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

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9

General Discussion and Future Perspectives

Cardiovascular disease (CVD) is the first cause of death in the Western world and its prevalence is increasing in Eastern Europe and developing countries.¹ Lipid abnormalities, including increased levels of apolipoprotein (apo)B-containing lipoproteins and decreased levels of high-density lipoprotein (HDL), are important CVD risk factors. In the past decades the understanding of lipid metabolism has increased tremendously, particularly by the development of several mouse models that are genetically modified (*i.e.* transgenic mice and knockout mice) with respect to genes involved in lipid metabolism. The research described in this thesis was designed to gain further insight into the roles of apoCI, lipoprotein lipase (LPL), and cholesteryl ester transfer protein (CETP) in lipid metabolism. The major conclusions and implications of our findings as well as the future perspectives will be discussed in this chapter.

Role of LPL in Lipid Metabolism

LPL is crucially involved in the hydrolysis of triglycerides (TG) in apoB-containing lipoproteins. Many factors have been shown to influence LPL activity, including apolipoproteins (*i.e.* apoCII, apoCIII, apoE, and recently also apoAV) and lipoprotein binding sites and receptors (*i.e.* heparan sulphate proteoglycans [HSPG], VLDL receptor, and CD36) (for schematic representation see Fig. 1). In this thesis, we have identified apoCI as an additional factor that inhibits LPL activity. This apoCI-mediated inhibition of LPL can explain the hypertriglyceridemia that is observed in *APOC1* transgenic mice.² Previously, it was thought that the hypertriglyceridemia in these mice was caused by inhibition of the apoE-mediated uptake of lipoprotein remnants. It was suggested that apoCI would either displace or mask apoE, thereby preventing the uptake of remnants via the low-density lipoprotein receptor (LDLr) or the LDLr related protein (LRP). However, we have now demonstrated that the hyperlipidemic effect of apoCI still exists in *apoE*^{-/-} and *lrp-ldlr^{-/-}vldlr^{-/-}* mice, and is therefore not dependent on apoE or these receptors. Although our experimental setup does not exclude potential additional hyperlipidemic effects of apoCI via apoE and the receptors, these effects are minor as compared to LPL inhibition since apoE-deficiency in mice does not cause marked hypertriglyceridemia.

Which mechanisms may underlie the apoCI-mediated inhibition of LPL? ApoCII is needed in very small quantities to catalyze LPL-mediated lipolysis. Theoretically, apoCI might reduce the LPL-activity by reducing the amount of apoCII on VLDL particles. However, this is unlikely since the amount of apoCII on VLDL particles is similar in *APOC1* transgenic mice as compared to wild-type controls.³ Nevertheless, apoCI might not displace but mask apoCII, thereby possibly hampering the binding of apoCII to LPL, which has been proposed to be essential to provide LPL with a high local concentration of TG near the active site of LPL.⁴ It is also unlikely that apoCI reduces the LPL-mediated lipolysis by decreased binding of the lipoprotein particles to the negatively charged heparan sulphate proteoglycans (HSPG), thereby preventing the lipoproteins to come in close proximity with LPL. ApoCI is highly positively charged due to a high arginine/lysine content, which would thus even be expected to increase the binding affinity for HSPG. Indeed, VLDL from *APOC1* transgenic mice bound equally well to heparin-Sepharose as VLDL from wild-type mice.³ Anyway, the LPL-inhibito-

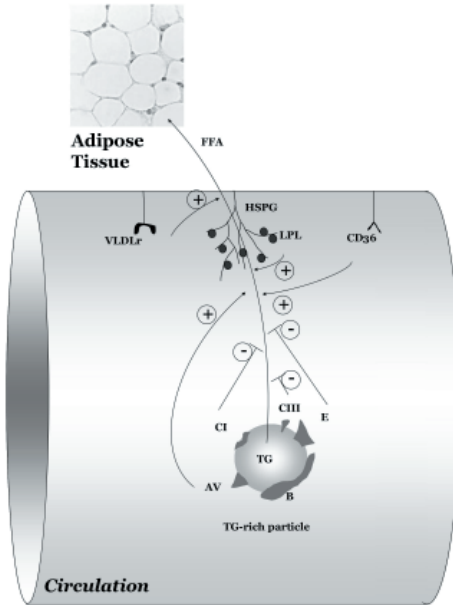


Figure 1. Schematic representation of regulators of obesity. CI, CIII, E, B, AV, apolipoproteins CI, CIII, E, B, AV; FFA, free fatty acids; HSPG, heparan sulphate proteoglycans; LPL, lipoprotein lipase; TG, triglycerides; VLDLr, very low-density lipoprotein receptor.

ry properties of apoCI do not appear to involve HSPG binding, since we showed that apoCI inhibits the LPL-mediated lipolysis *in vitro* in the absence of HSPG, which has also been reported by others^{5,6} A new potential explanation for the apoCI-mediated inhibition of lipolysis is presented by the recent finding that apoAV enhances LPL-mediated lipolysis.^{7,8} Although the exact mechanism of LPL activation by apoAV is still under debate, it probably involves positioning of TG-rich lipoprotein particles in close proximity to the HSPG where LPL resides. Although displacement of apoAV by apoCI is unlikely, as apoAV is tightly anchored into the lipoproteins due to its hydrophobic nature, masking of the apoAV-domain(s) that are important in LPL activation could result in inhibition of LPL. Nevertheless, if the apoCI-mediated inhibition of LPL occurs via apoAV, it is unlikely to be the only mechanism, since apoCI still inhibits LPL in an *in vitro* system where only apoCII as a co-factor is present. ApoE has also been reported to inhibit LPL activity. Its positive arginine residues appeared to be involved in LPL inhibition, since elimination of their charges by modification with cyclohexadione completely abolished the inhibitory effects of apoE,⁹ probably by bringing about substrate dissociation from LPL. It is conceivable that apoCI acts in a similar way as apoE. If this is the case, neutralization of the positively charged lysine residues in apoCI may also prevent the inhibitory effect on LPL.

Population-based studies in humans showed that increased levels of apoCI protein in plasma, due to the *HpaI* promoter polymorphism,¹⁰ were correlated with increased plasma TG.¹¹ Our findings that apoCI inhibits LPL *in vitro* and in mice indicate that there might be a causal relationship between higher apoCI plasma levels and higher plasma triglycerides in humans. ApoCI may also be a genetic factor that modulates type III hyperlipidemia in homozygote *APOE**2 carriers. Most of the subjects with type III

hyperlipidemia are *APOE*2*-homozygotes, whereas only 10% of the *APOE*2* homozygotes develops the hyperlipidemia. Therefore, additional genetic and/or environmental factors are needed to induce hyperlipidemia, and it is thus tempting to speculate that apoCI is one of them. We have demonstrated that increased levels of apoAV reduced the hypertriglyceridemia in *APOE*2* knockin mice. Similarly, apoCI might present a factor leading to hypertriglyceridemia in *APOE*2/2* individuals. To address these hypotheses, studies are needed to determine the apoCI plasma levels in large hyperlipidemic cohorts, or *APOE*2/E*2* cohorts with and without hyperlipidemia, to investigate the possibility of increased levels of apoCI as an additional factor in (*APOE*2*-related) hyperlipidemia.

Reduced LPL activity in mice (*i.e.* by deficiency for LPL, the VLDLr, or CD36) results in raised plasma TG levels, decreased generation of FFA, concomitant with a lean phenotype and resistance to diet-induced obesity.¹²⁻¹⁴ In reverse, mice lacking apoCI-II, a potent inhibitor of LPL, are more susceptible to develop diet-induced obesity.¹⁵ Thus, it seems that there is a positive correlation between LPL activity and body weight. However, it is likely that over a long-term period the same amount of TG are hydrolyzed by inhibited LPL as compared to more active LPL, which would generate similar amounts of fatty acids (FA). It can be speculated though that the short-term excess of FA generated by more active LPL, stimulates FA-uptake in adipose tissue, whereas slow lipolysis of TG may result in usage of FA rather than storage. In line with our observation that apoCI is an inhibitor of LPL activity, overexpression of *APOCI* is associated with protection against diet-induced obesity.¹⁶ In addition to the fact that apoCI reduces LPL activity *per se*, apoCI also appears to bind FA (M. Westerterp, unpublished results), which may prevent FA from being taken up by underlying tissues. It would be interesting to know whether the factors that modulate LPL activity in mice, including apoCI, are also predictive for the development of obesity in humans. This can be investigated by correlation studies between *e.g.* plasma apoCI levels, plasma TG levels, and body-mass-index (BMI) in a large group of healthy individuals, adjusted for confounding factors such as food intake and physical inactivity. For example, these associations could be studied in carriers of the apoCI *HpaI* promoter polymorphism, which have increased plasma levels of apoCI as compared to suitable controls. These studies are important since hyperlipidemia increases the risk of CVD development, whereas prevention of obesity decreases this risk. Both hyperlipidemia and prevention of obesity are caused by decreased LPL activity, at least in mice, thus a delicate balance should probably be maintained.

Role of CETP in Lipid Metabolism

Whether CETP activity is either pro- or anti-atherogenic remained a matter of debate (Fig. 2).¹⁷⁻²⁰ As studies in humans are associative and the effect of CETP deficiency on lipid metabolism and atherosclerosis in humans gave conflicting results, we addressed the role of CETP in mice that are naturally deficient for CETP. *APOE*3-Leiden (E3L)* mice have a moderate hyperlipidemia resulting in a lipoprotein profile that appeared to resemble that of humans.^{21,22} In addition, *E3L* mice respond to lipid lowering therapies

such as statins and fibrates.²³ We found that expression of CETP by *E3L* mice resulted in severely aggravated atherosclerosis.

Several other mouse models expressing CETP have been described with respect to atherosclerosis development, but they also gave conflicting results. CETP expression in *APOC3*-transgenic mice²⁴ and *LCAT*-transgenic mice,²⁵ reduced atherosclerosis. These mice are not able to hydrolyze TG and to deliver HDL-cholesteryl esters to the liver, resulting in the accumulation of large TG-rich particles and large CE-rich HDL particles, respectively. This is not comparable to the human situation, where mainly LDL accumulates. Therefore, the results obtained in these models can not be used as indicators of the effect of CETP expression on atherosclerosis in humans. *ApoE*^{-/-} and *ldlr*^{-/-} mice

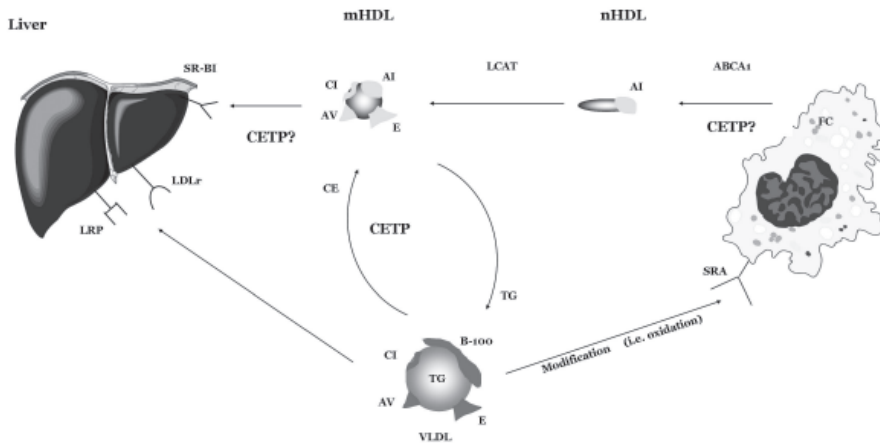


Figure 2. Schematic, simplified representation of human reverse cholesterol transport. CETP mediates the exchange of neutral lipids between apoB-containing lipoproteins and HDL. In addition CETP seems to decrease cholesterol efflux from macrophages, at least via SR-BI. Recent data indicate that cell-bound CETP may be involved in the hepatic uptake of HDL-cholesteryl esters.⁵⁵ ABC, ATP-binding cassette transporter; AI, AV, B-100, CI, E: apolipoproteins AI, AV, B-100, CI, E; CETP, cholesteryl ester transfer protein; LCAT, lecithin:cholesterol acyl transferase; LDLr, low-density lipoprotein receptor; LRP, LDLr related protein; mHDL, mature HDL; nHDL, nascent HDL; SRA, scavenger receptor class A; SR-BI, scavenger receptor class B type I.

displayed more severe atherosclerotic lesions upon CETP expression.²⁶ Although these last models support our findings, they are quite extreme as compared to the human physiology. Apart from its crucial role in uptake of lipoproteins, apoE also plays an important role in the apoE-mediated cholesterol efflux from macrophages. In mice, the turnover of VLDL is more rapid than in humans, which at least partly explains the low levels of LDL in mice as compared in humans. However, by completely deleting either apoE (*i.e.* *apoE*^{-/-} mice) or the LDL receptor (*i.e.* *ldlr*^{-/-} mice), the clearance of apoE- and apoB-containing particles is virtually completely blocked. Since in *E3L* mice, the uptake of apoB-containing lipoproteins is attenuated but not blocked, our model expressing CETP most closely resembles the human situation as compared to the other mouse models. In general it seems that, when the expression of CETP provides an ad-

ditional pathway to excrete the accumulated TG or cholesteryl esters, as observed in *APOC3*²⁴ and *LCAT* transgenic mice,²⁵ respectively, it will be anti-atherogenic. However, if the receptor-mediated hepatic uptake of apoB-containing lipoproteins is retarded, accumulation of cholesterol in these lipoproteins by the action of CETP will be atherogenic. Based on our findings it is likely that CETP constitutes a pro-atherogenic factor in humans.

It is tempting to speculate whether beneficial effects regarding atherosclerosis development can be expected from the recently developed CETP inhibitors. One of the major concerns regarding CETP inhibition is the possibility that it results in a reduction of the reverse cholesterol transport (RCT). This has been supported by the fact that subjects deficient in CETP show an increase in HDL-cholesterol levels,²⁷ which suggests an important role for the (V)LDL-pathway in the removal of HDL-cholesterol in humans and is not operative in the absence of CETP. In addition, the efflux of peripheral cholesterol towards the remodelled HDL may be decreased, thereby further reducing the RCT. It is promising that we found that CETP expression or atorvastatin- and fenofibrate-mediated inhibition of CETP did not hamper the total flux of HDL-cholesteryl esters in mice. Inhibition of CETP by torcetrapib in *CETP.E3L* mice dose-dependently increased HDL levels (W. de Haan, H.M. Princen, L.M. Havekes, P.C.N. Rensen, unpublished results), which is in line with the increases in HDL that were found upon treatment of humans with either torcetrapib or JTT-705.²⁸⁻³¹ To take a more closer look on the effects of CETP inhibition on RCT, the efflux of macrophage-cholesterol and subsequent excretion via the liver into the bile should be studied in *CETP.E3L* mice according to the method by Zhang *et al.*³² However, it should be kept in mind that HDL-cholesterol is very efficiently cleared to the liver via SR-BI in mice, whereas mice do normally not express CETP to shuttle cholesterol towards apoB-containing lipoproteins. In rabbits, the clearance of HDL-cholesteryl esters from plasma is only partially mediated by the direct uptake of HDL-CE, as 25-70% is cleared after CETP-dependent transfer to apoB-containing lipoproteins.^{33,34} Turnover studies demonstrated that also in rabbits CETP inhibition did not compromise the removal of HDL-cholesteryl esters from plasma.³⁴ In humans, it has been shown that the CE output from plasma is even almost solely facilitated via apoB-containing lipoproteins, whereas selective and holo-particle uptake of HDL-cholesteryl esters by the liver could not be detected.³⁵ This suggests that CETP-mediated CE transfer might constitute a major pathway in humans, with only a small contribution of selective HDL-CE uptake,³⁵ which is in sheer contrast with the predominant involvement of SR-BI in selective uptake of HDL-cholesteryl esters in mice. Kinetic studies in humans by Brousseau *et al.*³⁶ showed that the torcetrapib-mediated inhibition of CETP (120 mg/day or 120 mg twice a day) did not alter the fecal concentrations of neutral sterols and bile acids in subjects with low HDL-cholesterol (<40 mg/dl). Thus, also in humans the RCT pathway seems not to be compromised by partial CETP inhibition, indicating that HDL is still functional with respect to cholesterol efflux from macrophages and cholesterol transport back to the liver. In addition, it might be speculated that other properties of HDL than efflux (*e.g.* anti-oxidative, anti-inflammatory, antithrombotic, antiapoptotic properties) are altered by the inhibition of CETP. Although information in humans is scarce, administration

of JTT-705 showed an improvement in antioxidative status of HDL in a small group of subjects with low HDL (n=19).³⁷ Taken together, the fact that HDL-cholesteryl ester flux is not hampered by CETP inhibition, in addition to increased levels of HDL-cholesterol and a possible improvement in antioxidative status of HDL, holds promise for therapies based on CETP inhibition. Administration of CETP inhibitors to *CETP.E3L* mice should provide valuable information whether inhibition of CETP is indeed anti-atherogenic and HDL from these mice should be fully characterized with respect to size, type, and anti-atherogenic properties.

Statins and fibrates are currently the most widely prescribed drugs to reduce CVD risk by improving the lipoprotein profile. In addition to major reductions in LDL-cholesterol and TG levels, respectively, these drugs increase HDL-cholesterol levels by 5-15%.³⁸⁻⁴² We showed administration of atorvastatin and fenofibrate increased HDL levels in *CETP.E3L* mice but not in *E3L* mice, which was related to the drug-mediated inhibition of CETP activity. Thus, a reduction in CETP expression can, at least partly, explain the elevation in HDL-levels that is observed after treatment with these drugs in humans. Strikingly, subjects with CVD carrying the rare CETP TaqIB promoter polymorphism, resulting in higher plasma CETP activity and a more atherogenic lipid profile, have more benefit from statin treatment compared to controls, and the progression of atherosclerosis was slowed down.^{43,44} This suggests not only that the level of CETP activity is physiologically important for the benefit that can be obtained with statins, but also that CETP inhibition might be an important strategy to improve CVD risk.

The first trials of CETP inhibitors with clinical endpoints are eagerly awaited, and will probably be reported in 2007.⁴⁵ Based on those data, it should be possible to estimate the importance of CETP inhibition for CVD risk reduction in humans. Until then, studies with CETP inhibitors in *CETP.E3L* mice can provide valuable information regarding the effects that can be expected in humans.

ApoCI as a Potential Therapeutic Target

ApoCI is the sole HDL apolipoprotein responsible for the inhibition of CETP both *in vitro* and *in vivo*.^{46,47} *CETP* transgenic mice that overexpress human *APOC1* display decreased specific CETP activity.⁴⁸ Since CETP inhibition is generally thought to be desirable for reduction of CVD risk, apoCI may be a lead in the search for CETP inhibitors. However, it was disappointing that absolute levels of CETP activity increased in *CETP.APOC1* mice, due to the apoCI-induced hyperlipidemia. Similarly, pilot data indicated that adenoviral transfer of human *APOC1* in *CETP.E3L* mice result in hypertriglyceridemia, without an effect on CETP activity as judged by the failure to increase HDL-cholesterol (unpublished data C.C. van der Hoogt, M. van Santen, L.M. Havekes, K. Willems van Dijk, P.C.N. Rensen). To develop an apoCI-based therapy to inhibit CETP, it would be of great interest to identify the domains within apoCI that are responsible for the hyperlipidemic as well as the CETP inhibitory effects.

ApoCI consists of two amphipathic α -helix structures (N-terminal residues 7-29; C-terminal residues 38-52), separated by a flexible, unstructured spacer (Fig. 3).⁴⁹ To date, two groups of researchers claimed to have identified the domain in apoCI that is

in vivo evidence whether parts of apoCI might induce CETP inhibition but not yet the undesired hypertriglyceridemic effects. This could be established by administration of well-designed adenoviral vectors expressing the desired part(s) of apoCI, to CETP expressing mice. In case we can show that such an apoCI-fragment induces CETP inhibition without development of hyperlipidemia, the fragment might either be considered as potential drug or as a lead to design new CETP inhibitors.

Concluding Remarks

Although it should be realized that several factors determining the risk for CVD, such as many genetic factors, are hard to treat, dyslipidemia is a treatable risk factor. This research showed that apoCI is an inhibitor of LPL, thereby preventing obesity. However, overexpression of apoCI induces hypertriglyceridemia, which may be considered atherogenic. On the other hand, apoCI is known as the endogenous inhibitor of CETP. If a domain could be identified within apoCI that is responsible for the inhibition of CETP, but does not result in hypertriglyceridemia, such a domain may be considered as potential drug or as a lead to design new CETP inhibitors. In this thesis we tried to get more insight into the roles of apoCI, LPL, and CETP in lipid metabolism. The data illustrate that 1) the activity of LPL is crucially determined by the relative abundance of apolipoproteins like apoCI, apoAV, apoCIII, and apoE, 2) CETP presents a pro-atherogenic factor in mice resembling human lipid distribution over lipoproteins, and 3) at least in *CETP.E3L* mice, a reduction in CETP activity is the cause of fenofibrate- and atorvastatin-induced increase in HDL-cholesterol.

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