). Studies on the interaction of apoE3 and apoE4 with hepatic clearance receptors show that VLDL isolated from APOE3 and APOE4 knockin mice have a normal binding capacity of their VLDL to the mouse LDLr as compared to mouse apoE. The non-LDLr-mediated VLDL clearance is impaired by human apoE3 and apoE4 as compared to mouse apoE(197). Thus, structural differences between mouse and human apoE cause increased susceptibility to hyperlipidemia.

7. ApoE-associated hyperlipidemia and adenovirus mediated gene transfer in mice

The role of the apoE gene in lipoprotein metabolism is intensively investigated by adenovirus mediated gene transfer, using recombinant adenovirus vectors(198,199,200). The adenoviral vectors used are mostly derived from serotype 2 or 5. These vectors lack the E1A region that makes the vector replication defective. Via homologous recombination the gene of interest is inserted in the E1 region of the vector. To amplify the vectors, the E1 functions are complemented in trans using specific E1-expressing cell lines such as 293, 911 or PerC6 cell lines(201,202,198). Administration of adenovirus to mice via the tail vain will result in highly efficient uptake by the liver, more than 90% of administered dose will be taken up by the liver(203). Adenoviral vectors can infect non-dividing cells and do not integrate in the genome of the host cell. In mice, the peak expression level of the transgene is at day

4 and 5 after injection of the virus into the tail vein, reducing to background levels at day 10-14. The loss of expression is due to downregulation of the promotor and an immune response directed against the adenoviral vector and the transfected cells(204,205).

Expression of APOE3

ApoE-deficient mice are hyperlipidemic due to a hampered hepatic clearance of remnant lipoproteins. Also, these mice have a reduced secretion of VLDL-TG in the circulation(37,206). Studies using adenovirus mediated gene transfer in apoE-deficient mice demonstrated that moderate expression of human APOE3 reduces the hyperlipidemia(79,207,208,209). Both, the hepatic clearance and the VLDL-TG secretion are restored by moderate levels of APOE3(37,210). Overexpression of APOE3 results in a dose dependent increase in the VLDL-TG secretion rate(37,79,156) and a dose-dependent inhibitory effect on the hydrolysis rate(76,77). This results in accumulation of TG-rich VLDL particles in the circulation and hyperlipidemia. In case of overexpression of APOE, the enhanced hepatic clearance cannot compensate for the hyperlipidemic effect of apoE via the stimulated VLDL-TG production and inhibited lipolysis rate(79).

Expression of APOE4

Expression of APOE4 has a very similar effect in mice as compared to APOE3. Overexpression of APOE4 induces more severe hyperlipidemia in mice than APOE3(156,174). The effect on VLDL-TG production is the same for apoE4 as for apoE3(174). Also, ApoE4 has an equal effect on the lipolysis rate and hepatic clearance as apoE3(209). Our group performed further analysis on the structure-function relation of apoE4 via generation of truncated variants of apoE4. Whereas overexpression of full length APOE4 induces severe hyperlipidemia in apoe-deficient mice, the variant lackin the C-terminal domain due to truncation at aminoacid 202 (APOE4-202) reduces the hyperlipidemia in these mice(156). Absence of an increase in the VLDL-TG secretion upon expression of APOE4-202 as compared to full length APOE4 was underlying this lipid decreasing effect. The reduction of the hyperlipidemia in apoE-deficient mice after expression of APOE4-202 and APOE4-185 indicate that the N-terminal 158 aminoacids are sufficient for efficient hepatic

clearance(211). The C-terminal aminoacids 260-299 contribute to the hyperlipidemic effect of APOE4 expression in apoE-deficient mice(212).

Expression of APOE2

Injection of a moderate dose of adenovirus expressing APOE3 reduces hyperlipidemia in apoe-deficient mice. APOE2(158) shows an impaired ability to mediate clearance of remnant lipoproteins and does not reduce the hyperlipidemia in apoE-deficient mice(209). The secretion of VLDL-TG by the liver is equally induced by overexpression of APOE2(158) and APOE3(174). Importantly, the apoE2-carrying VLDL particles are not efficiently hydrolysed by LPL in presence of apoE2(158)(174,187,213), resulting in accumulation of TG-rich VLDL particles. Thus the APOE2-induced hyperlipidemia is a combined effect of poor VLDL-TG hydrolysis and remnant clearance.

8. Outline of thesis

In VLDL metabolism, the balance in production of the VLDL-TG by the liver, the hydrolysis of VLDL-TG by LPL at the capillary endothelium and the hepatic uptake of the remnant particles is important to maintain normal lipid levels. A dysfunction of one aspect of the VLDL metabolism influences the whole metabolism and might lead to hyperlipidemia. ApoE affects all three main aspects of VLDL metabolism. The aim of this thesis is to analyze the role of apoE in the VLDL metabolism and gain insight in the beneficial and adverse effects of apoE in relation to the structure and function of apoE. To gain more insight in the role of the N- and C-terminal domain of apoE in receptor mediated clearance, we expressed apoE4 and the truncated variant apoE4-202 in mice carrying apoE2 as a transgene (chapter 2). Chapter 3 and 4 focus on LPL-mediated VLDL-TG hydrolysis and the importance of lipolysis in apoE-induced hyperlipidemia. In chapter 3 we address the question whether an accelerated LPL-mediated VLDL-TG hydrolysis rate (via apoCIII-deficiency) is capable to reduce the hyperlipidemia induced by apoE4 overexpression. APOE2 is a LDLr-binding defective variant of apoE and interacts

less efficient with LPL that is bound to the surface of the vessel wall. We investigated whether stimulation of LPL-activity reduces APOE2-associated hyperlipidemia. The LPL activity in APOE2 knock-in mice was stimulated directly and indirectly via its modulators apoCIII and apoAV (chapter 4). To study the role of apoE in hepatic uptake of lipoproteins, we generated *LRP$^{lox/lox}$.Ldlr-/-.Apoe-/-* mice. In these mice the role of apoE in LRP-mediated lipid metabolism was investigated via deficiency and overexpression of apoE(chapter 5). In chapter 6, the results of our studies as presented in this thesis and future perspectives are discussed.

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Chapter 2

Hyperlipidemia in APOE2 transgenic mice is ameliorated by a truncated apoE variant lacking the C-terminal domain.

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Abstract

Familial Dysbetalipoproteinemia (FD) associated with the apolipoprotein E2 (APOE2) genotype is a recessive disorder with low penetrance. Here we have investigated whether additional expression of full-length APOE3, APOE4 or a truncated variant of APOE4 (APOE4-202) can reduce APOE2-associated hyperlipidemia in a mouse model for FD. This was achieved using adenovirus mediated gene transfer to mice transgenic for human APOE2 and deficient for endogenous *Apoe* (APOE2.*Apoe-/-* mice). The hyperlipidemia of APOE2.*Apoe-/-* mice was readily aggravated by APOE3 and APOE4 overexpression. Only a very low dose of APOE4 adenovirus ($2x10^8$ pfu) was capable of reducing the serum cholesterol and TG levels. Expression of higher doses of APOE4 was associated with an increased VLDL-TG production rate and accumulation of TG-rich VLDL in the circulation. In contrast, a high dose ($2x10^9$ pfu) of AdAPOE4-202 reduced the lipid levels in APOE2.*Apoe-/-* mice. Despite the absence of the C-terminal lipid-binding domain, APOE4-202 is capable of binding to lipoproteins and mediates hepatic uptake. Moreover, overexpression of APOE4-202 in APOE2.*Apoe-/-* mice does not aggravate their hypertriglyceridemia. These results extend our previous analyses of APOE4-202 expression in *Apoe-/-* mice and demonstrate that apoE4-202 functions even in the presence of clearance defective apoE2. Thus, apoE4-202 is a safe and efficient alternative to full-length apoE in therapeutic applications.

Introduction

ApoE plays a central role in the metabolism of chylomicron and VLDL remnants. It functions as a ligand for receptor-mediated endocytosis(1), has an inhibitory effect on the lipolysis of VLDL-TG (2,3,4,5) and stimulates hepatic secretion of VLDL-TG(6,7). Mutations in APOE are associated with familial dyslipidemia (FD) that is characterized by an accumulation of chylomicron and VLDL remnants in the circulation and results in premature atherosclerosis(8). Most patients with FD are homozygous carriers of APOE2 (Arg158->Cys)(9). ApoE2 is one of the three major isoforms of apoE in the human population. The other two are apoE3 (Cys112;

Arg158) and apoE4 (Cys112->Arg)(10). In vitro, apoE2 binds poorly to the LDL receptor(11), which *in vivo* is a major route for lipoprotein remnant clearance.

Hepatic overexpression of APOE isoforms in *Apoe-/-* mice using adenovirus mediated gene transfer indicates that moderate levels of APOE3 and APOE4 expression lead to a normalization of the hypercholesterolemia and a dose-dependent but moderate increase in serum TG levels(12,13,14). Higher levels of hepatic overexpression of apoE3 and apoE4 lead to massive hypertriglyceridemia, even to the extent that the hypercholesterolemia is not reduced any more. A major factor contributing to the hyperlipidemia is the apoE-mediated increase in the VLDL-TG production rate(12,13,15,16). Hepatic overexpression of apoE2 does not reduce hypercholesterolemia of *Apoe-/-* mice at any expression level(14). We have shown recently in *Apoe-/-* mice that the hypertriglyceridemic effect of APOE overexpression can be abrogated by deletion of the C-terminal lipid-binding domain (amino acids 203-299) of apoE (apoE4-202). Injection of a very high dose of up to $1*10^{10}$ pfu adenovirus expressing APOE4-202 in *Apoe-/-* mice did not result in a rise in the serum triglyceride levels and normalized the hypercholesterolemia. These studies demonstrated that the N-terminal 202 amino acids of apoE4 could associate with lipoproteins lacking apoE and mediate hepatic clearance, without affecting the VLDL-TG production rate.

Since FD associated with apoE2/E2 phenotype is a recessive disease, it can be anticipated that additional expression of a low dose of an APOE isoform with normal binding affinity to the LDL receptor will ameliorate the phenotype. However, in FD the steady state serum levels of cholesterol, TG and apoE2 are elevated, and the adverse effect of APOE overexpression on the VLDL-TG production rate could also overrule the positive effects of increased hepatic clearance. In the present study, the effects of hepatic overexpression of APOE3, APOE4 and APOE4-202 are investigated in a mouse model for FD, the APOE2 transgenic mouse. Similar to the human situation, the APOE2 transgenic mouse model is only hyperlipidemic in the absence of functional apoE, i.e. on the *Apoe-/-* background (the APOE2.*Apoe-/-* mouse). Our data indicate that the hyperlipidemia of APOE2.*Apoe-/-* mice is extremely sensitive to hepatic overexpression of full length APOE3 and APOE4. Only a very low dose of adenovirus carrying APOE4 could reduce the hyperlipidemia in these mice, whereas higher doses of Ad-APOE4 aggravated the hyperlipidemia. In

contrast, a high dose of Ad-APOE4-202 reduced the lipid levels in APOE2.*Apoe-/-* mice. Thus, even in the presence of elevated serum apoE levels, apoE4-202 can restore the clearance of VLDL remnants, without increasing serum TG levels.

Methods

Generation and analysis of transgenic mice

Transgenic mice expressing human apoE2(Arg158->Cys) in the absence of endogenous *Apoe* have been described previously(17). The transgenic status of the breeding offspring was determined by polymerase chain reaction analysis as described. For these experiments, female mice were fed a regular mouse diet (SRM-A: Hope Farms, Woerden, The Netherlands). The mice were housed under standard conditions in conventional cages and given free access to food and water. For adenovirus mediated gene transfer experiments, mice were transferred to filter-top cages, placed in a designated room and allowed to adapt for at least 5 days. All animal experimentation protocols were approved by the Committee on Animal Experimentation of the Leiden University Medical Center.

Adenovirus Transfections

The generation and expansion of recombinant adenoviruses, expressing apoE3, apoE4 and apoE4 truncated at amino acid 202 under control of a Cytomegalovirus promotor have been described(12). For *in vivo* administration, the virus was purified twice via CsCl gradient centrifugation and dialyzed against dialysis buffer (consisting of 25 mmol/L Tris, 137 mmol/L NaCl, 5 mmol/L KCl, 0.73 mmol/L NaH_2PO_4, 0.9 mmol/L $CaCl_2$, and 0.5 mmol/L $MgCl_2$, pH 7.45) and finally dialysis buffer supplemented with sucrose (50 g/L). For storage, aliquots of 150µl virus were frozen at -80°C. Routine virus titers of the stocks varied from $1x10^{10}$ to $1x10^{11}$ plaque-forming units (pfu) per milliliter. At least 3 days before adenovirus injection, basal serum lipid (t=0) values were measured. At day 0, mice were injected into the tail vein with recombinant adenovirus diluted with PBS to a total volume of 200 µL. To prevent sequestration of low doses of Ad-APOE by liver Kupffer cells and to achieve a more linear dose response relation, mice were pre-injected with Ad-LacZ(18). The mice

that received the low doses of $2x10^8$ and $5x10^8$ pfu Ad-APOE4 were pre-injected with respectively $8x10^8$ and $5x10^8$ pfu Ad-LacZ 4 hours before administration of the apoE expressing virus. Up to 10 days after adenovirus administration, blood samples of 100µl were drawn from the tail vein of 4 hour fasted mice or, when a larger volume of serum was required, blood samples were collected by orbital puncture.

Lipid, lipoprotein and ApoE Measurements

Total serum cholesterol and triglyceride (TG) levels were measured enzymatically with commercially available kits: respectively Boehringer Mannheim 236691 and Sigma Chemical Co 337-B.

Lipoprotein fractions were separated using density gradient ultracentrifugation. Prior to, and 4 days after infection with Ad-apoE4 or Ad-apoE4-202, sera of individual mice were pooled to a volume of 100µl. After centrifugation, the gradient was fractionated in fractions of 0.5 ml. The fractions were analyzed enzymatically for cholesterol and TG, as described above. Human apoE concentrations were determined by sandwich ELISA as described previously(19).

Characterization of VLDL

The VLDL fractions (d < 1.006 g/mL) were analyzed for protein content by the method of Lowry et al.(20). Furthermore, total and free cholesterol, TG and phospholipid content were determined enzymatically, using commercially available kits (236691 and 310328, Boehringer-Mannheim; 337-B, Sigma Chemicals; and 99054009, Wako Chemicals, respectively).

Hepatic VLDL TG production

At day six after infection with adenovirus, the mice were fasted for 4 hours and then intravenously injected with 500 mg/kg Triton WR 1339 (Sigma) as described(21). Blood samples of Ad-APOE4 and Ad-APOE4-202 infected mice were collected 1 and 60 min. after Triton injection. Serum TG concentrations were measured enzymatically, as described above. The hepatic VLDL-TG production rate was measured as the accumulation of serum TG after Triton injection and expressed as mg/dL/min.

Results

Serum lipid, lipoprotein and apoE levels after adenovirus mediated gene transfer of full-length and truncated APOE in APOE2.*Apoe-/-* transgenic mice

Full-length APOE3, APOE4 and the truncated variant APOE4-202 were expressed in female APOE2.*Apoe-/-* mice using adenovirus mediated gene transfer. Serum cholesterol and TG levels were followed for up to 10 days after virus injection. Upon administration of a high dose of $2x10^9$ pfu of adenovirus expressing full-length apoE3 (Fig. 1A), the serum cholesterol levels did not change (20,0±3,7 to 20.7±5.6mM p=0,84). Serum TG level rose from 2.3±0.7 to 24.8±11.0 mM (p<0,01) at day 5, but progressively decreased at days 7 and 9 to the level of day 0 (Fig. 1D). Injection of lower doses of adenovirus ($5x10^8$ or $1x10^9$ pfu) expressing full-length apoE4 (Fig. 1B and E) resulted in hypertriglyceridemia, combined with non-significant changes in cholesterol levels at day 5 after injection. Only the lowest dose of $2x10^8$ pfu of adenovirus expressing full-length apoE4 resulted in improvement of the hyperlipidemia at day 5 after injection (cholesterol from 13,8±1,7 to 2,2±0,7mM (p<0,01); TG from 3,1±2,2 to 0,7±0,3mM, p<0,05). In contrast, expression of $2x10^9$ pfu of adenovirus expressing the truncated apoE4-202 variant resulted in a significant reduction of serum cholesterol levels from 13,1±3,2 to 3,8±1,1 mM (p<0,01) and did not raise the serum TG level in these mice at any time point after injection (Fig. 1C and F).

To determine the effects of the apoE variants on the individual lipoprotein classes, serum samples were subjected to density gradient ultra-centrifugation. After fractionation, the lipid levels in the fractions were measured. The lipoprotein profile of untreated APOE2.*Apoe-/-* mice shows that cholesterol is mainly distributed over the VLDL and IDL/LDL fractions (Fig. 2A). The tremendous increase in serum TG levels after adenovirus mediated expression of APOE4 is mainly confined to the VLDL fraction (Fig. 2B). Most of the cholesterol is present in the VLDL fractions and some in the HDL fractions. Furthermore, no clear IDL/LDL peak is detected. Upon expression of apoE4-202 (Fig. 2C), the VLDL and IDL fractions decreased significantly, whereas the HDL peak remained similar. The ratio VLDL cholesterol to HDL cholesterol decreased about 5 times as compared to the non-infected mice.

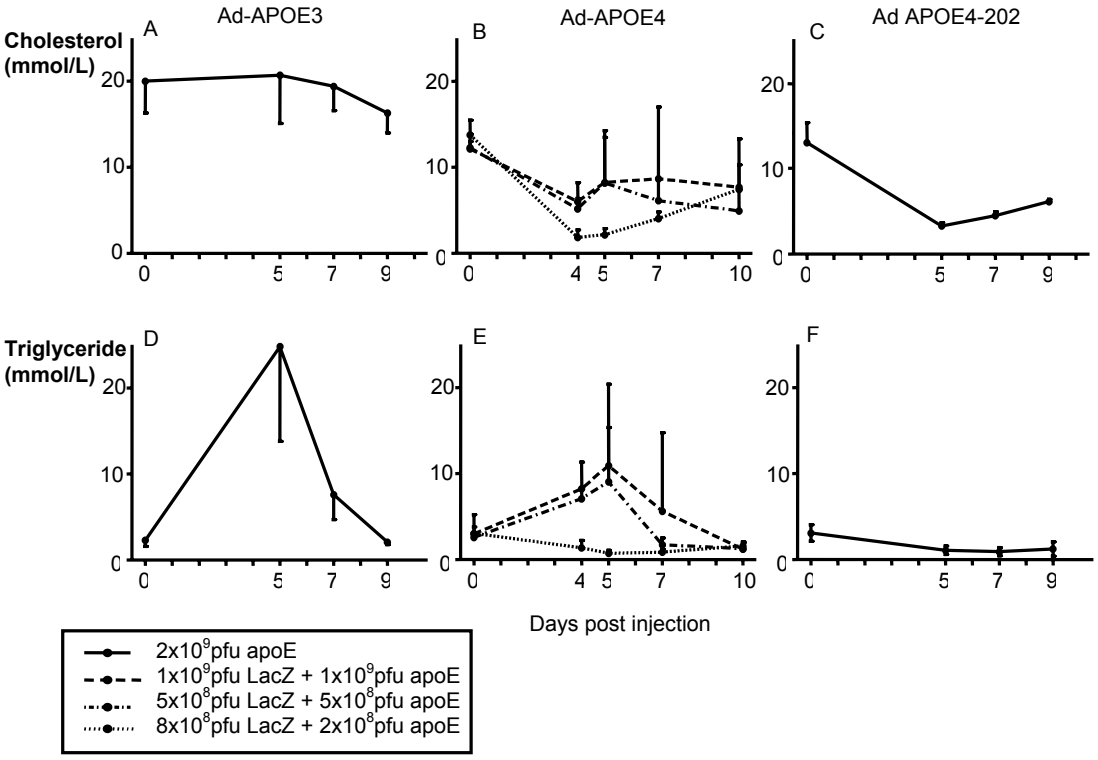

Figure 1. Serum cholesterol, TG and apoE levels after adenovirus-mediated gene transfer of APOE3, APOE4 and APOE4-202 to APOE2.*Apoe-/-* transgenic mice. Female mice (n=5 per group) were injected with $2x10^9$ pfu Ad-APOE3 (A and D), increasing doses of $2x10^8$, $5x10^8$ and $1x10^9$ pfu of Ad-APOE4 (B and E), or $2x10^9$ pfu Ad-APOE4-202 (C and F). Mice injected with the low doses of Ad-APOE4 ($2x10^8$ and $5x10^8$ pfu) were pre-injected with respectively $8x10^8$ and $5x10^8$ pfu Ad-LacZ. Serum cholesterol (A, B and C) and TG values (Fig. D, E and F) were measured up to 10 days after injection and represented as mean ± SD.

ApoE levels in these fractions were measured by sandwich ELISA. In APOE2.*Apoe-/-* mice (Fig. 2D), apoE was mostly present in the VLDL and IDL/LDL fractions. Expression of APOE4 resulted in a considerable amount of apoE in the HDL and bottom fractions (Fig. 2E), the latter indicating the presence of unbound apoE. Furthermore, abudant apoE was present in the VLDL fractions. Expression of apoE4-202 resulted in a significant amount of apoE in the bottom and HDL fractions (Fig. 2F), but ApoE was also clearly present in the VLDL fractions.

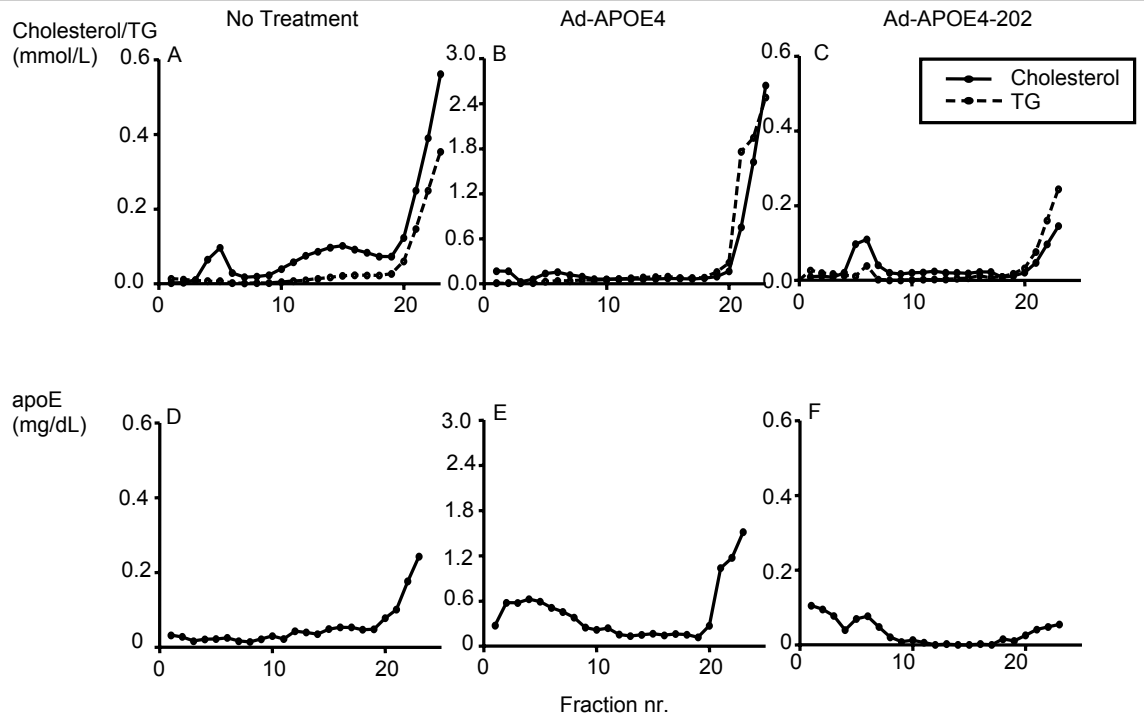

Figure 2. Distribution of cholesterol, TG and apoE in lipoprotein fractions after adenovirus-mediated gene transfer of APOE4 and APOE4-202 to APOE2.*Apoe-/-* transgenic mice. Female mice (n=5 per group) were left untreated (A and D), injected with $2x10^9$ pfu Ad-APOE4 (B and E), or injected with $2x10^9$ pfu Ad-APOE4-202 (C and F). Four days post-injection, sera of 5 fasted mice per group were pooled and lipoproteins were separated by density gradient ultracentrifugation. After fractionation, the individual fractions were analyzed for cholesterol and TG (A, B and C) and apoE content (D, E and F).

VLDL composition

The lipid composition of the VLDL fractions isolated by density gradient ultracentrifugation was determined (Fig. 3). Expression of apoE4 in APOE2.*Apoe-/-* mice resulted in an increase in TG content of circulating VLDL from 46% to 71% (Fig. 3 A and B). This latter level approaches the TG content of nascent VLDL (76%, Fig. 3 D). After expression of apoE4-202 the TG content of circulating VLDL increased from 46% to 61% (Fig. 3 A and C).

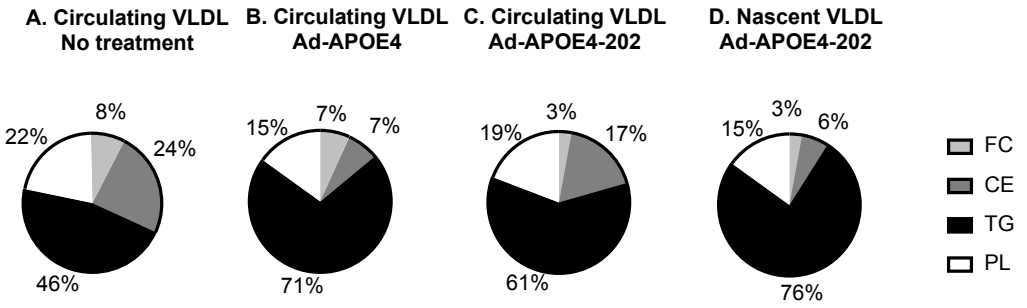

Figure 3. VLDL composition following adenovirus-mediated gene transfer of APOE4 and APOE4-202 to APOE2.*Apoe-/-* transgenic mice. Female mice (n=5 per group) were left untreated (A), injected with $2x10^9$ pfu Ad-APOE4 (B), or $2x10^9$ pfu Ad-APOE4-202 (C and D). For the analysis of circulating VLDL (A, B and C), sera were collected from fasted mice four days post-injection and pooled. For the analysis of nascent VLDL (D), on day 6 following injection with Ad-APOE4-202, fasted mice were injected with Triton WR 1339, and sera were collected 1 hour after Triton injection. VLDL was isolated by density gradient ultracentrifugation. Lipid composition was determined in w/w % of total lipids (FC, free cholesterol; CE, cholesteryl ester; TG, triglyceride; PL, phospholipid).

Hepatic VLDL production

The VLDL-TG production rate after apoE4 and apoE4-202 overexpression in APOE2.*Apoe-/-* mice was determined after injection with Triton WR1339 (Fig. 4). Triton WR1339 blocks VLDL-TG lipolysis and VLDL remnant clearance. Expression of apoE4 in APOE2.*Apoe-/-* mice resulted in a 10-fold increase in the VLDL-TG production rate, whereas expression of apoE4-202 did not affect the VLDL-TG production rate.

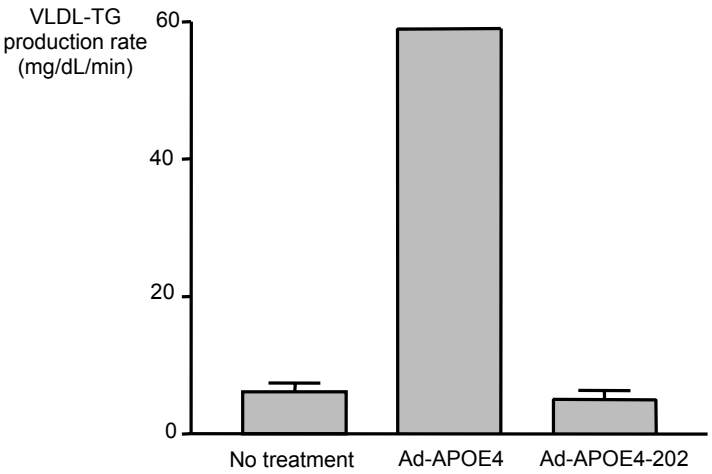

Figure 4. Hepatic VLDL-TG production in APOE2.*Apoe-/-* mice injected with Ad-APOE4 or Ad-APOE4-202. Female mice were left untreated (n=5), injected with $2x10^9$ pfu Ad-APOE4 (n=1), or $2x10^9$ pfu Ad-APOE4-202 (n=5). VLDL-TG production was measured six days post-injection with the virus expressing the APOE4 or APOE4-202. Fasted serum TG level was determined 1 and 60 minutes after Triton WR 1339 injection. The VLDL-TG secretion rate was calculated by the increase in TG content as a function of time and expressed as mg/dL/min. Values are represented as mean ± SD.

Discussion

FD is characterized by elevated levels of VLDL and chylomicron remnants in the serum of patients and an increased risk for premature atherosclerosis. The majority of patients with FD are homozygous for the E2 allele of apoE. The association of the APOE2/E2 phenotype with FD is at least partly explained by the observation that apoE2 binds poorly to the LDL receptor *in vitro*, which is a major route of lipoprotein remnant uptake *in vivo*. Thus, it can be anticipated that additional expression of APOE with normal binding affinity to the LDL receptor will reduce the hyperlipidemia associated with the recessive FD disease. We have previously generated a transgenic mouse line expressing the human APOE2 gene(17). In the presence of endogenous mouse apoE, APOE2 mice do not develop hyperlipidemia.

However, in the absence of mouse apoE, VLDL size remnants accumulate in the serum of these APOE2.*Apoe-/-* mice and serum lipid and apolipoprotein levels are highly reminiscent of the FD phenotype. Using this mouse model for FD, we now show that hepatic overexpression of full length APOE3 or APOE4 leads to a dose-dependent induction of hypertriglyceridemia (Fig. 1). Only a very low level of APOE4 expression reduced the hyperlipidemia of the APOE2.*Apoe-/-* mouse. Higher expression levels induced hypertriglyceridemia, without reducing serum cholesterol levels. In contrast, the hyperlipidemia of APOE2.*Apoe-/-* mice is reduced significantly by high levels of APOE4-202 expression.

We have shown previously in *Apoe-/-* mice that adenovirus-mediated overexpression of full-length apoE4 resulted in hypertriglyceridemia at high doses of adenovirus and resulted in rescue of the hyperlipidemia at moderate doses of adenovirus(12,16). Similar to the observations in the APOE2.*Apoe-/-* mice, the truncated apoE4-202 variant reduced the hyperlipidemia in *Apoe-/-* mice at high expression levels. Thus, APOE4-202 does not have a hypertriglyceridemic effect at high expression levels in either mouse model. Moreover, apoE4-202 can associate with lipoproteins both in the absence of endogenous mouse apoE and in the presence of binding-defective human apoE2 and mediate hepatic lipoprotein uptake. Interestingly, the lowest dose of Ad-APOE4 that rescues the hyperlipidemia of *Apoe-/-* mice ($5x10^8$ pfu) (12) does result in hypertriglyceridemia in APOE2.*Apoe-/-* mice. The higher sensitivity of the APOE2.*Apoe-/-* mice is in line with the fact that the hypertriglyceridemic effect of APOE4 is gene dose dependent and that the starting level of APOE gene expression in the APOE2 mice is higher.

It is interesting to note that the reduction of hyperlipidemia in the APOE2.*Apoe-/-* mice was achieved with a very low dose ($2x10^8$ pfu) of Ad-APOE4 combined with pre-injection of Ad-LacZ ($1.8x10^9$ pfu). Without pre-injection of adenovirus, doses below $5x10^8$ pfu give extremely low levels of gene expression, a dose of $5x10^8$ pfu gives low levels of gene expression and a dose of $2x10^9$ pfu gives very high levels of gene expression. Tao et al. have reported that this non-linear adenovirus dose-response is caused by the initial sequestration and non-functional infection of adenovirus vectors by the kupffer cells in the liver(18). It was shown that pre-injection with a control adenovirus vector rendered the adenovirus dose-response linear, a finding we have confirmed (data not shown). The data presented

in Figure 1 also show that by pre-injection with adenovirus, even very low doses of adenovirus result in productive gene expression.

In the *Apoe-/-* mice, APOE4 overexpression had a major impact on the VLDL-TG production rate(12,15). The dramatically increased VLDL-TG production rate in the APOE2.*Apoe-/-* mouse (Fig. 4) is obviously in line with this observation. Neither in the *Apoe-/-* mice, nor in the APOE2.*Apoe-/-* mice did APOE4-202 overexpression result in an increase in the VLDL-TG production rate. The mechanism of the increase in the VLDL-TG production rate, which is apparently mediated via the C-terminal 97 amino acids of apoE, remains to be determined. It has been speculated that apoE is involved in one of the last steps of de-novo VLDL synthesis, the intracellular addition of TG to the nascent VLDL particle(22). It is possible that the C-terminal lipid-binding domain of apoE stabilizes the TG-content of the nascent VLDL particle and that overexpression of full-length apoE thus enhances VLDL-TG secretion.

As compared to nascent VLDL, the VLDL sized particles that accumulate in untreated APOE2.*Apoe-/-* mice are relatively depleted of TG (76% versus 46%, Fig. 3). The TG content of circulating VLDL is affected by both hepatic VLDL production and uptake, but also by peripheral VLDL-TG lipolysis. In APOE2.*Apoe-/-* mice, VLDL is poorly cleared resulting in a prolonged circulation time and thus prolonged exposure to lipoprotein lipase (LPL). This is believed to result in the observed relatively low TG content of circulating VLDL in APOE2.*Apoe-/-* mice. Overexpression of APOE4 in the APOE2.*Apoe-/-* mice resulted in an accumulation of circulating VLDL that had a relatively high TG content (71%). This could be explained by the increased VLDL-TG secretion rate upon APOE4 overexpression. However, it has also been demonstrated *in vitro* that the rate of LPL-mediated VLDL-TG lipolysis is inhibited by apoE in a dose-dependent manner(3,4,5). At high levels of hepatic APOE4 expression, the relative apoE content of the circulating VLDL is high (Fig. 2E). Thus, it cannot be excluded that inhibition of VLDL-TG lipolysis contributes to the high TG level of the circulating VLDL particles in APOE2.*Apoe-/-* mice overexpressing APOE4.

The TG content of circulating VLDL after overexpression of APOE4-202 (61%) is increased as compared to circulating VLDL from untreated APOE2.*Apoe-/-* mice (46%) (Fig. 3), but is below that of circulating VLDL from wild-type mice (69%) (21). As shown in Figure 4, the hepatic VLDL-TG production rate does not contribute to

this effect, since it remains unchanged after overexpression of APOE4-202. The relative TG-enrichment of the circulating VLDL in APOE2.*Apoe-/-* mice after overexpression of APOE4-202 is likely caused by decreased residence times in the circulation (and thus shorter exposure to LPL) as a result of enhanced VLDL particle uptake. The enhanced VLDL particle uptake is demonstrated by the lowering in serum lipid levels (Fig. 1 and 2). However, APOE2.*Apoe-/-* mice overexpressing APOE4-202 do accumulate a modest amount of VLDL in serum (Fig. 2C) and the VLDL-TG content is below that of wild type level. Thus it can be concluded that the residence time of VLDL containing both APOE2 and APOE4-202 is somewhat prolonged as compared to wild-type VLDL.

At high hepatic expression levels of APOE4, the circulating VLDL contains abundant apoE4 (Fig. 2 B and E), which in principle would be an excellent substrate for LDL receptor mediated uptake. A tiny amount of APOE expression can rescue the hyperlipidemia of *Apoe-/-* mice(6,23). However, both in the present study in APOE2.*Apoe-/-* mice and previously in *Apoe-/-* mice(12), apparently, higher levels of APOE expression and thus higher levels of apoE on the VLDL do not compensate the increase in VLDL production rate with an increase in VLDL clearance. It thus seems likely that apoE- and TG-enriched VLDL is a relatively poor substrate for hepatic clearance, which is in agreement with our previous results(16). Mechanistically this could be explained if the apoE molecules on apoE and TG-rich particles have a decreased affinity for hepatic receptors such as the LDL receptor. There is evidence to indicate that high particle apoE content(24), size(25) and/or the lipid content(26) affect the conformation of the receptor-binding domain of apoE. Thus, in addition to increased VLDL production and likely inhibition of VLDL-TG lipolysis, a decreased clearance rate as a result of decreased receptor binding affinity could contribute to the hyperlipidemia observed after APOE overexpression.

ApoE is an attractive intervention target for conventional pharmacological or gene therapeutic treatment of dyslipidemia's that are characterized by increased serum levels of VLDL and VLDL remnants, such as FD. The data presented here in APOE2.*Apoe-/-* mice and previously in *Apoe-/-* mice indicate that apoE affects lipoprotein metabolism in a dose-dependent manner. Low levels of hepatic APOE expression are associated with reduction of the hyperlipidemia, whereas higher levels are associated with serious aggravation of the hyperlipidemia. Our data

indicate that in a dyslipidemic situation, such as in APOE2 associated FD, additional expression of full-length APOE may readily aggravate the hyperlipidemia. The relatively small therapeutic window makes additional or induced expression of full-length APOE a risky treatment. However, the insight that the hyperlipidemic effect can be circumvented by deletion of the C-terminal domain, combined with the observation that this truncated apoE variant is functional in the presence of APOE2, provides novel avenues for the application of apoE variants as therapeutic proteins.

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Chapter 3

ApoC-III deficiency prevents hyperlipidemia induced by apoE overexpression.

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Abstract

Adenovirus-mediated overexpression of human apoE induces hyperlipidemia by stimulating the VLDL-TG production rate and inhibiting the LPL-mediated VLDL-TG hydrolysis rate. Since apoCIII is a strong inhibitor of TG hydrolysis, we questioned whether *Apoc3*-deficiency might prevent the hyperlipidemia induced by apoE overexpression *in vivo*. Injection of $2x10^9$ pfu AdAPOE4 caused severe combined hyperlipidemia in *Apoe-/-* mice (TG from 0.7±0.2 to 57.2±6.7 mM; TC from 17.4±3.7 to 29.0±4.1 mM) that was confined to VLDL/IDL sized lipoproteins. In contrast, *Apoc3*-deficiency resulted in a gene-dose-dependent reduction of the apoE4-associated hyperlipidemia (TG from 57.2±6.7 mM to 21.2±18.5 and 1.5±1.4 mM; TC from 29.0± 4.1 to 16.4±9.8 and 2.3±1.8 mM in *Apoe-/-*, *Apoe-/-.Apoc3+/-* and *Apoe-/-.Apoc3-/-* mice, respectively). In both *Apoe-/-* mice and *Apoe-/-.Apoc3-/-* mice, injection of increasing doses of AdAPOE4 resulted in an up to 10-fold increased VLDL-TG production rate. However, *Apoc3*-deficiency resulted in a significant increase in the uptake of TG-derived fatty acids from VLDL-like emulsion particles by white adipose tissue, indicating enhanced LPL activity. *In vitro* experiments showed that apoCIII is a more specific inhibitor of LPL-activity than apoE. Thus, *Apoc3*-deficiency can prevent apoE-induced hyperlipidemia associated with a 10-fold increased hepatic VLDL-TG production rate, most likely by alleviating the apoE-induced inhibition of VLDL-TG hydrolysis.

Introduction

The level of circulating plasma VLDL and VLDL-remnants is determined by both the hepatic production rate of VLDL, the conversion rate of VLDL to VLDL-remnants, and their clearance rate. The VLDL-TG hydrolysis rate is a crucial step in the formation of VLDL-remnants that can be efficiently cleared from the plasma by the liver. Both apoE and apoCIII have long been recognized as modulators of plasma VLDL-TG levels. Plasma levels of apoCIII and apoE are positively correlated with plasma TG levels in human studies(1,2,3,4,5) and apoCIII has been shown to inhibit LPL-mediated TG-hydrolysis *in vitro*(6,7). Likewise, apoE has been shown to inhibit

LPL-mediated TG hydrolysis *in vitro* and *in vivo*(8,9,10,11). In addition, apoE increases the production rate of VLDL-TG in a gene-dose-dependent manner(12,13,14). However, apoE also functions as a ligand mediating the receptor-mediated uptake of VLDL-remnants.

We have previously reported that high levels of apoE expression obtained via adenovirus-mediated transfer of APOE result in hyperlipidemia, characterized by increased plasma cholesterol and TG levels. This was associated with an increased VLDL-TG production rate and a reduced LPL-mediated VLDL-TG hydrolysis rate(13). Apparently, the increased production of VLDL and their hampered conversion to remnants is not compensated for by an increased apoE-mediated hepatic clearance. We and others have also previously demonstrated that *Apoc3*-deficiency prevents the postprandial hypertriglyceridemia induced by an intragastric olive oil load(15,16)**,** indicative for an enhanced lipolysis of chylomicrons. In the current study we hypothesize that *Apoc3*-deficiency may also prevent the apoE-induced combined hyperlipidemia.

To investigate the role of apoCIII in APOE-induced hyperlipidemia, low and high doses of AdAPOE4 were injected into *Apoe*-deficient mice, in the presence and absence of endogenous *Apoc3*. The APOE4 isoform was selected since low expression levels rescue the Apoe-/- fenotype, but high expression levels seem to induce a more pronounced hyperlipidemia as compared to the APOE3 isoform(17,18). We here report that *Apoc3*-deficiency gene-dose-dependently prevents the hyperlipidemia induced by apoE4 overexpression. Since *Apoc3*-deficiency did not affect the VLDL-TG production rate, and did result in an enhanced uptake of TG-derived FFA by adipose tissue, we conclude that *Apoc3*-deficiency alleviates the block in the formation of lipoprotein remnants from apoE-rich and TG-rich VLDL.

Experimental procedures

Mouse studies

ApoE knockout (*Apoe-/-*) mice, that have been generated previously(19), were intercrossed with apoCIII-knockout (*Apoc3-/-*) mice (obtained from the Jackson

Laboratories, Bar Harbor, ME) to generate *Apoe-/-.Apoc3+/-* and *Apoe-/-.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 transfection, 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.

Adenoviral transfection

The recombinant adenoviral vector, expressing the human APOE4 gene and the green fluorescent protein (GFP) gene under control of a cytomegalovirus (CMV)-promoter (AdAPOE4) was generated as described(17). A LacZ-expressing recombinant adenovirus (AdLacZ) was used for control virus treatment. The recombinant adenoviruses were propagated in the human embryonic retina cell line 911 and/or human embryonic kidney cell line 293 as described(20,21). The viruses were purified via ultracentrifugation in a CsCl gradient, followed by dialysis and titration.

For *in vivo* administration, $0.5x10^9$ to $2x10^9$ plaque-forming units (pfu) adenovirus, adjusted to 200 µL with PBS, was injected into the tail vein of female mice. To achieve a linear dose-response of AdAPOE4 virus, by saturating the uptake of virus particles by Kupffer cells(22), mice were preinjected with $0.5x10^9$ pfu AdLacZ at 3 h before injection of the virus of interest.

Plasma lipid and lipoprotein analysis

Two days before and 5 days after adenovirus injection, blood samples of approximately 50 µL were drawn from the tail vein of 4 h-fasted mice. Plasma triglyceride (TG) and total cholesterol (TC) levels were measured enzymatically, using commercially available kits (Sigma). Lipoprotein fractions were separated using fast protein liquid chromatography (FPLC). Hereto, a plasma pool obtained from each group of mice before adenovirus injection and 5 days after adenovirus injection was diluted 5 to 10 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). Elution fractions of 50 µL were collected and assayed for TG and TC levels, as described above. Human ApoE levels were determined by sandwich ELISA as described

previously(23).

Characterization of VLDL

At 5 days after injection of AdLacZ ($2x10^9$ pfu) or AdAPOE4 ($1x10^9$ or $2x10^9$ pfu), *Apoe-/-* and *Apoe-/-Apoc3-/-* mice were fasted for 4 hours and serum was isolated. Sera from each group of mice were pooled and VLDL (d < 1.006 g/ml) was isolated by density gradient ultracentrifugation. The VLDL was enzymatically analyzed for total and free cholesterol, TG, and phospholipid content, using commercially available kits (236691 and 310328, Boehringer-Mannheim; 337-B, Sigma Chemicals; and 99054009, Wako Chemicals). The protein content was determined by the method of Lowry et al. (24)

Hepatic VLDL-TG secretion

The hepatic VLDL-TG secretion rate in *Apoe-/-* and *Apoe-/-.Apoc3-/-* mice was measured 5 days after infection with AdLacZ ($2x10^9$ pfu) or AdAPOE4 ($0.5x10^9$ and $1x10^9$ pfu). Four hours fasted mice were anesthetized with a mixture of vetranquil/dormicum/fentanyl (6.25/6.25/0.3125 mg/kg mouse) and intravenously injected with 500 mg/kg Triton WR 1339 (Sigma) as described (10,25). Blood samples were drawn via the tail vein at 1, 10, 20, 30, 60, 90, and 120 min after Triton injection, and plasma TG concentrations were measured as described above. The hepatic VLDL-TG secretion rate was determined by the increase in plasma TG concentration after subtraction of the TG concentration in the plasma samples at 1 min after Triton injection.

Preparation of VLDL-like emulsion particles

VLDL-like TG-rich emulsion particles were prepared according to the sonication and ultracentrifugation procedure of Redgrave and Maranhao(26). Hereto, 100 mg total lipid at a weight ratio triolein (Fluka): egg yolk phospatidylcholine (Lipoid E PC 98%, from Lipoid, Ludwigshafen, Germany): lysophosphatidylcholine (Sigma): cholesteryl oleate (Sigma): cholesterol (Sigma) of 70: 22.7: 2.3: 3.0: 2.0, supplemented with 200 µCi of glycerol tri[9,10(n)-^{3}H]oleate ([^{3}H]triolein, TO, Amersham Biosciences), was sonicated using a Soniprep 150 (MSE Scientific Instruments, UK) at 10 µm output as described(27). An emulsion fraction containing

80 nm-sized emulsion particles was obtained by consecutive density gradient ultracentrifugation steps exactly as described(28). The TG content of the emulsions was determined as described above.

Tissue distribution of VLDL-like emulsion-derived triglycerides *in vivo*

Fed *Apoe-/-* and *Apoe-/-.Apoc3-/-* mice were anesthesized with a mixture of vetranquil/dormicum/fentanyl (6.25/6.25/0.3125 mg/kg mouse) and their abdomens were opened. 200 µL of [^{3}H]TO-labeled VLDL-like emulsion particles was administered via the vena cava inferior at a dose (1.0 mg of TG) that exceeded the endogenous plasma TG content in both experimental groups. At 10 min after injection of the emulsion particles, the liver, heart, spleen, and aliquots of hindlimb muscle, gonadal white adipose tissue (gWAT), perirenal white adipose tissue (pWAT), and intestinal white adipose tissue (iWAT) were isolated, dissolved in Soluene (Perkin Elmer) at 60°C and counted in 10 mL of Ultima Gold. The ^{3}H-activity in the tissues was corrected for wet organ weight.

In vitro LPL activity assay

The effect of apoCIII and apoE on LPL activity was determined essentially as described(9). First, [^{3}H]TO-labeled emulsion particles (0.5 mg/mL TG) were incubated with purified human apoCIII (Academy Biomedical Company, Houston, USA) or recombinant human apoE in 75 µL of PBS (30 min at 37°C). Subsequently, 0.1 M Tris.HCl pH 8.5, with or without 5% (v/v) heat-inactivated human serum as source of apoCII, was added to a total volume of 200 µL. At *t*=0, LPL (purified bovine milk LPL, 3300 U/mg, Sigma), final concentration 3.5 U/mL, was added in 200 µL of 120 mg/mL free fatty acid-free BSA (Sigma) as [^{3}H]oleate acceptor. After 30 minutes, the [9,10-^{3}H]oleate that was generated during lipolysis was extracted. Hereto, 50 µL samples were added to 1.5 mL CH_3OH: $CHCl_3$: heptane: oleic acid (1410: 1250: 1000: 1, v/v/v/v) and 0.5 mL 0.2 N NaOH to terminate lipolysis. ^{3}H-radioactivity in 0.5 mL of the aqueous phase obtained after vigorous mixing and centrifugation (10 min at 1000 *g*) was counted in 5 mL of Ultima Gold (PerkinElmer Life Sciences). Recovery of [9,10-^{3}H]oleate in the aqueous phase following organic extraction was corrected for a 78.0±0.9% recovery of [1-^{14}C]oleate internal standard.

Statistical analysis

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

Results

Plasma lipid and lipoprotein levels after injection of AdAPOE4

To determine the role of apoCIII in apoE-induced hyperlipidemia, *Apoe-/-*, *Apoe-/-.Apoc3+/-* and *Apoe-/-.Apoc3-/-* mice were injected with increasing doses of AdAPOE4. Plasma TG and TC levels were measured 5 days after adenovirus injection (Fig 1). In *Apoe-/-* mice, a moderate dose of AdAPOE4 ($1x10^9$ pfu) resulted in plasma apoE levels of 10-40 mg/dL and significantly reduced plasma TC levels and moderately increased plasma TG levels, whereas a high dose ($2x10^9$ pfu AdAPOE4) resulted in plasma apoE levels of 40-60 mg/dL and a substantial increase of both plasma TG and TC levels. In contrast, in *Apoe-/-.Apoc3-/-* mice, injection of both the moderate and the high dose of AdAPOE4 resulted in near wild-type plasma TG and TC levels and plasma apoE levels of 3-10 mg/dL for both doses. Thus absence of apoCIII prevents the apoE-induced hyperlipidemia. This effect of *Apoc3*-deficiency is *Apoc3*-gene-dose dependent, since injection of the high dose of AdAPOE4 in *Apoe-/-.Apoc3+/-* mice resulted in plasma apoE levels of 10-25 mg/dL, a moderate hypertriglyceridemia and unchanged plasma cholesterol levels.

The effect of a high dose of $2x10^9$ pfu AdAPOE4 on the distribution of lipids over the lipoprotein fractions was measured after lipoprotein separation via FPLC (Fig 2). The APOE4-induced change in plasma TG and TC was predominantly confined to the VLDL fractions in all types of mice, measured as area under the curve in FPLC lipoprotein profiles. Five days after expression of a high dose of $2x10^9$ pfu AdAPOE4, *Apoe-/-* mice showed an increase in VLDL-TG from 0.8 to 61.2 mM. The VLDL-TC levels also increased from 13.3 to 28.4 mM. *Apoe-/-.Apoc3-/-* mice have low VLDL-TG levels although they increased from 0.2 to 0.8 mM. The VLDL-TC levels decreased from 8.5 to 0.6 mM. *Apoe-/-.Apoc3+/-* mice showed an intermediate response in VLDL lipid levels after expression of AdAPOE4. The VLDL-TG levels

increased from 0.14 to 21.6 mM and the VLDL-TC levels decreased from 14.8 to 11.4 mM.

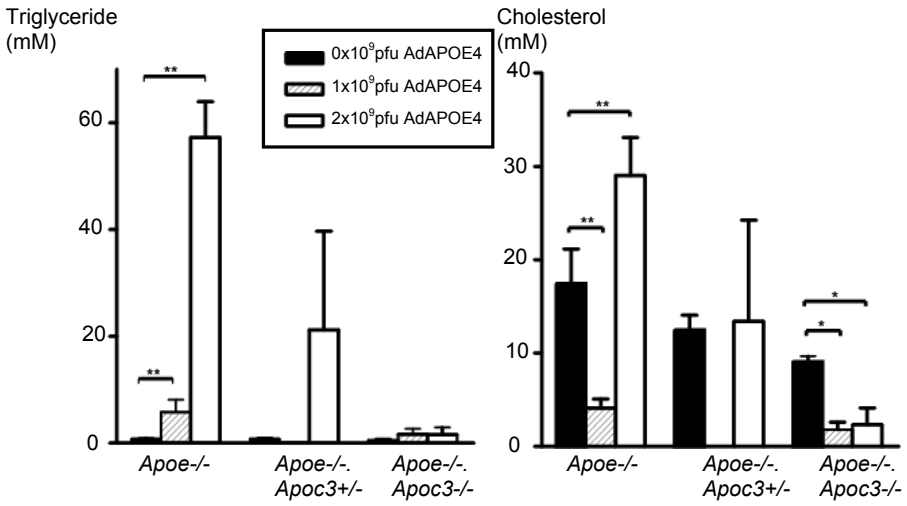

Figure 1. Plasma triglyceride and cholesterol levels of *Apoe-/-*, *Apoe-/-.Apoc3+/-*, and *Apoe-/-.Apoc3-/-* mice injected with adAPOE4. Five *Apoe-/-* and *Apoe-/-.Apoc3-/-* mice were injected with $1x10^9$ (dashed bars) or $2x10^9$ pfu (open bars) of AdAPOE4. *Apoe-/-.Apoc3+/-* were injected with a dose of $2x10^9$ pfu (open bar) of AdAPOE4 only. As control virus treatment, 5 *Apoe-/-*, *Apoe-/-.Apoc3+/-*, and *Apoe-/-.Apoc3-/-* mice were injected with $2x10^9$ pfu AdLacZ (indicated as $0x10^9$ pfu AdAPOE4, black bars). At day 5 after virus injection, fasted plasma samples were measured for triglyceride (A) and cholesterol (B) concentration, represented as means ± SD. * $p<0.05$; ** $p<0.005$.

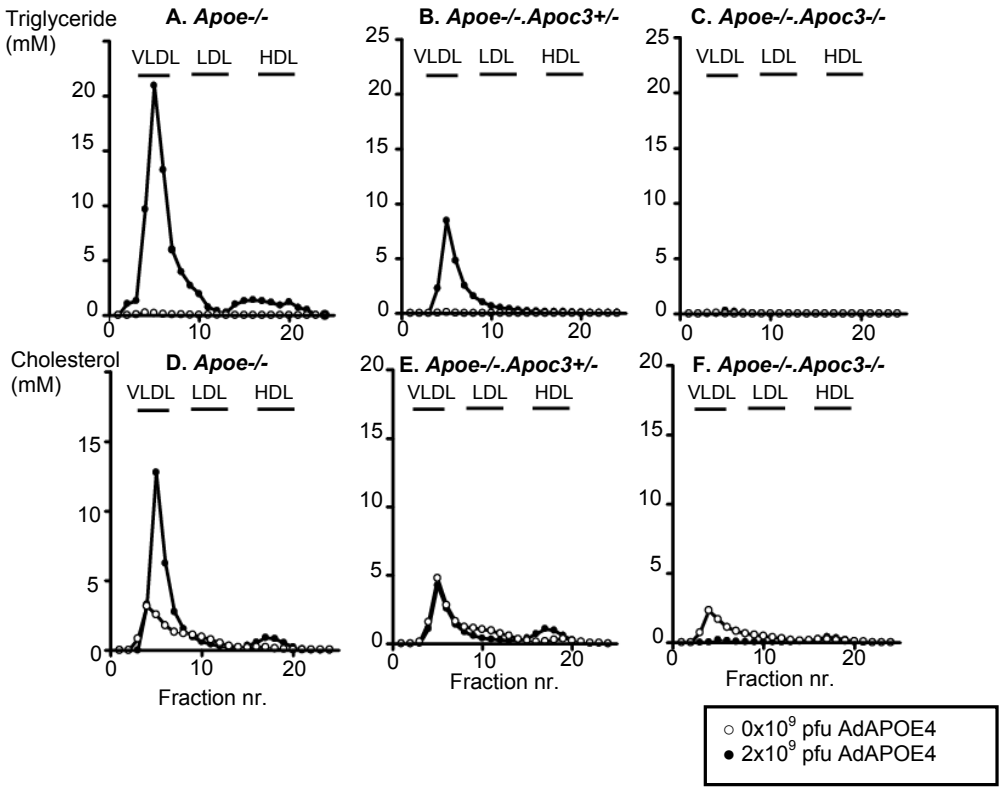

Figure 2. Lipoprotein profiles of *Apoe-/-*, *Apoe-/-Apoc3+/-*, and *Apoe-/-Apoc3-/-* mice injected with AdAPOE4. Plasma pools of 4 h fasted *Apoe-/-* (A,D), *Apoe-/-Apoc3+/-* (B,E), and *Apoe-/-Apoc3-/-* (C,F) mice, obtained before (○) and 5 days after injection of $2x10^9$ pfu AdAPOE4 (●), were subjected to FPLC. The separate lipoprotein fractions were assayed for triglyceride (A-C) and cholesterol (D-F) levels.

VLDL composition

Sera from 4 hours fasted *Apoe-/-* and *Apoe-/-.Apoc3-/-* mice, at day 5 after injection of $2x10^9$ pfu AdLacZ, $1x10^9$ pfu AdAPOE4 or $2x10^9$ pfu AdAPOE4 were pooled and VLDL was isolated via ultracentrifugation. The lipid composition and

protein content of the VLDL was determined (Table I). Strikingly, the TG level of VLDL in *Apoe-/-* and *Apoe-/-.Apoc3-/-* mice was unchanged after identical doses of AdAPOE4. At a high dose of $2x10^9$ pfu AdAPOE4 the VLDL in *Apoe-/-.Apoc3-/-* mice contained less CE in the particle and more PL on the surface of the VLDL than in Apoe-/- mice after equal treatment. This indicates that this VLDL particle is decreased in size, although this is a small effect. The total VLDL protein content in *Apoe-/-.Apoc3-/-* mice after $2x10^9$ pfu AdAPOE4 was 6-fold lower than in *Apoe-/-* mice, indicating that *Apoe-/-.Apoc3-/-* mice accumulate less VLDL in their blood circulation.

Apoe-/-	**TG**	**CE**	**FC**	**PL**	**protein**		***Apoe-/-.Apoc3-/-***	**TG**	**CE**	**FC**	**PL**	**protein**
AdLacZ($2x10^9$pfu)	12.7	40.6	25.9	20.8	0.06		**AdLacZ($2x10^9$pfu)**	6.1	54.4	11.9	27.6	0.05
AdAPOE4($1x10^9$pfu)	64.1	8.8	6.9	20.3	0.04		**AdAPOE4($1x10^9$pfu)**	67.6	7.1	6.5	18.8	0.02
AdAPOE4($2x10^9$pfu)	64.7	17.5	7.0	10.8	1.57		**AdAPOE4($2x10^9$pfu)**	66.4	11.3	4.0	18.3	0.26
The VLDL triglyceride (TG), cholesteryl ester (CE), free cholesterol (FC), and phospholipid (PL) content was measured as % of total w/v. VLDL protein was measured in mg/ml.												

Table I. VLDL composition in *Apoe-/-* and *Apoe-/-.Apoc3-/-* mice after adenovirus treatment.

Hepatic VLDL-TG secretion after injection of AdAPOE4

To evaluate whether absence of apoCIII prevents the apoE-induced hyperlipidemia by decreasing the VLDL production rate, we determined the hepatic VLDL-TG secretion rate. To this end, Triton WR 1339 was injected i.v. to block peripheral TG clearance, and the VLDL-TG secretion rate was calculated from the increase in plasma TG levels.

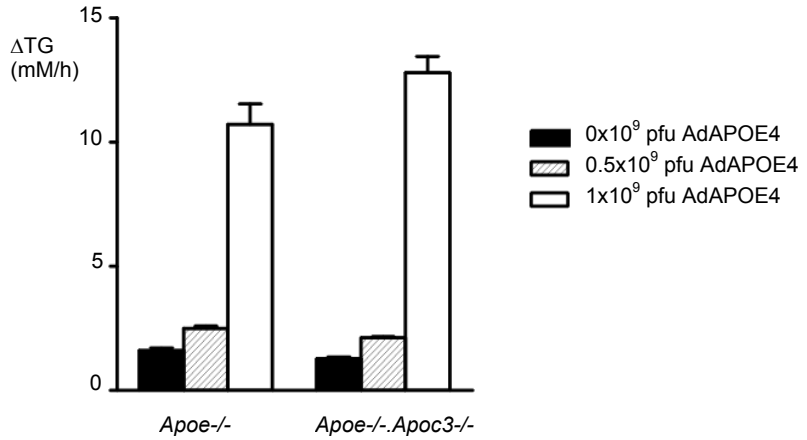

Figure 3. Hepatic VLDL-triglyceride production rate in *Apoe-/-* and *Apoe-/-Apoc3-/-* mice injected with AdAPOE4. *Apoe-/-* and *Apoe-/-Apoc3-/-* mice were injected with AdLacZ (black bars, indicated as $0x10^9$ pfu AdE4, n=4), $0.5x10^9$ pfu AdAPOE4 (dashed bars, n=4), or $1x10^9$ pfu AdAPOE4 (open bars, n=2). At day 5 after virus injection, 4 h fasted mice were injected with Triton WR 1339. The VLDL-TG production rate was determined as the increase in plasma TG concentration during 2 h after Triton injection. Values are represented as ΔTG in mM/h ± SD.

In both *Apoe-/-* and *Apoe-/-.Apoc3-/-* mice injected with control LacZ virus, the hepatic VLDL-TG secretion was low, as reported previously for *Apoe-/-* mice(9).

After AdAPOE4 injection, the VLDL-TG secretion rate increased dose-dependently, as evidenced by a more pronounced increase in plasma TG after Triton treatment. A dose of $0.5x10^9$ pfu AdAPOE4 increased the VLDL-TG production rate to that observed in wild-type mice(13), whereas a dose of $1x10^9$ pfu increased the VLDL-TG secretion rate approx. 10-fold (Fig 3). Since in both *Apoe-/-* and *Apoe-/-.Apoc3-/-* mice the VLDL-TG secretion rate was increased significantly and to a similar extent by high levels of apoE expression, it was concluded that *Apoc3*-deficiency does not affect the VLDL production rate.

Tissue distribution of VLDL-like emulsion-derived triglycerides *in vivo*

To investigate if *Apoc3*-deficiency leads to an enhanced rate of LPL-mediated VLDL-TG hydrolysis *in vivo*, *Apoe-/-* and *Apoe-/-.Apoc3-/-* mice were given bolus injections of [^{3}H]TO-labeled emulsion particles. To achieve a high availability of [^{3}H]TO-labeled particles to peripheral LPL, the mice were given a high dose of 1 mg TG that exceeded the endogenous plasma TG content of both groups of mice. The tissue-specific uptake of [^{3}H]oleate was determined at 10 minutes after bolus injection. No strain-specific differences were observed with respect to the uptake of [^{3}H]TO-derived activity by liver, heart, spleen, and skeletal muscle. In contrast, the uptake of ^{3}H-activity by white adipose tissue (WAT) was significantly higher in *Apoe-/-.Apoc3-/-* versus *Apoe-/-* mice, reaching statistical significance for gonadal WAT (3.2-fold; $p<0.05$) and intestinal WAT (2.4-fold; $p<0.05$) (Fig. 4).

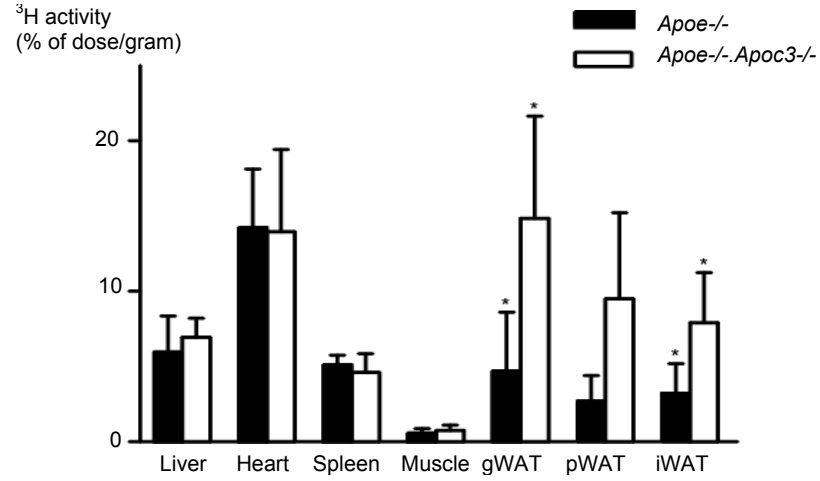

Figure 4. Tissue distribution of triglyceride-derived fatty acids from VLDL-like emulsion particles. Five *Apoe-/-* (black bars) and *Apoe-/-.Apoc3-/-* (open bars) mice were intravenously injected with glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles. At 10 min after bolus injection, the indicated organs were isolated, dissolved, and the uptake of [^{3}H]oleate was determined as % of the injected dose per gram wet tissue weight, represented as means ± SD. * $p<0.05$. gWAT, pWAT, and iWAT denote gonadal, perirenal, and intestinal white adipose tissue, respectively.

These data indicate that *Apoc3*-deficiency results in a more efficient peripheral LPL-mediated TG hydrolysis.

LPL-activity in presence of apoCIII or apoE

Both apoE and apoCIII have been shown to inhibit LPL-mediated TG-hydrolysis. To determine the inhibitory potency of apoCIII versus apoE, the activity of LPL-mediated TG-hydrolysis in the presence of varying amounts of apoCIII or apoE was determined *in vitro*.

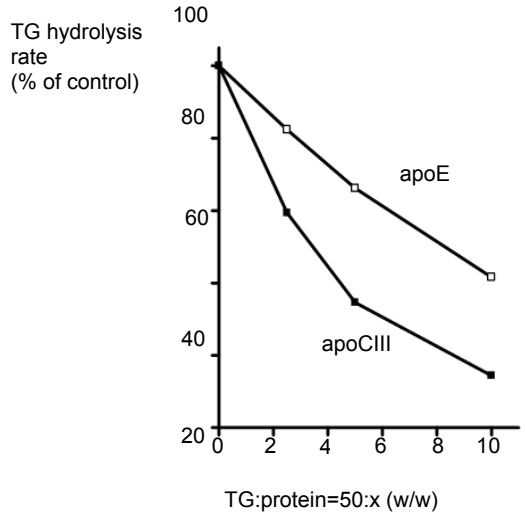

Figure 5. Effect of apoCIII and apoE on LPL-mediated triglyceride hydrolysis *in vitro*. Glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles were incubated (30 min at 37°C) with apoCIII (■) or apoE (□) at TG:protein = 50: 0, 2.5, 5, and 10 weight ratios. At *t*=0, LPL was added and [^{3}H]oleate was extracted as described. The reaction velocities in the presence of apoCIII and apoE were calculated as %[^{3}H]oleate release per min and expressed relative to control incubations (100%).

Pre-incubation of VLDL-like emulsion particles with apoCIII resulted in a dose-dependent inhibition of the release of [^{3}H]oleate from the emulsion-incorporated glycerol tri[^{3}H]oleate, reaching 86% inhibition at a TG: apoCIII ratio of 50:10 (w/w) (Fig 5). In agreement with previous observations(9), apoE also dose-dependently inhibited LPL activity. However, the inhibitory efficacy of apoCIII is higher than that of apoE.

Discussion

We and others have shown previously that high levels of adenovirus-mediated expression of apoE result in hyperlipidemia. This effect is due to a dramatic increase of the VLDL-TG production rate(12,13,18,29) and inhibition of LPL-mediated VLDL-TG-hydrolysis, caused by enrichment of VLDL with apoE(8,9). Our current data show that deletion of *Apoc3* gene-dose-dependently reduces the hyperlipidemia induced by high doses of AdAPOE4 (Fig 1). Neither the VLDL composition (table I) nor the apoE-induced increase in the VLDL-TG production rate was affected by the absence of apoCIII (Fig. 3). However, absence of apoCIII clearly increases the rate of peripheral TG-hydrolysis as indicated by an enhanced uptake of fatty acids from [^{3}H]TO by white adipose tissue (Fig. 4). These data indicate that the apoE-induced inhibition of VLDL-TG hydrolysis can be alleviated by elimination of endogenous apoCIII. To determine whether apoCIII is a more potent inhibitor of VLDL-TG hydrolysis than apoE, *in vitro* lipolysis experiments were performed (Fig 5). Indeed apoCIII seems a more potent inhibitor of VLDL-TG hydrolysis than apoE. Thus, we conclude that the absence of apoE-induced hyperlipidemia in *Apoe-/-.Apoc3-/-* mice is explained by the alleviation of the apoE-induced inhibition of VLDL-TG lipolysis.

The effect of apoCIII on VLDL production is somewhat controversial. Overexpression of APOC3 in transgenic mice has been associated with both an increased VLDL-TG secretion rate(30) and in a different transgenic line, with no change in the VLDL-TG secretion rate(31). Hirano *et al.*(32) have reported an increased VLDL-TG secretion rate in *Apoc3*-deficient mice. However, we have found that endogenous *Apoc3*-deficiency does not affect the VLDL-TG production rate neither in absence or presence of endogenous *Apoe*(15). Moreover, the apoE-

induced increase in the VLDL-TG secretion is also not affected by presence or absence of apoCIII (Fig. 3). Thus, although the discrepancy with respect to this effect of apoCIII on hepatic VLDL-TG production between various research groups remains to be resolved, we have consistently and repeatedly found no effect of *Apoc3*-deficiency on VLDL-TG production rates.

The VLDL particles circulating in the plasma of *Apoe-/-* and *Apoe-/-.Apoc3-/-* mice have a very similar TG content after treatment with AdAPOE4 (Table I). Also, there was no effect of *Apoc3*-deficiency on the composition of nascent VLDL isolated after triton injection in mice treated with $5x10^8$ or $1x10^9$ pfu AdAPOE4 (data not shown). Importantly, the VLDL protein content after injection of $2x10^9$ pfu AdAPOE4 is 6-fold higher in *Apoe-/-* mice as compared to *Apoe-/-.Apoc3-/-* mice. This indicates that *Apoc3*-deficiency prevents the accumulation of APOE-rich VLDL particles in the circulation (Table I, (13)).

It has been demonstrated that apoE dose-dependently inhibits LPL-mediated TG-hydrolysis *in vitro*(8,9,13). In the *Apoe-/-* mice injected with a high dose of AdAPOE4, it is more than likely that circulating apoE-rich particles are poor LPL substrates, and thereby remain very TG-rich. This is also supported by the positive correlation between plasma apoE levels and plasma TG levels in both transgenic mice and in humans(10,33). The mechanism underlying the inhibition of LPL by excess apoE has been proposed to be displacement of apoCII from the particle, which is an essential cofactor of LPL(10). However, our current data indicate that apoCII may not be the rate-limiting factor. It seems likely that nascent VLDL produced in apoE-overexpressing *Apoe-/-* and *Apoe-/-.Apoc3-/-* mice is equally rich in apoE and thus that the exclusion pressure for apoCII will be equal on particles from both mouse lines. Nevertheless, the hypolipidemic phenotype of *Apoe-/-.Apoc3-/-* mice injected with a high dose of AdAPOE4 is illustrative for an efficient TG-hydrolysis and clearance of subsequently formed remnants. Thus in the absence of apoCIII, the apoCII particle level does not seem to be limiting for efficient LPL-mediated TG-hydrolysis.

The mechanism responsible for LPL inhibition by apoCIII is similarly not known. It has been proposed that apoCIII acts as a direct non-competitive inhibitor of LPL(34). However, attempts to identify the domain in the apoCIII protein responsible for direct LPL inhibition have yielded conflicting results(6,7). Whether apoCIII affects

LPL activity via direct protein-protein interaction (with apoCII and/or LPL), or indirectly by for example inhibiting the binding of TG-rich particles to endothelial surfaces where LPL is active remains to be determined. If the interaction of apoCIII with LPL is non-specific or based on steric hindrance, then the size of the protein and the occupation of the VLDL particle surface would determine the inhibitory effect on LPL. We incubated VLDL-like emulsion particles with equal amounts of apoCIII and apoE, based on TG to apolipoprotein weight/weight ratio (Fig.5). At equal weight/weight ratio and thus presumably equal apolipoprotein occupation of the particle surface, apoCIII inhibits LPL-mediated TG hydrolysis to a greater extent than apoE. This indicates that apoCIII is indeed a specific and efficient inhibitor of LPL *in vitro*. Interestingly, the absence of an effect on plasma TG levels even after significant overexpression of apoE in *Apoe-/-Apoc3-/-* mice (Fig.1) could also indicate that apoCIII and excess apoE act in a supra additive manner to inhibit LPL-mediated TG hydrolysis *in vivo*.

The mechanism underlying the enhanced VLDL-TG clearance by *Apoc3*-deficiency *in-vivo* was investigated by injecting the mice with radio labeled TG-rich emulsion particles. Previously, it has been shown that these emulsion particles rapidly acquire apolipoproteins in the blood circulation. Also, these TG-rich emulsion particles are processed by LPL and cleared by hepatic lipoprotein receptors similar to nascent chylomicron and VLDL particles(9,27). The size of the emulsion particles was similar to that reported for nascent VLDL particles(14). In a previous study, we have specifically focused on the plasma kinetics of VLDL-TG in *Apoe-/-* and *Apoe-/-.Apoc3-/-* mice(15). Mice were given an intravenous bolus injection with glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles which contained 150 µg TG. This resulted in a significantly faster decay of label from plasma due to *Apoc3*-deficiency. In the present study, we focused on the effect of *Apoc3*-deficiency on local LPL activity in the periphery. To achieve an optimal availability of LPL substrate, mice were given a bolus injection of glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles which contained a total of 1 mg TG (Fig. 4). Due to the excess of TG that was administered, the plasma levels of the label remained very high in both *Apoe-/-.Apoc3-/-* and *Apoe-/-* mice (data not shown). However, the increased [^{3}H]oleate uptake by white adipose tissues clearly indicated a higher local LPL activity in *Apoe-*

/-.Apoc3-/- mice. Thus, *Apoc3*-deficiency results in both an enhanced clearance of VLDL-TG from the plasma and subsequent uptake by adipose tissue.

ApoE and TG-rich VLDL particles are poor substrates for receptor-mediated hepatic clearance. After processing by LPL, the VLDL particles will become smaller, thereby increasing the affinity of apoE for hepatic clearance receptors(28). We have shown that *Apoc3*-deficiency stimulates the LPL-mediated TG hydrolysis, thereby stimulating the remnant formation. This might lead to a faster hepatic clearance of these particles, accompanied by a higher rate of apoE-mediated remnant clearance. This was confirmed by the lower circulating apoE levels in *Apoe-/-Apoc3-/-* mice as compared to *Apoe-/-* mice after equal treatment with AdAPOE4. Also, the VLDL-cholesterol levels were low in *Apoe-/-Apoc3-/-* mice after AdAPOE4 treatment, without increasing the LDL-cholesterol levels, indicating enhanced clearance of VLDL-remnants. However, from our current analyses we can not exclude the possibility that apoCIII influences the hepatic clearance of VLDL remnants directly(35).

Numerous studies have reported associations between plasma apoCIII levels, plasma TG levels, and premature cardiovascular disease, both in healthy and patient populations(1,36,37,38,39). Our current results demonstrate that *Apoc3*-deficiency has the potential to alleviate the hyperlipidemia associated with a 10-fold increased VLDL-TG production rate. Since peripheral uptake of fatty acids is increased by *Apoc3*-deficiency we conclude that the processing of VLDL into remnant particles is stimulated, which leads to a faster remnant particle clearance by the liver. These data thus confirm the notion from human association studies that apoCIII is a potentially powerful molecule in modulating plasma TG levels and thus in modulating predisposition to cardiovascular disease.

Acknowledgements

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Chapter 4

ApoE2-associated hyperlipidemia is amelio-rated by increased levels of apoAV, but unaffected by apoCIII-deficiency.

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Submitted

Abstract

ApoE2-associated hyperlipidemia is characterized by a disturbed clearance of apoE2-enriched VLDL-remnants. Since excess apoE2 inhibits lipoprotein lipase (LPL)-mediated triglyceride (TG)-hydrolysis *in-vitro*, we investigated whether direct or indirect stimulation of LPL activity *in-vivo* reduces the apoE2-associated hyperlipidemia. Hereto, we studied the role of LPL and two potent modifiers, the LPL-inhibitor apoCIII and the LPL-activator apoAV in *APOE2*-knockin (APOE2) mice.

Injection of heparin in APOE2 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 apoE2-enriched particles can serve as substrate for LPL. Indirect activation of LPL activity via deletion of apoCIII in APOE2 mice did neither affect plasma TG nor TC levels, whereas overexpression of *Apoa5* did reduce plasma TG by 81% and plasma TC by 41%.

In conclusion, the combined hyperlipidemia in APOE2 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 APOE2 mice. These data indicate that changes in apoAV levels have a dominant effect over changes in apoCIII levels in the improvement of APOE2-associated hyperlipidemia.

Introduction

ApoE2-associated hyperlipidemia is characterized by increased plasma levels of chylomicron and VLDL remnants and is associated with xanthomatosis and premature atherosclerosis(1). ApoE2 has a single aminoacid substitution (Arg158→Cys) as compared with the common apoE3 variant, resulting in a low binding affinity for the LDLR(2),(3). *In-vivo,* this is associated with impaired hepatic clearance of VLDL and chylomicron remnant particles(4), resulting in increased plasma TG and TC levels. Simultaneously, apoE2 accumulates in plasma leading to an increase in apoE-mediated inhibition of LPL-mediated TG hydrolysis(5). It has

been postulated that both impaired remnant clearance and impaired remnant generation via lipolysis contribute to the hyperlipidemia associated with apoE2(5).

We and others have found that VLDL obtained from hyperlipidemic patients homozygous for APOE2 is a relatively poor substrate for LPL-mediated lipolysis(6). Two potent modifiers of LPL activity have been described, apoAV and apoCIII, that are encoded in same gene cluster on chromosome 11(7). *In-vitro* and *in-vivo* mouse studies indicate that apoAV stimulates LPL-mediated TG hydrolysis and that apoCIII inhibits this process(8,9,10,11,12). Overexpression of apoAV in mice reduces plasma TG levels via stimulation of LPL activity(13) and overexpression of apoCIII results in increased plasma TG levels via inhibition of LPL(14). Studies in *Apoc3*-knockout mice show accelerated LPL-mediated TG hydrolysis(15,16). Deficiency in apoAV in both mice and humans is associated with hypertriglyceridemia(17,18,19).

In the present study, we have investigated the role of LPL-mediated TG-hydrolysis in apoE2 associated hyperlipidemia *in-vivo*. Direct stimulation of LPL activity in APOE2 knockin (APOE2) 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 APOE2 associated hyperlipidemia.

Methods

Adenoviral constructs

The adenoviral vector expressing active LPL (AdLPL) was kindly provided by Dr. Santamarina-Fojo(20). The generation of the adenoviral vectors expressing apoAV (Ad*Apoa5*), the control empty vector (AdEmpty) and β-galactosidase (AdLacZ) have been described(13,8). Expansion, purification and titration of the adenoviral vectors were performed as described previously(21). Before *in-vivo*

administration, the adenoviral vectors were diluted to a dose of $5x10^8$ pfu in 200 μl sterile PBS.

Mouse models

APOE2 knockin mice, carrying the human APOE2 gene in place of the mouse *Apoe* gene have been described previously(22). These mice were backcrossed 8 times with C57BL/6 mice to achieve a more homogenous genetic background and subsequently intercrossed to obtain homozygous APOE2 mice. *Apoc3-/-* mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and intercrossed with APOE2 mice to obtain APOE2, APOE2.*Apoc3+/-* and APOE2.*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 APOE2 mice at the age of 13-18 weeks were selected for injection with AdLPL. A dose of $5x10^8$ 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 capillairy tubes (Hawksley, Sussex, England).

Female APOE2 mice between the age of 13 and 18 weeks were injected with a dose of $5x10^8$ pfu of Ad-*apoa5* or $5x10^8$ of empty vector (AdEmpty). Three hours prior to this virus injection, the mice were injected with $5x10^8$ pfu AdLacZ to saturate the uptake of viral particles by hepatic Kupffer cells(23). 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(24). The circulating human apoE level in homozygous APOE2 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, AKTA-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 APOE2 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 APOE2, APOE2.*Apoc3+/-* and APOE2.*Apoc3-/-* mice aged 13 to 20 weeks. The mice were fasted overnight 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 was determined over the period of 6 h.

Statistical analysis

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

Results

Effect of increased LPL activity on lipid levels in APOE2 mice

I.V. injection of heparin results in activation of LPL and its release from the endothelial surfaces. Stimulation of LPL activity in APOE2 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 mice decreased 28% ($p<0.05$, n=4).

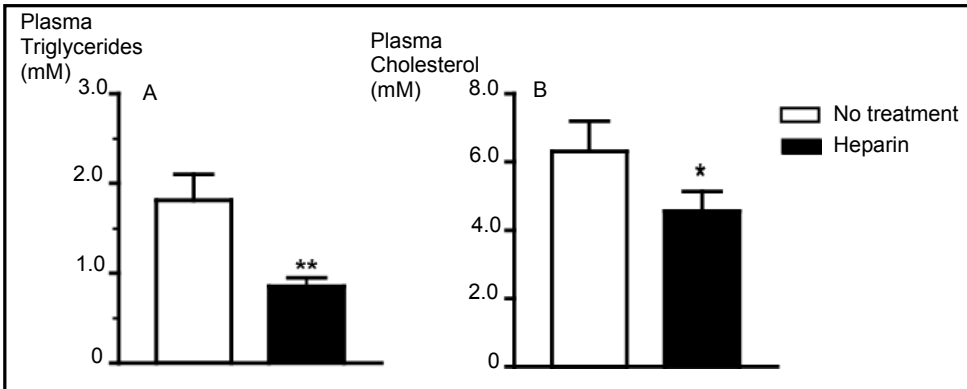

Figure 1. Plasma lipid levels of APOE2 mice after heparin treatment. Fasted APOE2 mice were injected with heparin. Before (open bars) and 1 hour after injection (black bars), plasma samples were obtained and assayed for triglyceride (A) and cholesterol (B). The values are represented as means ± SD for n=4 mice per group. * $p<0.05$, ** $p<0.005$.

APOE2 mice were injected with adenovirus expressing LPL to determine the effect on hyperlipidemia (fig 2). At day 5 after injection of $5x10^8$ pfu AdLPL, APOE2 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 APOE2-containing VLDL particles by overexpression of LPL (data not shown).

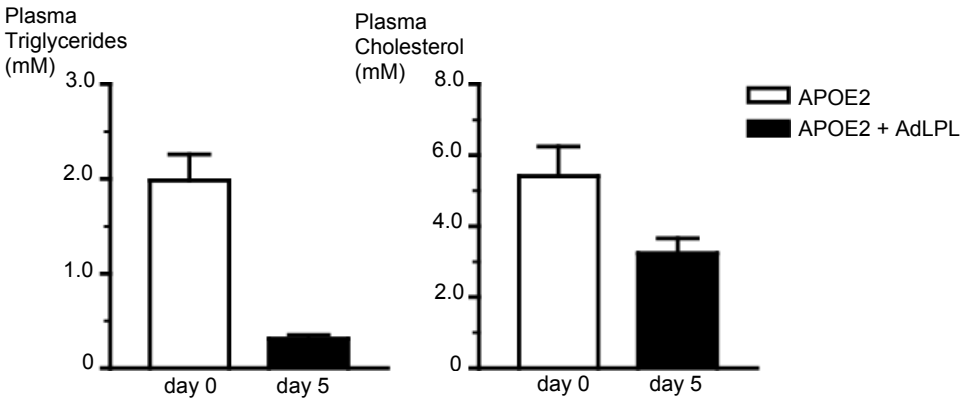

Figure 2. Plasma lipid levels of APOE2 mice injected with AdLPL. APOE2 mice were injected with $5x10^8$ pfu AdLPL. Before (open bars) and at day 5 after adenovirus injection (black bars), fasted plasma samples were assayed for triglyceride (A) and cholesterol (B). Values are represented as means ± SD for n=3 mice per group.

Effect of apoCIII-deficiency on lipid levels in APOE2 mice

The main endogenous inhibitor of LPL, apoCIII, was deleted from the genetic background of APOE2 mice by crossbreeding with *Apoc3* knockout mice. The effect of *Apoc3*-deficiency on APOE2-associated hyperlipidemia was investigated in APOE2 mice heterozygous or homozygous deficient for the endogenous *Apoc3* gene (fig 3). Surprisingly, the plasma TG levels were not different between APOE2, APOE2.*apoc3*+/- and APOE2.*apoc3*-/- mice. Also, the TC levels were not affected by *Apoc3*-deficiency in the presence of APOE2. No differences in plasma lipid levels were found between male and female mice (data no shown). The distribution of TG and TC over the lipoprotein fractions was measured after separation via FPLC. No differences were observed between APOE2, APOE2.*apoc3*+/- and APOE2.*apoc3*-/- mice (data not shown).

To further analyse the effect of apoCIII-deficiency in APOE2 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 APOE2 carrying mice was not different (APOE2 AUC 5.8; APOE2.*apoc3*+/- AUC 5.5 and APOE2.*apoc3*-/- mice AUC 4.3 mM/6 h, n.s. for n=5 mice per group, data not shown).

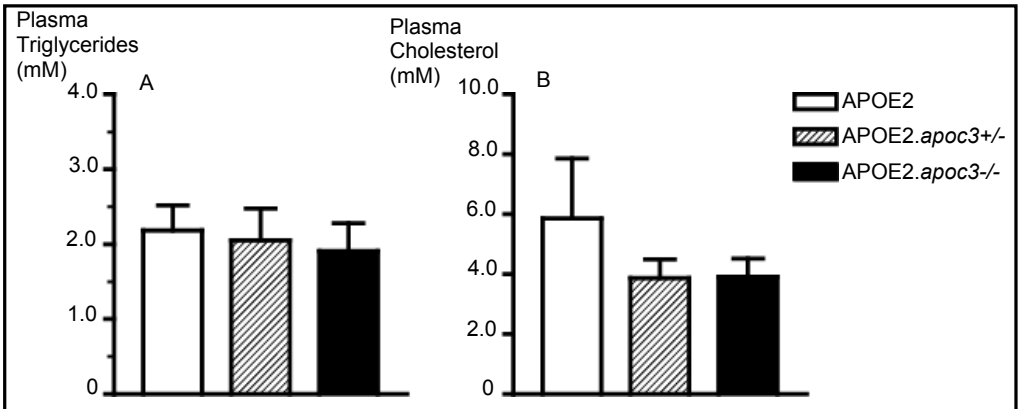

Figure 3. Plasma lipid levels of APOE2 mice deficient for *apoc3*. Fasted plasma samples were obtained from APOE2 mice (open bars), APOE2.*apoc3*+/- mice (hatched bars) and APOE2.*apoc3*-/- mice (black bars). The samples were assayed for triglycerides (A) and cholesterol(B). Values are represented as mean ± SD for n=5 mice per group .

Effect of adenovirus-mediated expression of *Apoa5* on lipid levels in APOE2 mice.

The activator of LPL, apoAV, was expressed in APOE2 mice via a recombinant adenoviral vector. Injection of a moderate dose of Ad*Apoa5* ($5x10^8$ 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).

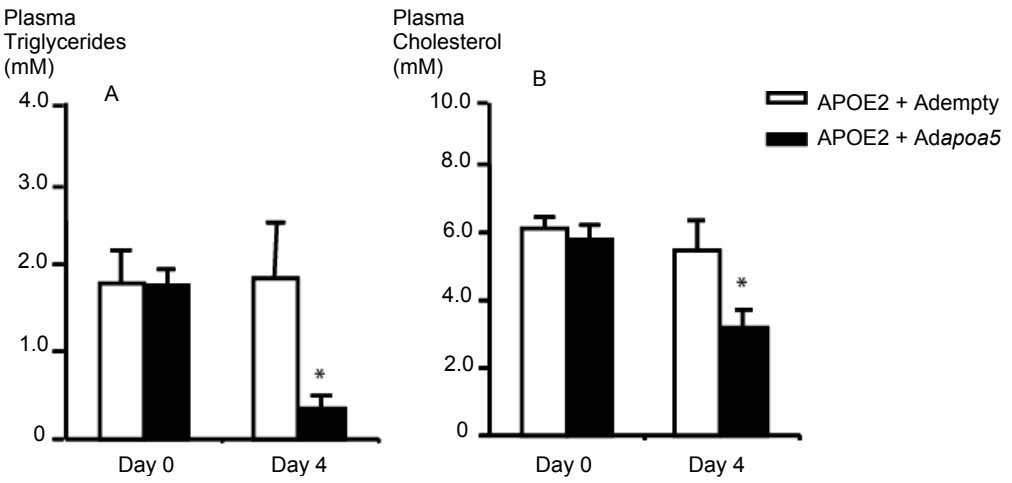

Figure 4. Plasma lipid levels of APOE2 mice injected with Ad*apo*A5. APOE2 mice (n=5 per group) were injected consecutively with AdLacZ ($5x10^8$ pfu) and Ad*apoa5* or AdEmpty ($5x10^8$ pfu). Before injection (□) and at 4 days after injection (■), fasted plasma was collected from the individual mice and assayed for triglyceride (A) and cholesterol (B). Values are represented as means ± SD. * $p<0.05$.

Discussion

In the current study, we have addressed the hypothesis that alleviating the apoE2-mediated inhibition of lipolysis can reduce the apoE2-associated hyperlipidemia. Using the APOE2 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 APOE2 mice (fig 1). Likewise, injection of adenovirus expressing LPL in APOE2 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. *Apoa5* overexpression did reduce the APOE2-associated hyperlipidemia in APOE2 knock-in mice (fig 4). In contrast, the APOE2-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 APOE2 associated hyperlipidemia. The indirect stimulation of LPL activity via apoAV overexpression but not apoCIII-deficiency ameliorates the APOE2-associated hyperlipidemia. We conclude that apoAV is apparently dominant over apoCIII in the improvement of APOE2-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(25,26,27). This can at least partially explain the hypertriglyceridemia that is found in APOE2-associated 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 apoE2-rich lipoprotein particles(5). However, this is difficult to reconcile with the observation that indirect stimulation of LPL activity via apoAV overexpression ameliorates the APOE2-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 apoE2 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 sulphate proteoglycans (HSPG) via apoE(28). It has been shown that apoE2 is partly defective in the association with HSPG(29) and this could also explain part of the apoE2-associated hypertriglyceridemia. In agreement with this hypothesis, it has been found *in-vitro* that VLDL obtained from APOE2 homozygous FD patients is effectively lipolysed by LPL in solution, but poorly lipolysed by HSPG-bound LPL(6). Thus, apoE2-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 apoE2-associated hyperlipidemia. Intriguingly, this explanation is also in line with the observation that additional apoAV rescues the apoE2-associated hyperlipidemia. It has recently been found that the LPL-activating effect of apoAV involves enhanced binding to HSPG(10,30). Thus additional apoAV on the TG-rich particle apparently overcomes the apoE2-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(14,31). 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 apoE2-containing lipoproteins to the liver via an LPL-mediated bridging effect(32). 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(16). This hyperlipidemia is caused by an apoE4-induced increase in VLDL-production and simultaneous apoE4-mediated inhibition of VLDL-TG lipolysis(33). 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(16). To our surprise, *Apoc3*-deficiency did not affect the hyperlipidemia or lipoprotein lipid distribution (data not shown) in APOE2 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 APOE2 mice on *Apoc3* deficient or wild type backgrounds. The absence of a hypolipidemic effect of *Apoc3*-deficiency in APOE2 mice indicates that the defect in APOE2-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 APOE2 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(8), whereas others have found no effects of apoAV on VLDL production in neither APOA5 transgenic mice(34) nor in *apoa5-/-* mice(17). Intriguingly, in the APOE2 mice, we did not observe differences in the VLDL-TG secretion rate between Ad*Apoa5* 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(35,19,36,37,38,39). 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(7,34). Our current data clearly indicate that apoAV and apoCIII affect different steps in the conversion of TG rich lipoproteins to remnants. Moreover, within the context of APOE2-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

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Chapter 5

The role of apoE in LDL receptor related protein-mediated lipid metabolism.

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Abstract

Here, we have investigated the role of apoE in LDL-receptor related protein (LRP) mediated lipoprotein metabolism. To this end, we further characterized mice deficient for the hepatic LRP on a LDL receptor and apoE double deficient background (*LRP-/-.Ldlr-/-.Apoe-/-* and *Ldlr-/-.Apoe-/-* mice) and used adenovirus mediate gene transfer to re-introduce apoE. In the absence of the LDL receptor and apoE, deletion of the hepatic LRP resulted in a paradoxical decrease of plasma cholesterol (TC) and triglyceride (TG) levels. Since the LRP can function as a clearance receptor for lipoprotein lipase (LPL), the tissue-specific LPL activity was investigated using TG-rich particles labelled in the FA-moiety. This experiment showed a decreased rate of FA uptake by white adipose tissue of *Ldlr-/-.Apoe-/-* mice as compared to *LRP-/-.Ldlr-/-.Apoe-/-* mice, indicative of locally decreased lipolysis. Thus in absence of apoE and the LDL receptor, the hepatic LRP functions as a LPL clearance receptor and down-regulates peripheral LPL activity. Re-introduction of apoE in *Ldlr-/-.Apoe-/-* mice resulted in a significant hypertriglyceridemia but did not affect the plasma TC level. Overexpression of apoE in *LRP-/-.Ldlr-/-.Apoe-/-* mice resulted in an aggravated and significant further increase in both plasma TG and TC levels as compared to *Ldlr-/-.Apoe-/-* mice. These data indicate that the hepatic LRP prevents part of the apoE-induced hyperlipidemia and thus functions as an apoE-dependent lipoprotein receptor. Thus, in the absence of the LDL receptor and apoE, the LRP clearly functions as a regulator of LPL activity, whereas apoE expression unveals the function of LRP as a lipoprotein receptor.

Introduction

The LRP is a cell-surface receptor that is mainly expressed in hepatocytes, monocytes and smooth muscle cells(1). In the liver, the LRP functions as a clearance receptor for a large variety of ligands in the circulation, including LPL, HL and several factors that play a role in the coagulation cascade(2,3,4,5,6,7,8,9). The most prominent role of the LRP in lipoprotein metabolism is the removal of lipoprotein remnants(10). *In-vitro*, the LRP binds apoE-rich chylomicron remnants(11) and

internalizes these particles via receptor-mediated endocytosis (12). In the presence of the LDL receptor, the LRP has a relatively minor contribution to the hepatic uptake of chylomicron and VLDL remnants(13,14). However, when LDL receptor expression is decreased, the LRP prevents the accumulation of remnant lipoproteins(15,16,17) and thus plays an important role as a backup lipoprotein receptor. In addition, the LRP can mediate cell surface clustering and hepatic sequestration of chylomicron remnants (18).

We have recently found that atherogenesis in mice deficient for apoE and the LDL receptor is aggravated by the absence of the hepatic LRP(19). Paradoxically, deletion of the hepatic LRP from *Ldlr-/-.Apoe-/-* mice was associated with a decrease in plasma lipid levels and thus the effect of the LRP on atherosclerosis was independent of the effect of the LRP on lipid metabolism. The pro-atherogenic effect of the LRP was explained by the plasma accumulation of pro-atherogenic ligands such as coagulation factor VIII, von Willebrand factor, and tissue-type plasminogen activator(19). The hypolipidemic effect of the LRP was explained by increased plasma levels of LPL in the circulation. Surprisingly, this was not accompanied by increased post-heparin LPL activity.

In the current study we have further investigated the role of the LRP in relation to apoE in lipid metabolism. We have addressed the functional consequences of hepatic LRP absence on LPL activity in peripheral tissues and find that this activity is increased in *LRP-/-.Ldlr-/-.Apoe-/-* mice as compared to *Ldlr-/-.Apoe-/-* mice. These data show that the LRP can regulate the circulating level and peripheral activity of LPL. Re-introduction of apoE in *LRP-/-.Ldlr-/-.Apoe-/-* and *Ldlr-/-.Apoe-/-* mice via adenovirus mediated gene transfer illustrated the role of the hepatic LRP as a hepatic apoE-dependent backup lipoprotein receptor. Thus, the LRP plays a complex role in lipoprotein metabolism by affecting LPL level and activity and by functioning as an apoE-dependent lipoprotein receptor.

Methods

Mice

Mx1-CRE.*LRP*$^{lox/lox}$*.Ldlr-/-.Apoe-/-* mice and *LRP*$^{lox/lox}$*.Ldlr-/-.Apoe-/-* littermates were obtained as descibed(19). To induce LRP-deficiency in the liver, the mice were 3 times intra-peritoneal injected with pI:pC (total 750μg, Sigma, St. Louis, MO), with an interval of one day. These mice are indicated as *LRP-/-.Ldlr-/-.Apoe-/-* mice. The control group that lacks the Mx1-CRE transgene (indicated as *Ldlr-/-.Apoe-/-* mice) was also treated with pI:pC. At least 10 days after pI:pC treatment, the mice were used for experiments, at an age of 4 to 6 months. 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 transfection, 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.

Plasma lipid and lipoprotein analysis

Blood samples for lipid determinations were drawn form the tail vein of mice after 4 hours fasting. Plasma TG and TC levels were measured enzymatically using commercially available kits (Sigma, St. Louis, MO). Plasma apoE levels were measured by sandwich ELISA as described(20). Lipoprotein fractions were separated using fast protein liquid chromatography (FPLC). Hereto, a plasma pool of each group of mice was diluted 5 times in PBS and 50 μL was injected onto a Superose 6 column (3.2 x 30 mm, AKTA-system, Pharmacia, Uppsala, Sweden). Elution fractions of 50 μL were collected for lipid determinations.

Tissue distribution of VLDL-like emulsion-derived fatty acids *in-vivo*

80 nm sized emulsion particles were prepared as described(21). The TG content of the emulsions was determined with the Roche Molecular Biochemicals enzymatic kit for triacylglycerols. Fed *Ldlr-/-.Apoe-/-* and *LRP-/-.Ldlr-/-.Apoe-/-* mice were anesthetized with a mixture of vetranquil/dormicum/fentanyl (6.25/6.25/0.3125 mg/kg mouse) and their abdomens were opened. 200 μL of [^{3}H]TO-labeled VLDL-like emulsion particles was administered via the vena cava inferior at a dose (2.0 mg of

TG) that exceeded the endogenous plasma TG content in both experimental groups. At 10 min after injection of the emulsion particles, the liver, heart, spleen, and aliquots of hindlimb muscle, gonadal white adipose tissue (gWAT), perirenal white adipose tissue (pWAT) and intestinal white adipose tissue (iWAT) were isolated, dissolved in Soluene (Perkin Elmer) at 60°C and counted in 10 mL of Ultima Gold. The ^{3}H-activity in the tissues was corrected for wet organ weight.

Adenovirus-mediated expression of APOE4

The recombinant adenoviral vector, expressing the human APOE4 gene and the green fluorescent protein (GFP) under control of a cytomegalovirus (CMV)-promoter (AdAPOE4) was generated as described(22). A LacZ expressing recombinant adenovirus (AdLacZ) was used for control virus treatments. The recombinant adenoviruses were propagated in the human embryonic retina cell line 911 and/or human embryonic kidney cell line 293 as described(23,24). The viruses were purified via ultracentrifugation in a CsCl gradient, followed by dialysis and titration(25).

For *in-vivo* administration, $5x10^{8}$ plaque-forming units (pfu) AdAPOE4, adjusted to 200 µL with sterile PBS, was injected into the tail vein of female mice. To achieve a linear dose-response of AdAPOE4 virus by saturating the uptake of virus particles by Kupffer cells(26), all mice were preinjected with $5x10^{8}$ pfu AdLacZ at 3 h before injection of AdAPOE4. Two days before and 5 days after adenovirus injection, blood samples of approximately 50 µL were drawn from the tail vein of 4 h-fasted mice for lipid determinations.

Statistical Analysis

Data were analyzed using the non-parametric Mann-Whitney test. In case a serie of data was obtained from the same group of mice, the paired t-test was used. P-values less than 0.05 were regarded as statistically significant.

Results

Plasma lipid and lipoprotein levels

Plasma lipid levels were determined in and *Ldlr-/-.Apoe-/-* and *LRP-/-.Ldlr-/-.Apoe-/-* mice. In line with previous data, the plasma lipid levels were significantly lower in mice lacking the hepatic LRP(19). The plasma TG levels decreased 29% and the TC levels 33% due to LRP deficiency, $p<0.05$ (fig 1). The lipid lowering effect of LRP-deficiency was mainly confined to the VLDL-sized lipoprotein fraction, but also to LDL sized lipoproteins. No changes were observed in the HDL sized fractions (data not shown).

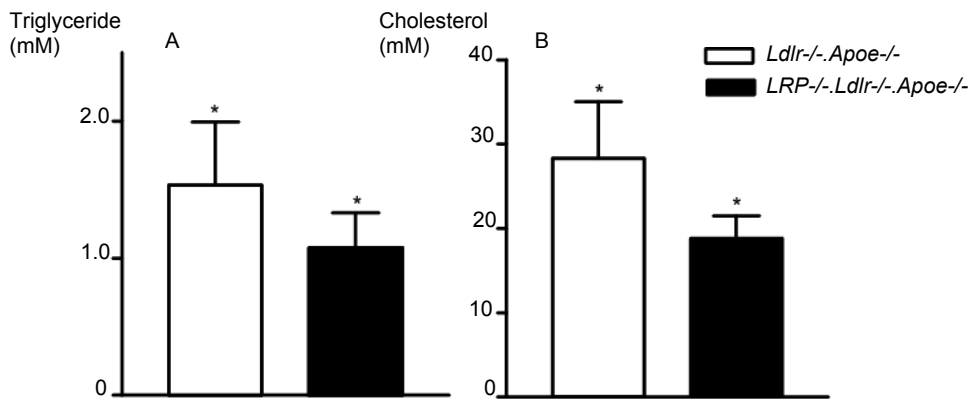

Figure 1. Plasma lipid levels in *Ldlr-/-.Apoe-/-* and *LRP-/-.Ldlr-/-.Apoe-/-* mice. Fasted plasma samples from *Ldlr-/-.Apoe-/-* (open bars) and *LRP-/-.Ldlr-/-.Apoe-/-* mice (black bars), n=7 mice per group, were analyzed for triglyceride (A) and cholesterol (B). Values are represented as means ± SD. * indicates $p<0.05$.

Tissue distribution of emulsion-derived triglycerides in-vivo

As explanation for the decrease in plasma TG levels upon deletion of LRP, the accumulation of LPL in circulation has been put forward(19). To analyze the effect of LRP-deficiency on LPL activity, mice were given a bolus injection of [^{3}H]triolein-labeled VLDL-like emulsion particles and the tissue-specific uptake of [^{3}H]FA was determined. The plasma decay of the label was not affected by LRP-deficiency in *Ldlr-/-.Apoe-/-* mice. Also, no significant differences were observed with respect to

the uptake of [^{3}H]triolein-derived activity by liver, heart, spleen and skeletal muscle. However, the uptake of ^{3}H-activity by white adipose tissue (WAT) was elevated in *LRP-/-.Ldlr-/-.Apoe-/-.* versus *Ldlr-/-.Apoe-/-* mice, reaching statistical significance for perirenal WAT (4.5-fold; *P*<0.05) and intestinal WAT (2.5-fold; *P*<0.05), (fig 2). These data indicate that hepatic LRP-deficiency on the *Ldlr-/-.Apoe-/-* background leads to a higher peripheral LPL activity in white adipose tissue.

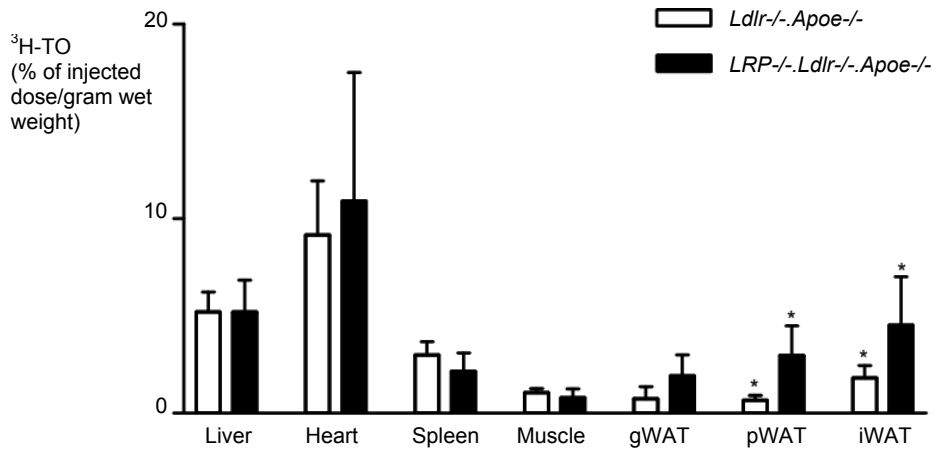

Figure 2. Tissue distribution of VLDL-like emulsion particles derived fatty acids. Fed *Ldlr-/-.Apoe-/-* (open bars, n=5) and *LRP-/-.Ldlr-/-.Apoe-/-* (black bars, n=5) mice received an intravenous bolus of [^{3}H]triolein-labeled emulsion particles (2.0 mg of TG). At 10 min after injection, mice were killed and the tissue distribution of ^{3}H radioactivity was determined. All of the values are represented as means ± S.D. * indicates p < 0.05. *gWAT, pWAT*, and *iWAT* denote gonadal, perirenal, and intestinal white adipose tissue, respectively.

Plasma lipid and lipoprotein levels after overexpression of AdAPOE4

To investigate the role of apoE in the LRP-mediated lipoprotein metabolism, *Ldlr-/-.Apoe-/-* and *LRP-/-.Ldlr-/-.Apoe-/-* mice were injected with adenovirus vectors expressing APOE4 (AdAPOE4). Plasma TG, TC and apoE levels were measured before and 5 days after virus injection. Administration of a dose of $5x10^8$ pfu AdAPOE4 in *Ldlr-/-.Apoe-/-* mice resulted in an 24-fold increased plasma TG levels,

p<0.05 (fig 3A), but did not affect TC levels (fig 3B). In *LRP-/-.Ldlr-/-.Apoe-/-* mice, the TG levels increased 65-fold (p<0.05) and the TC levels increased 2.2-fold (p<0.05). Also, the apoE levels were 1.9-fold (p<0.05, fig 3C) higher in *LRP-/-.Ldlr-/-.Apoe-/-* as compared to *Ldlr-/-.Apoe-/-* mice after identical injection of AdAPOE4 virus, indicating a slower clearance of apoE from plasma in absence of LRP.

The contribution of individual lipoprotein classes to the changes in total plasma lipid levels was determined after separation on a FPLC. The increase in plasma lipid levels was mainly confined to VLDL sized lipoproteins for both TG and TC levels (data not shown).

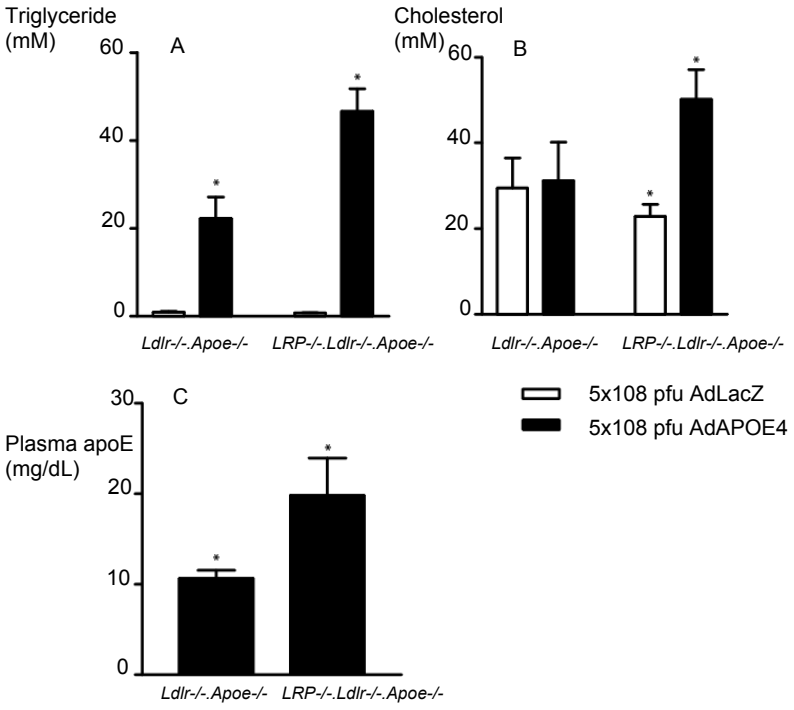

Figure 3. Effect of AdAPOE4 expression on plasma lipid levels. *Ldlr-/-.Apoe-/-* (n=4 mice per group) and *LRP-/-.Ldlr-/-.Apoe-/-* (n=4 mice per group), were injected with $5x10^8$ pfu (black bars) or $5x10^8$ pfu AdLacZ as control virus treatment (open bars). At day 5 after virus injection, fasted plasma samples were analyzed for triglyceride (A) and cholesterol (B), and the AdAPOE4 injected mice were analysed for plasma apoE concentration (C). Values are represented as means ± SD. * indicates p<0.05.

Discussion

In the absence of the LDL receptor and apoE, deletion of LRP paradoxically reduces plasma lipid levels in mice(19). This is accompanied by an increase in circulating LPL mass, but not in total heparin-releasable LPL-activity. We further investigated the TG metabolism in these mice. In line with previously reported data(19), the plasma TG and TC levels were significantly lower in mice lacking the LRP on the *Ldlr-/-.Apoe-/-* background (fig 1). We hypothesized that the difference in plasma lipid levels between *Ldlr-/-.Apoe-/-* and *LRP-/-.Ldlr-/-.Apoe-/-* mice is not caused by a change in total LPL activity, but by a change in local, tissue-specific LPL activity. This was addressed using TG-rich emulsion particles labeled in TG moiety with [^{3}H]-triolein. A significantly higher local uptake of fatty acids in white adipose tissue was indeed observed in *LRP-/-.Ldlr-/-.Apoe-/-* mice as compared to *Ldlr-/-.Apoe-/-* mice, indicating a higher local activity of LPL (fig 2). Analysis of the plasma TG increase in response to a post-prandial lipid load (olive oil gavage), similarly indicated enhanced LPL activity in the absence of the hepatic LRP (data not shown).

The role of apoE in LRP-mediated lipoprotein metabolism was investigated by injecting a moderate dose of adenovirus expressing APOE4 in *Ldlr-/-.Apoe-/-* and *LRP-/-.Ldlr-/-.Apoe-/-* mice (fig 3). Compared to the effect of AdLacZ, AdAPOE4 induced an increase in TG levels in both *Ldlr-/-.Apoe-/-* mice as well as *LRP-/-.Ldlr-/-.Apoe-/-* mice. AdAPOE4 did not affect TC levels in *Ldlr-/-.Apoe-/-* mice, whereas the TC levels in *LRP-/-.Ldlr-/-.Apoe-/-* mice were increased. Comparing the effect of AdAPOE4 on lipid levels in the *Ldlr-/-.Apoe-/-* mice and the *LRP-/-.Ldlr-/-.Apoe-/-* mice, it is obvious that the apoE-induced hyperlipidemia is more severe in *LRP-/-.Ldlr-/-.Apoe-/-* mice, indicating that the LRP functions as lipoprotein receptor. Thus, the LRP plays a complex role in lipoprotein metabolism by affecting LPL level and activity and by functioning as an apoE-dependent lipoprotein receptor.

The role of the hepatic LRP as clearance receptor for LPL is in line with previous observations(19,4). The previously observed increase in circulating LPL mass, but not total heparin-releasable LPL activity is now complemented with the observation that local LPL activity in the WAT is increased. In relation to whole body muscle mass, WAT mass constitutes a relatively minor fraction and the contribution of heparin releasable LPL activity to total LPL activity is thus likely minor and explains

why the total heparin releasable LPL activity did not differ between *Ldlr-/-.Apoe-/-* mice and *LRP-/-.Ldlr-/-.Apoe-/-* mice. Whether local WAT LRP activity has been affected in the *LRP-/-.Ldlr-/-.Apoe-/-* mice has not been addressed in the current study. However, LRP is mainly expressed in vascular smooth muscle cells (VSMC's) and the MX1-Cre system that is used to delete the hepatic LRP reportedly fails to affect VSMC's(27). This implies that the local increase in LPL activity is caused by the hampered hepatic LPL clearance. At present we have no mechanistic explanation for an increase in WAT-specific LPL activity.

LRP deficiency on LDLr and apoE deficient background resulted in lower plasma TC levels as compared to LRP presence (fig 1). It has previously been reported that LPL can function as a bridge between lipoproteins and the cell surface to enhance binding of remnant particles. *In-vitro* studies suggest that LPL can mediate binding of VLDL to LRP and HSPG(28,29,30,31,32). If this explains the lower level of plasma cholesterol in *LRP-/-.Ldlr-/-.Apoe-/-* mice, the hypercholesterolemia of the *Ldlr-/-.Apoe-/-* mice should be reduced by an increase in plasma LPL level. However, we have found that injection of an adenovirus vector carrying LPL in *Ldlr-/-.Apoe-/-* mice resulted in a 65% decrease in plasma TG levels but no change in plasma TC levels (data not shown). Thus, in our mouse model, we could not stimulate hepatic cholesterol clearance via increased plasma LPL. Other studies have indicated that apoE is needed for LPL-stimulated hepatic uptake pathways(33), which is in line with our results. At present, we have no explanation for the lower plasma TC levels of *LRP-/-.Ldlr-/-.Apoe-/-* mice as compared *Ldlr-/-.Apoe-/-* mice.

Remnant lipoproteins that are enriched with apoE have an increased affinity for the LRP(11,34,35). We showed that mild overexpression of APOE4 on a *Ldlr-/-.Apoe-/-* background, induced a less severe hyperlipidemia in presence of LRP as compared to the absence of LRP (fig 3). In presence of mouse apoE, *Ldlr-/-* mice also have lower lipid levels as compared to *LRP-/-.Ldlr-/-* mice(20). Thus, in this genetic context, LRP also functions as an apoE-dependent lipoprotein receptor.

LRP has a different affinity for the various apoE variants. It was shown in isolated hepatocytes that LRP has a high affinity for apoE3 and apoE2 and a low affinity for apoE*3-L(30). We analyzed this *in-vivo* by using APOE2(158)**Apoe-/-.Ldlr-/-* and APOE*3-L.*Apoe-/-.Ldlr-/-* mice. These mice were injected with adenovirus

expressing active LPL. This resulted in a 70% decrease in plasma TG levels in the APOE2(158) carrying mice and a 75% decrease in APOE3L carrying mice. Surprisingly, the TC levels did not decrease in both groups of mice (data not shown). This indicates that, in mice lacking LDL receptor, the hepatic clearance via LRP is not efficient via the apoE variants APOE2 and APOE3L, whereas mouse apoe and APOE4 do have a detectable lipid reducing effect.

In this study, we showed that LRP plays a dual role in lipid metabolism. In the absence of the LDL receptor and apoE, LRP-deficiency results in a reduction of the hyperlipidemia and an increase in local LPL activity in white adipose tissue. In the presence of apoE, LRP functions as a hepatic lipoprotein clearance receptor and prevents the apoE-induced hyperlipidemia. Our data add insight into one of the roles of this extremely versatile receptor and demonstrate that the net result of functional changes in the LRP protein are difficult to predict.

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Chapter 6

General Discussion

&

Future Perspectives

In this thesis, the role of apoE in VLDL metabolism has been addressed. ApoE influences at least three aspects of VLDL metabolism: VLDL secretion by the liver, lipolytic processing of VLDL-TG in the circulation and hepatic VLDL-remnant clearance. Both the level of apoE expression as well as the specific apoE variant play a role in the net effect of apoE on VLDL metabolism. The beneficial and the adverse effects of apoE in relation to the structure and function of apoE in VLDL metabolism has been addressed in the current thesis.

Hepatic apoE expression increases VLDL-TG production in a gene-dose dependent manner(1,2,3,4). It has previously been reported by our laboratory that adenovirus mediated gene transfer of a C-terminally truncated variant of APOE4 (APOE4-202), fails to increase VLDL-TG production, yet rescues the hyperlipidemia of *Apoe*-deficient mice(2). This is surprising, since the C-terminal domain of apoE is considered important for association of apoE to lipids and lipoproteins. Nevertheless, the apoE4-202 variant was found to associate with lipoproteins, indicating that the N-terminal domain is sufficient for this interaction.

To explain the effect of apoE on VLDL-TG production, it has been suggested that the C-terminal domain of apoE is involved in the assembly of TG in nascent-VLDL particles by stabilizing the pre-VLDL particle(5). This would be in agreement with the more lipophylic nature of the C-terminal domain of apoE. Recently, Kypreos et al have mutated a series of lipophylic amino acids in the full-length apoE-protein and found that this variant also did not increase VLDL-TG production(6). Thus, the lipophylic amino acids in the C-terminal domain of apoE are likely involved in the generation of TG-rich nascent VLDL particles. It will be of considerable interest to define the minimum set of lipophylic amino acids in the C-terminal domain of apoE required to increase VLDL-TG production.

In **chapter 2**, the effect of full-length and truncated apoE4 on the clearance of VLDL containing the apoE2(158) variant was investigated. Although the amino acid variation lies outside the LDL receptor binding domain of apoE (amino acids 136-150), apoE2(158) displays a decreased binding affinity to the LDL receptor. The APOE2(158) mutation has been hypothesized to interfere with the formation of a salt bridge in the N-terminal domain of apoE that is presumed important for the correct

conformation of the LDL receptor-binding domain of ApoE(7). Since APOE2(158)-associated hyperlipidemia is inherited as a recessive trait, APOE4 and APOE3 expression should rescue the phenotype of homozygous APOE2(158) carriers.

To address the role of additional apoE in APOE2(158)-associated hyperlipidemia, we have used mice carrying APOE2(158) as a transgene on an *Apoe*-deficient background, as well as mice carrying APOE2(158) in place of the endogenous *Apoe* (APOE2 knockin mice). Both mouse strains are hyperlipidemic, whereas in humans homozygous carriers of APOE2(158) do not necessarily develop hyperlipidemia. This discrepancy might result from the expression level of APOE2(158) in our models, although circulating apoE2 levels in these mice are very similar to human levels. The most likely explanation for the complete penetrance of the hyperlipidemic trait is the homogeneous genetic background of the mice (C57/Bl6), that apparently is predisposed towards hyperlipidemia. The latter could be explained by differential mouse-specific expression of for example modifier genes. Whether some mouse specific mechanism renders mice more susceptible to APOE2-associated hyperlipidemia and whether this mechanism also differentiates between susceptible and resistant humans remains to be determined.

In **chapter 2** it is shown that only very low expression of APOE4 reduces the hyperlipidemia in APOE2-transgenic mice. Moderate and high levels of APOE4 aggravate the hyperlipidemia. The APOE2-transgenic mice are more sensitive to develop hyperlipidemia as compared to *Apoe*-deficient mice upon expression of APOE4. The underlying mechanisms are similar; an increased hepatic VLDL-TG secretion rate and decreased lipolysis of VLDL-TG, both resulting in accumulation of TG-rich circulating VLDL. The restored hepatic clearance via APOE4 apparently is not able to compensate for these effects. In contrast to AdAPOE4, moderate and high doses of AdAPOE4-202 reduced the APOE2-associated hyperlipidemia. These data indicate that APOE4-202 is efficient in clearing VLDL particles that are enriched with APOE2.

The apparent inability of additional apoE4 on the circulating VLDL particles to increase hepatic clearance and overcome the increased VLDL production and decreased lipolysis may also be caused by a deficiency in binding of this apoE4 to the LDL receptor. The conformation of the N-terminal receptor-binding domain

depends on the lipid content, size and apoE occupation of the particle(8,9,10). It may well be possible that the composition of the particles that accumulate in the APOE2 mice overexpressing apoE is not conductive for efficient apoE4-mediated LDL receptor clearance. In this regard it seems likely that the C-terminal domain, which is lacking in APOE4-202, functions as a particle-sensor for the N-terminal domain of apoE and determines via allosteric interaction the conformation of the LDL-receptor binding region. Thus, absence of the C-terminal domain in APOE4-202 would result in an open conformation of the N-terminal domain, associated with increased affinity for hepatic receptors and thereby stimulating the clearance of apoE and TG rich VLDL particles that are normally poorly cleared(1) (fig 1).

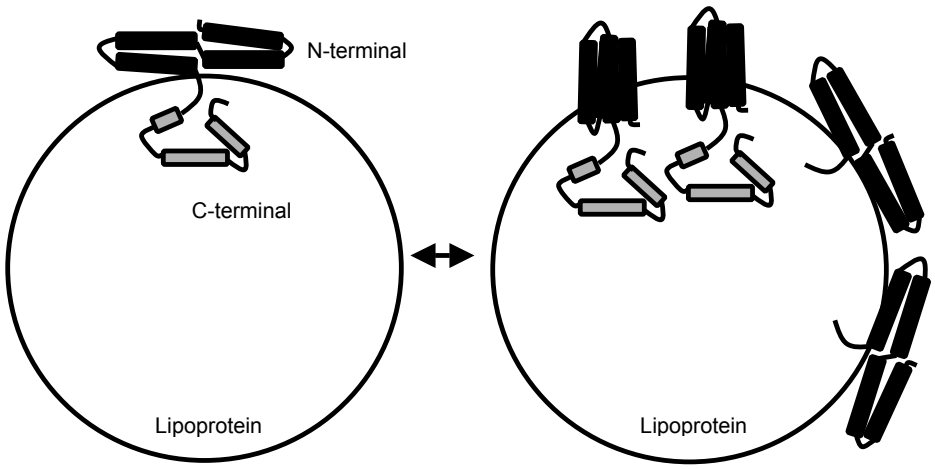

Figure 1. Conformation of apoE. Adapted from Saito et al. JBC 2001. Left: N-terminal four-helix bundle assumes open conformation at low apoE surface concentration. The receptor binding domain of apoE is now accessible for interaction with hepatic clearance receptors. Right: N-terminal four-helix bundle assumes closed conformation at high apoE surface concentration and the receptor binding domain is inaccessible. Presumably, deletion of the C-terminus opens the conformation of the N-terminus and stimulates hepatic clearance.

This hypothesis is supported by the observation that the truncated variant of APOE2 (APOE2-202) also reduces APOE2-associated hyperlipidemia(11). Whether specific amino acids in the C-terminal domain of apoE can be defined that function as particle-sensor in the circulation and whether these differ from the amino acids that are responsible for the increase in VLDL-TG production remains to be determined. The conformational flexibility of the receptor binding domain of apoE and its role in VLDL metabolism might be further investigated with the use of high resolution imaging. Techniques like solution NMR(12,13) allow analysis of the dynamic conformation full length apoE as well as mutated and truncated variants on lipoprotein particles of different lipid and apolipoprotein composition.

Both overexpression of APOE and APOE2-associated hyperlipidemia are characterized by circulating VLDL particles that are rich in TG and apoE and are a relatively poor substrate for LPL. To address the role of LPL activity in hyperlipidemia, in **chapters 3 and 4**, we have modulated LPL activity via specific apolipoproteins that activate or inhibit LPL activity. Two potent modifiers of LPL activity are apoCIII and apoAV. ApoCIII is an important inhibitor of LPL(14,15). Deficiency of apoCIII has been shown to increase LPL activity in mice(16). This results, both on a wildtype, as well as on an *Apoe*-deficient background, in decreased plasma TG levels. ApoAV has a stimulating effect on LPL mediated hydrolysis of TG-rich lipoproteins(17,18). ApoAV-deficiency is associated with increased plasma TG levels, whereas APOA5 overexpression is associated with decreased plasma TG levels (19,20,21,22).

In **chapter 3**, APOE4-associated hyperlipidemia was induced via adenovirus mediated gene transfer of APOE4. This results in an approximately 10-fold increased VLDL-TG production. Despite this considerable increase in VLDL-TG production, endogenous *Apoc3*-deficiency prevented the development of hyperlipidemia. *In-vitro* analysis substantiated that apoCIII is a more specific inhibitor of LPL activity as compared to apoE. It is possible that in presence of excess apoE, the inhibitory effect of apoCIII on LPL is supra-additive. By removing apoCIII, this exaggerated inhibition would be relieved. However, also in the absence of apoE, removal of apoCIII accelerates LPL activity(16), indicating that apoCIII does not require apoE to inhibit

LPL acitivity. This indicates that a putative direct interaction between apoE and apoCIII explains only part of the mechanism of inhibition of LPL activity by apoCIII.

In addition to the effect of apoCIII on LPL-mediated VLDL-TG hydrolysis, apoCIII has been shown to inhibit hepatic clearance of VLDL particles(23). Thus, also the hepatic clearance might be stimulated in case of *Apoc3*-deficiency and contribute to the prevention of hyperlipidemia induced by APOE overexpression.

In **chapter 4**, the role of LPL-mediated VLDL-TG hydrolysis in APOE2-associated hyperlipidemia was further investigated. The primary defect in APOE2-associated hyperlipidemia is a disturbed hepatic binding and clearance of VLDL and chylomicron particles. This results in accumulation of apoE2 in circulation and inhibition of LPL-mediated VLDL-TG hydrolysis. *In-vitro* studies on the interaction between HSPG-bound LPL and apoE2-containing VLDL particles indicated a reduced interaction and reduced rate of lipolysis(24). Surprisingly, *Apoc3*-deficiency did not reduce the APOE2-associated hyperlipidemia, whereas it was very efficiently in reducing APOE4-induced hyperlipidemia. In contrast with *Apoc3*-deficiency, activation of LPL via adenovirus mediated overexpression of APOA5 did reduce the APOE2-associated hyperlpidemia. ApoAV has been suggested to accelerate hydrolysis of TG-rich VLDL particles by guiding these particles to HSPG-bound LPL(25,26). This mechanism would thus overcome the defect of apoE2-containing VLDL in association with HSPG-bound LPL. Alternatively, apoAV could have a direct effect on LPL or apoCII(20). One possibility for such a direct effect is apoAV-mediated stabilization of the active LPL-dimer(27).

The mechanism of apoCIII-mediated inhibition of LPL is poorly characterized. It has been suggested that apoCIII is able to directly influence LPL as a non-competitive inhibitor(28). The C-terminal domain of apoCIII is involved in lipid binding, whereas the N-terminus contains the LPL inhibitory properties(29,14). It is of interest to note that low doses of apoAV can reverse the inhibition of LPL activity by apoCIII(18). From this observation and the results in chapter 4, we conclude that apoCIII and apoAV affect LPL activity via distinct mechanisms. To further analyse the mechanism of interaction between apoAV, apoCIII and LPL, an *in-vitro* assay with HSPG bound-LPL, as was set up by de Man et al.(24), might be very useful. Extending this assay for lipoprotein particles carrying different apolipoprotein

compositions and apoE variants, or, VLDL isolated from different mouse models, would help understanding the role of apoAV and apoCIII in the lipolysis of APOE2-carrying lipoprotein particles.

Our mouse studies on LPL modulating apolipoproteins indicate that the lipolysis of VLDL-TG is a key process in reducing hyperlipidemia associated with apoE variants. In humans the association between apoE-associated hyperlipidemia and LPL activity has also been indicated (24,30,31,32). Furthermore, in APOE2 homozygous patients, polymorfisms in apoC3 and apoAV have been associated with increased expression of hyperlipidemia(33,34). If stimulation of LPL activity via apolipoproteins like apoCIII and apoAV is a helpful tool to reduce hyperlipidemia in patients needs further investigation.

In absence of the LDLr, the hepatic clearance of lipoprotein remants is dependent on backup pathways like LRP and HSPG. These pathways are only efficient in presence of apoE(35). In addition to apoE, LRP has a wide range of ligands. Some of these ligands, like LPL and HL, directly influence lipid metabolism(36). On a LDLr and apoE-deficient background, absence of LRP leads to an accumulation of LPL mass in the circulation and a reduction in plasma TG and TC levels(37). Further analysis of the role of LPL in *LRP.Ldlr-/-.Apoe-/-* and *Ldlr-/-.Apoe-/-* mice revealed that the local LPL activity is indeed enhanced in absence of LRP (**chapter 5**). Next to the effects on local LPL activity, the accumulation of LPL mass in the circulation due to absence of LRP might also influence the hepatic clearance of lipoproteins via the so-called bridging effect of LPL. This bridging occurs due to HSPG-bound LPL that stimulates the binding of lipoproteins to the cell surface and increases the hepatic clearance via hepatic receptors(38,39). To analyze the role of LPL in LRP-mediated hepatic clarance, LPL was expressed via adenovirus mediated gene transfer. This results in efficient hydrolysis of TG(40), as measured by significantly reduced TG levels in both *LRP.Ldlr-/-.Apoe-/-* and *Ldlr-/-.Apoe-/-* mice. The cholesterol levels were not reduced in these mice. Apparently, in the absence of apoE, increased hepatic uptake via an LPL-dependent bridging effect does not occur, which is in line with other studies (41).

In presence of endogenous apoE, the LRP contributes to lipid metabolism as a backup receptor. This is most obvious in the absence of the LDLr(42,43). In the

space of disse, remnant particles are enriched with apoE, thereby increasing their affinity for the LRP. Overexpression of apoE also stimulates the clearance via LDLr and LRP(44,45). In **chapter 5**, we show that overexpression of APOE4 leads to less extreme hyperlipidemia in presence of LRP on an *Ldlr-/-* background as compared to mice that lack the LRP on an *Ldlr-/-* background. Both VLDL cholesterol and TG levels were lower in the presence of the LRP. Our data support the notion that part of the apoE-induced hyperlipidemia is prevented by enhanced clearance via LRP.

Although apoE enrichment in the space of Disse is postulated to stimulate hepatic clearance, data in chapters 2 and 3 indicate that the conformation of apoE might change upon saturation of lipoprotein particles with apoE. This means that the receptor binding domain is not longer accessible for hepatic receptor binding(10). Since apoE enrichment in the space of disse stimulates particle uptake via LRP, we speculate that or the apoE enrichment in the space of disse does not reach saturation of the particle, or the particle undergoes changes in the space of disse, thereby changing the behaviour and the conformation of apoE on the particle. Alternatively, LRP may not depend on an open conformation of the LDL receptor binding domain of apoE for binding to the LRP.

The development of hyperlipidemia is a complex process affecting many steps in lipid metabolism. Since lipid metabolism is an integral part of the energy homeostasis, disturbances in either will affect both. It has been shown that the increased lipolysis as observed in *Apoc3*-deficient mice is associated with an increased propensity to become obese and insulin resistant on a high fat diet(46). Thus, the price to be paid for a decrease in plasma lipid levels is in apoC3-deficient mice an increase in the susceptibility to type 2 diabetes. Thus, care should be taken in efforts to modulate plasma lipid levels via lipolysis. Alternatively, the truncated variant of apoE to enhance hepatic clearance thus far is not associated with adverse effects. In case of apoE2-associated hyperlipidemia, this variant seems to compensate for the binding defect, without affecting other aspects of VLDL metabolism. However, it may be obvious that additional insight in the specific functions of the various domains of apoE is required.

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Summary

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Nederlandse Samenvatting

Summary

Hyperlipidemia is an important health problem in Western society, due to its strong association with diseases like diabetes and obesitas. Disturbances in lipid metabolism might result from our dietary habits as well as genetic variation. Patients suffering from Familial Dysbetalipoproteinemia have a genetic defect in apolipoprotein E (apoE), resulting in accumulation of very low density lipoprotein (VLDL) particles in blood circulation.

In the current thesis, we investigated the structure of apoE and it's influence on VLDL metabolism and hyperlipidemia. Several aspects of the VLDL metabolism are affected by apoE. The liver produces VLDL particles that are secreted into the blood circulation. In this process, apoE stimulates the assembly of triglycerides (TG) into the VLDL particles and increases the production of TG-rich VLDL. After secretion of the VLDL into the blood circulation, the TG in the VLDL particle are hydrolysed by LPL to release free fatty acids to muscle and adipose tissue. This process is inhibited by apoE on the VLDL particle. The TG-depleted VLDL remnants are a good substrate for receptor-mediated uptake by the liver. Again, apoE on the VLDL remnant particle plays a dominant role in the hepatic uptake.

The primary defect in apoE2-associated hyperlipidemia is a poor binding of the apoE2 variant to hepatic clearance receptors. This leads to accumulation of apoE2 in the blood circulation and secondarily to inhibition of the LPL-mediated VLDL-TG hydrolysis. Adenovirus mediated expression of APOE4 (AdAPOE4) in APOE2 transgenic mice ameliorates the hyperlipidemia only at very low expression levels (**chapter 2**). At higher expression levels of APOE, these mice are very sensitive to severe hyperlipidemia, indicating that the stimulated hepatic clearance via apoE4 cannot compensate for the lipid raising effect of increased secretion of TG-rich VLDL and decreased lipolysis of VLDL-TG. A variant of APOE4, truncated at aminoacid 202 (APOE4-202), lacks the C-terminal domain of the protein and does not stimulate the secretion of TG-rich VLDL. Expression of AdAPOE4-202 in APOE2-transgenic mice reduced the hyperlipidemia to wild-type levels. The hepatic secretion of VLDL-TG in APOE2-transgenic mice was not stimulated by APOE4-202. Thus, APOE4-202 lacks the lipid raising effects of full lenght of apoE and is able to restore the apoE2-associated hampered clearance of VLDL particles.

Apoe-deficient mice are severely hyperlipidemic and overexpression of APOE4 in these mice actually aggravates the hyperlipidemia by increasing the secretion rate of VLDL-TG and inhibiting the hydrolysis of VLDL-TG. At moderate levels of APOE4 expression, the plasma lipid levels of *Apoe*-deficient mice are normalized. Apparently, under these circumstances the apoE4-mediated increased hepatic clearance can compensate for the hyperlipidemic effects of APOE4 expression. In **chapter 3**, the hypothesis was investigated that increasing the LPL-mediated VLDL-TG hydrolysis can compensate at least part of the hyperlipidemic effects induced by APOE4 overexpression. This hypothesis was addressed via the LPL inhibitor apoC3. *Apoc3*-deficiency in mice leads to decreased lipid levels both on a wild-type as well as on a *Apoe*-deficient background. This hypolipidemic effect is due to accelerated lipolysis of VLDL-TG. Chapter 3 shows that *Apoc3*-deficiency is able to prevent APOE4-induced hyperlipidemia. The VLDL-TG secretion was increased by APOE4 expression to a similar extent in presence and absence of apoC3. Further analysis of the LPL-activity shows an increased uptake of fatty adids by adipose tissue due to *Apoc3*-deficiency. *In-vitro* assays show a higher specificity of apoCIII for LPL as compared to apoE. Thus, *Apoc3*-deficiency is a potent tool to stimulate VLDL-TG hydrolysis and even compensates for a 10-fold increased hepatic VLDL-TG secretion, resulting in prevention of APOE4-induced hyperlipidemia.

The role of lipolysis in ameliorating hyperlipidemia associated with defects in apoE was further investigated in **chapter 4**. ApoE2 is defective in binding to hepatic receptors, leading to hampered hepatic clearance of VLDL particles in subjects carrying APOE2. Furthermore, *in-vitro* studies indicate that apoE2 carrying particles are a poor substrate for heparan sulphate proteoglycan (HSPG)-bound LPL. In chapter 4, we addressed the question whether modulation of LPL activity can reduce the APOE2-associated hyperlipidemia. The LPL activity in APOE2 knockin mice was stimulated via heparin injection and adenovirus mediated expression of LPL. These treatments led to reduction of the plasma lipid levels. Subsequently, two potent modifiers of LPL, the LPL inhibitor apoCIII and the LPL activator apoAV were investigated. Surprisingly, stimulation of LPL activity via *Apoc3*-deficiency did not affect plasma lipid levels. In contrast, overexpression of apoAV did reduce the apoE2 associated hyperlipidemia. Thus, apoAV apparently interacts with LPL via a different mechanism as compared to apoC3. Furthermore, it can be concluded that stimulation

of LPL activity is indeed beneficial in reducing the APOE2-associated hyperlipidemia and even compensates for the defective hepatic clearance via apoE2.

The hepatic clearance of VLDL remnant particles mainly occurs via the low density lipoprotein receptor (LDLr). In case of malfunction of the LDLr, backup pathways via LDLr related protein (LRP) and HSPG take over the hepatic clearance. These backup pathways were investigated in **chapter 5**. We generated *LRP-/-.Ldlr-/-.Apoe-/-* and *Ldlr-/-.Apoe-/-* mice. We further investigated the surprising finding that LRP-deficiency on the LDLr and apoE double deficient background decreased the plasma lipid levels. In line with this observation, administration of TG-rich emulsion particles resulted in an increased uptake of fatty acids in adipose tissue of mice lacking LRP. These data indicate that LPL activity is increased in *LRP-/-.Ldlr-/-.Apoe-/-* as compared to *Ldlr-/-.Apoe-/-*. Thus, in the absence of apoE, LRP-deficiency has a lipid decreasing effect. Subsequently, apoE was re-introduced. Overexpression of APOE4 in *LRP-/-.Ldlr-/-.Apoe-/-* results in a more severe hyperlipidemia as compared to the same treatment in *Ldlr-/-.Apoe-/-* mice. Since apoE-enriched VLDL particles have affinity for LRP and this pathway is absent in *LRP-/-.Ldlr-/-.Apoe-/-* mice, the severity of the hyperlipidemia in these mice is likely caused by poor hepatic clearance. Thus, in the absence of the LDL receptor and apoE, the LRP functions as a regulator of LPL activity, whereas apoE expression unveals the function of LRP as a lipoprotein receptor.

In the current thesis, both the positive and the negative effects of apoE in VLDL metabolism have been addressed. Negative effects include a clearance defect associated with the APOE2 variant and an increased VLDL-TG production rate and decreased lipolysis induced by overexpression of APOE4. We find that a C-terminally truncated APOE4 variant can ameliorate the APOE2-associated hyperlipidemia, because this variant lacks the TG-increasing effects of full-length apoE. Furthermore, new insight has been gained in the role of LPL-mediated VLDL-TG hydrolysis in APOE-associated hyperlipidemia and the interplay between LPL, APOE and the backup lipoprotein receptor LRP.

Nederlandse Samenvatting

Hyperlipidemie is een belangrijk gezondheidsrisico in de westerse samenleving en direct gerelateerd aan diabetes en overgewicht. Zowel eetgewoontes als genetische afwijkingen kunnen zorgen voor een verstoord vetmetabolisme en ophoping van cholesterol en triglyceriden in het bloed. Patiënten die lijden aan Familiaire Dysbetalipoproteinemie hebben een genetisch defect in apolipoproteine E (apoE). ApoE is een eiwit dat een centrale rol speelt in het vetmetabolisme. ApoE bevindt zich op lipoproteine deeltjes die cholesterol en triglyceriden door het bloed vervoeren en zorgt voor interactie met enzymen en receptoren. Een genetisch defect in apoE zal resulteren in verhoogde niveaus van "very-low density lipoprotein" (VLDL) deeltjes in de bloedbaan.

In dit proefschrift hebben we de structuur van apoE onderzocht en de manier waarop dit het VLDL metabolisme en hyperlipidemie beinvloedt. ApoE beinvloedt verschillende stappen van het VLDL metabolisme. De lever produceert VLDL deeltjes en scheidt deze uit in de bloedbaan. ApoE stimuleert hierbij de opbouw van triglyceriden (TG) in het VLDL en verhoogt daarmee de productie van TG-rijk VLDL. Na uitscheiding van VLDL in de bloedbaan worden de TG in het VLDL gehydrolyseerd door het enzym lipoproteine lipase (LPL). De vrijgekomen vrije vetzuren wordt door spieren gebruikt voor energie of opgeslagen in vetweefsel. De hydrolyse van VLDL-TG wordt geremd door apoE op het VLDL. De ontstane TG-arme en relatief cholesterol rijke VLDL deeltjes worden naar de lever afgevoerd via receptor gemedieerde opname. ApoE medieert binding van VLDL aan leverreceptoren.

Het APOE gen komt in verschillende varianten voor die van elkaar verschillen in aminozuur samenstelling. Het meest voorkomende vorm is APOE3. Vergeleken met APOE3 heeft APOE2 een aminozuur substitutie op positie 158 (Arg -> Lys). Dragers van het APOE2 gen zijn gevoelig voor het ontwikkelen van hyperlipidemie. Dit is het gevolg van een slechte binding van APOE2 aan leverreceptoren die verantwoordelijk zijn voor klaring. De ophoping van APOE2 in de bloedbaan leidt tot remming van de LPL-gemedieerde hydrolyse van VLDL-TG. In **hoofdstuk 2** onderzoeken we of de hyperlipidemie in APOE2 transgene muizen kan worden verlaagd door expressie van APOE4 via een adenovirus. Het functioneren van APOE4 is vergelijkbaar met APOE3, het heeft dus een normale bindings capaciteit aan de leverreceptoren. We vonden dat

alleen zeer lage expressie niveaus van APOE4 de hyperlipidemie in APOE2 transgene muizen kan verminderen. Hogere expressie van APOE4 verergert de hyperlipidemie. De stimulatie van lever klaring via apoE4 kan dus niet de hyperlipidemie in APOE2 transgene muizen verhelpen. De effecten van APOE2 en APOE4 expressie hebben te veel een lipiden verhogende effect op andere delen van het VLDL metabolisme (zoals de remming op de lipolyse van VLDL-TG en de uitscheiding van VLDL door de lever). Een verkorte variant van APOE4 die getrunceerd is bij aminozuur 202 (APOE4-202) mist het C-terminus van het eiwit en stimuleert niet de uitscheiding van TG-rijk VLDL door de lever. Expressie van AdAPOE4-202 in APOE2 transgene muizen reduceerde de hyperlipidemie volledig. APOE4-202 mist dus het lipiden verhogende effect van APOE4 en herstelt bovendien de klaring van VLDL in APOE2 transgene muizen.

Apoe-deficiënte muizen hebben een ernstige hyperlipidemie. Overexpressie van APOE4 in deze muizen verergert de hyperlipidemie door een verhoogde uitscheiding van TG rijk VLDL door de lever en remming van de hydrolyse van VLDL-TG. Middelhoge expressie van APOE4 resulteert in normalisatie van de plasma lipiden niveaus. Blijkbaar kan onder deze omstandigheden de apoE4-gemedieerde lever klaring compenseren voor de lipiden verhogende effecten van APOE4 expressie. In **hoofdstuk 3** onderzochten we de hypothese dat stimulatie van LPL-gemedieerde hydrolyse van VLDL-TG tenminste gedeeltelijk kan compenseren voor de lipiden verhogende effecten van APOE4 overexpressie. Deze hypothese werd getest via de LPL remmer apoC3. *Apoc3*-deficiëntie leidt in muizen tot verlaging van lipiden niveaus zowel op wildtype als op *Apoe*-deficiënte achtergrond. Deze lipiden verlaging wordt veroorzaakt door versnelde lipolyse van VLDL-TG. Hoofdstuk 3 laat zien dat *Apoc3*-deficiëntie in staat is om APOE4-geinduceerde hyperlipidemie te voorkomen. De VLDL-TG uitscheiding werd in gelijke mate verhoogd door APOE4 expressie in aan- en afwezigheid van apoC3. Nader onderzoek naar de LPL activiteit laat een verhoogde opname van vrije vetzuren zien in vetweefsel, veroorzaakt door *Apoc3*-deficiëntie. *In-vitro* studie laat een hoger specificiteit van apoCIII dan apoE zien voor LPL. *Apoc3*-deficiëntie is dus een potente manier om VLDL-TG hydrolyse te stimuleren en compenseert zelfs voor een 10-voudig verhoogde uitscheiding van VLDL-TG door de lever, wat resulteert in de preventie van APOE4-geinduceerde hyperlipidemie.

De rol van lipolyse in het verlagen van de hyperlipidemie die geassocieerd is met een defect in apoE werd onderzocht in **hoofdstuk 4**. ApoE2 bindt slecht aan lever receptoren. Dit leidt tot verstoorde lever klaring van VLDL deeltjes in mensen die APOE2 tot expressie brengen. In de bloedvaten is LPL gebonden aan de vaatwand via heparan sulfaat proteoglycanen (HSPG). *In-vitro* studies laten zien dat apoE2 bevattende deeltjes een slecht substraat zijn voor LPL dat gebonden is aan HSPG. In hoofdstuk 4 beantwoorden we de vraag of modulatie van LPL activiteit de APOE2-geassocieerde hyperlipidemie kan verlagen. De LPL activiteit in APOE2 knockin muizen werd gestimuleerd via heparine injecties en via adenovirus gemedieerde expressie van LPL. Deze behandelingen leidden tot verlaging van de plasma lipiden niveaus. Vervolgens werden 2 potente regulators van LPL getest, nl. de LPL remmer apoCIII en de LPL activator apoAV. Stimulatie van LPL door *Apoc3*-deficiëntie beïnvloedde niet de plasma lipiden niveaus, terwijl overexpressie van apoAV wel de APOE2-geassocieerde hyperlipidemie verlaagde. Blijkbaar interacteert apoAV via een andere mechanisme met LPL dan apoC3. Ook kan worden geconcludeerd dat stimulatie van LPL activiteit gunstig is voor het verlagen van de APOE2-geassocieerde hyperlipidemie en zelfs compenseert voor de defectieve binding van apoE2 aan klaringsreceptoren in de lever.

De lever klaring van TG-arm VLDL gebeurt vnl. via de "low density lipoprotein receptor" (LDLr). Wanneer de LDLr niet goed functioneert wordt de klaring overgenomen door back-up mechanismen zoals de "LDLr related protein" (LRP) en HSPG. Deze back-up mechanismen werden onderzocht in **hoofdstuk 5**. We hebben *LRP-/-.Ldlr-/-.Apoe-/-* en *Ldlr-/-.Apoe-/-* muizen gegenereerd. We onderzochten in deze muizen het verrassende effect dat het ontbreken van klaringsreceptoren door LRP-deficiëntie de plasma lipiden niveaus verlaagt. In lijn met deze observatie resulteerde toediening van TG-rijke emulsie deeltjes in verhoogde opname van vrije vetzuren in vetweefsel van muizen zonder LRP. Deze data laten zien dat LPL activiteit is toegenomen in de muizen zonder LRP. In afwezigheid van apoE heeft LRP-deficientie dus een lipiden verlagende effect. Vervolgens hebben we apoE opnieuw geintroduceerd middels overexpressie van APOE4. Dit resulteerde in een verergerde hyperlipidemie in de muizen zonder LRP. In aanwezigheid van ApoE heeft LRP dus een duidelijke rol als klaringsreceptor en voorkomt extreme hyperlipidemie. Samenvattend functioneert LRP in afwezigheid van LDLr en apoE als regulator van

LPL activiteit, terwijl bij apoE expressie de rol van LRP als lipoproteine receptor naar voren komt.

In dit proefschrift zijn de positieve en negatieve effecten van apoE in VLDL metabolisme besproken. Negatieve effecten zijn een klaringsdefect dat is geassocieerd met APOE2, maar ook een toename van productie snelheid en verminderde lipolyse van VLDL door overexpressie van APOE4. We vonden dat wanneer het C-terminale domein van APOE4 wordt verwijderd, dit eiwit de APOE2-geassocieerde hyperlipidemie verlaagt. Deze getrunceerde variant van APOE4 heeft niet het lipiden verhogende effect van apoE. Verder hebben we nieuwe inzichten verkregen in de rol van LPL-gemedieerde VLDL-TG hydrolyse in APOE-geassocieerde hyperlipidemie en de interactie tussen LPL, apoE en de back-up lever receptor LRP.

Publications

Full papers:

Van Vlijmen BJ, Gerritsen G, Franken AL, Boesten LS, Kockx MM, Gijbels MJ, Vierboom MP, van Eck M, van de Water B, van Berkel TJ, Havekes LM. *Circ Res*. **2001** Apr 27;88(8):780-6. Macrophage p53 Deficiency Leads to Enhanced Atherosclerosis in APOE*3-Leiden Mice.

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Curriculum Vitae

De auteur van dit proefschrift werd geboren op 17 februari 1976 te Coevorden. Het VWO diploma werd in 1995 behaald aan het Greijdanus scholengemeenschap te Zwolle. In 1995 begon ze de studie Bio-Farmaceutische Wetenschappen aan de faculteit Wis- en Natuurwetenschappen van de Universiteit Leiden. Deze studie werd afgerond met een stage op de afdelingen Biofarmacie van de Universiteit Leiden en Vaat- en Bindweefsel Onderzoek van TNO Kwaliteit van Leven te Leiden. Dit onderzoek werd uitgevoerd onder leiding van Dr. B.J.M. van Vlijmen en betrof de rol van apoptose in de ontwikkeling van atherosclerose. Sinds september 2000 startte de functie van Assistent in Opleiding aan het Leids Universitair Medisch Centrum. In dit project werd onderzoek gedaan naar de structuur-functie relatie van apolipoproteine E in het VLDL metabolisme. Dit onderzoek werd uitgevoerd op de afdeling Humane en Klinische Genetica van het Leids Universitair Medisch Centrum onder leiding van Dr. Ir. J.A.P. Willems van Dijk. Verder was er een nauwe samenwerking met de afdeling Vaat- en Bindweefsel Onderzoek van TNO Kwaliteit van Leven, onder leiding van Prof. Dr. Ir. L.M. Havekes. De resultaten van dit door de Nederlandse Hartstichting gesubsidieerde onderzoek staan beschreven in dit proefschrift. Sinds mei 2005 is ze werkzaam op de afdeling Medical Affairs van Centocor B.V.