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Modulation of HDL metabolism : studies in APOE*3- Leiden.CETP mice

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Chapter 8

IDENTIFICATION OF A C-TERMINAL DOMAIN IN APOLIPOPROTEIN CI THAT INHIBITS CETP ACTIVITY BUT NOT LPL ACTIVITY, BY STRUCTURE-FUNCTION ANALYSIS

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Submitted

Abstract

Apolipoprotein CI (apoCI) is a highly positively charged plasma apolipoprotein of 57 amino acids and has a dual role in plasma lipoprotein metabolism and atherosclerosis. On one hand, apoCI inhibits the cholesteryl ester transfer protein (CETP), which increases the plasma level of anti-atherogenic high density lipoproteins (HDL). On the other hand, apoCI inhibits lipoprotein lipase (LPL), which increases proatherogenic (very) low density lipoproteins ((V)LDL). In this study, we show that the CETP-inhibitory function of apoCI resides in the C-terminal domain, whereas the C terminus of apoCI is not sufficient for the LPL-inhibitory function of apoCI. The C-terminal peptide apoCI₃₂₋₅₇ potently inhibited CETP activity, mainly caused by positively charged amino acids, with a negligible effect on LPL activity. Therefore, apoCI₃₂₋₅₇ may be an interesting lead in the search for novel CETP inhibitors as a new strategy to increase HDL thereby reducing cardiovascular risk.

Introduction

Apolipoprotein CI (apoCI) is the smallest known apolipoprotein. ApoCI consists of only 57 amino acids, arranged in two amphipathic helices connected by a flexible hinge region, and is unusually rich in positively charged lysine (K) and arginine (R) residues (21 mol%). ApoCI is mainly produced by hepatocytes and secreted into plasma, where it associates with predominantly anti-atherogenic high density lipoproteins (HDL), but also with atherogenic very low density lipoproteins (VLDL).¹

ApoCI has various functions in lipoprotein metabolism. ApoCI has been identified as the main endogenous HDL-associated inhibitor of cholesteryl ester transfer protein (CETP) activity,² probably because of its large amount of positively charged amino acids,³ which is regarded as an anti-atherogenic property. CETP is responsible for the transfer of cholesteryl esters (CE) from HDL to low density lipoproteins (LDL) and VLDL in plasma. In this way, CETP lowers HDL-cholesterol (C) and at the same time increases (V)LDL-C, thereby unfavourably modifying two risk factors for atherosclerosis. CETP inhibition is generally regarded as an effective new therapeutic strategy to increase HDL-C levels and reduce cardiovascular disease (CVD) risk.^{4,5} In fact, apoCI-deficiency in human CETP transgenic mice increases CETP activity and lowers HDL-C levels.⁶ Conversely, human apoCI overexpressing (APOC1) transgenic mice have reduced specific CETP activity.⁷ ApoCI could therefore be an interesting lead for a new generation of CETP inhibitors.

However, apoCI is also a major inhibitor of lipoprotein lipase (LPL),⁸ which is a pro-atherogenic property. LPL hydrolyses triglycerides (TG) within TG-rich lipoproteins such as VLDL, resulting in the liberation of fatty acids (FA) that can be stored in adipose tissue or used as energy supply for skeletal muscle and heart. Therefore, apoCI-deficient mice on a hyperlipidemic apoE-deficient background have decreased VLDL levels⁹ and decreased atherosclerosis.¹⁰ Conversely, APOC1 transgenic mice have severely increased plasma VLDL levels,⁸ and APOC1 mice on a hyperlipidemic apoE-deficient background have increased atherosclerosis.^{11,12} Because of apoCI-induced LPL inhibition, human apoCI overexpression in CETP transgenic mice not only reduces specific CETP activity, but also largely increases VLDL levels. The increase in VLDL levels consequently increases hepatic CETP gene expression, which precludes an increase in HDL-C resulting from CETP inhibition only.⁷

To investigate whether apoCI may represent a suitable lead for novel CETP inhibitors, we aimed in this study to identify by structure-function analysis the minimal CETP-inhibitory domain of apoCI without LPL-inhibitory activity. Studies in baboons with high HDL have identified the N-terminal domain apoCI₁₋₃₈ as the CETP inhibitor,^{13,14} whereas studies on the interaction of human apoCI with human CETP indicated that the C-terminal domain apoCI₃₄₋₅₄, rather than the N-terminal domain apoCI₄₋₂₅, inhibited CETP.³ Therefore, we

generated full-length apoCI as well as an array of N-terminal and C-terminal apoCI-derived peptides by solid-phase synthesis, and determined their effect on the activity of CETP as well as of LPL. To get more insight into the mechanism by which apoCI inhibits CETP activity, we also evaluated the contribution of the positively charged amino acids by replacing K and R residues by electroneutral alanine (A) residues.

Materials and Methods

ApoCI and apoCI peptides.

Full-length human apoCI (apoCI₁₋₅₇; purity >95%) was synthesized by the Protein Chemistry Technology Center (UT Southwestern Medical Center, Dallas, TX). ApoCI-derived peptides, without or with replacement of positively charged K and R by electroneutral A, were synthesized by the Peptide Synthesis Facility of the Department of Immunohematology and Blood Transfusion at the Leiden University Medical Center (Leiden, The Netherlands) by solid phase peptide synthesis on a TentagelS-AC (Rap, Tübingen, Germany) using 9-fluorenylmethoxycarbonyl/t-Bu chemistry, benzotriazole-1-yl-oxy-trispyrrolidino-phosphonium hexafluorophosphate/*N*-methylmorpholine for activation and 20% piperidine in *N*-methylpyrrolidone for fluorenylmethoxycarbonyl removal.¹⁵ The peptides were cleaved from the resin, deprotected with trifluoroacetic acid/water, and purified on Vydac C18. The purified peptides were analyzed by reverse-phase HPLC and their molecular masses were confirmed by MALDI-TOF mass spectrometry (purity >95%). The primary sequences of apoCI and apoCI-derived peptides are shown in Table 1.

Table 1. Primary sequences and isoelectric points (pI) of full-length human apoCI (apoCI₁₋₅₇) and apoCI-derived peptides.

ApoCI peptides	pI	Primary sequence
1-57	7.93	TPDVSSALDKLKEFGNTLED K ARELIS R IKQSELSAKMREWFSETF Q KV K EKLKIDS
1-20	4.27	TPDVSSALDKLKEFGNTLED
1-23	4.86	TPDVSSALDKLKEFGNTLED K A R
1-30	5.98	TPDVSSALDKLKEFGNTLED K ARELIS R IK
1-38	6.02	TPDVSSALDKLKEFGNTLED K ARELIS R IKQSELSAKM
46-57	9.53	F Q KV K EKLKIDS
35-57	9.40	SAKMREWFSETF Q KV K EKLKIDS
32-57	8.11	SELSAKMREWFSETF Q KV K EKLKIDS
32-57 A	5.00	SELSAAMAEWFSETF Q KV K EKLKIDS
32-57 B	4.25	SELSAKMREWFSETFQAVAEAL A IDS
32-57 C	3.45	SELSAAMAEWFSETFQAVAEAL A IDS

The positively charged basic amino acids Lys (K) and Arg (R) are represented in boldface.

CETP activity.

The effect of apoCI and apoCI-derived peptides on the CETP-mediated transfer of [³H]cholesteryl oleate (CO) from LDL to HDL was determined essentially as described.¹⁶ HDL, LDL and lipoprotein deficient serum (LPDS) were isolated from human plasma by ultracentrifugation, and LDL was radiolabelled by incubation with [³H]CO (Amersham Biosciences, Buckinghamshire, UK)-containing phosphatidylcholine vesicles in the presence of LPDS exactly as described.¹⁶ ApoCI and apoCI-derived peptides were incubated (6 h at 37°C) with [³H]CO-LDL (31.25 nmol total cholesterol) and HDL (12.5 nmol total cholesterol) in a total volume of 175 µL 0.1 M phosphate buffer, pH 7.4, in the presence of the 10 µL LPDS as CETP source and 8 mM of the lecithin-cholesterol acyltransferase (LCAT) inhibitor 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Sigma). After incubation, LDL was precipitated with 75 µL 0.1 M phosphate buffer and 25 µL 0.167 mM manganese chloride, and the supernatant was counted for HDL-associated [³H]CO in Ultima Gold (Perkin Elmer, Boston, MA, USA). CETP activity was calculated as nmol CE transfer/ mL/min and expressed as % of control.

LPL activity assay.

The effect of apoCI and apoCI peptides on the triacylglycerol hydrolase activity of LPL was assessed by determining their effect on the LPL-mediated hydrolysis of TG within VLDL-like emulsion particles,⁸ using either soluble LPL as described¹⁷ or heparan sulfate proteoglycan (HSPG)-bound LPL. Hereto, 0.25 µg/well HSPG (Sigma) was bound to 96-wells plates by overnight incubation at 4°C. Wells were blocked (1 h at 37°C) with 1% BSA in 0.1 M Tris.HCl pH 8.5, Bovine LPL (0.2 U/well; Sigma) was attached to HSPG by incubation (2 h at 37°C) in 12% BSA in 0.1 M Tris.HCl, pH 8.5. After washing, [³H]TO-labelled VLDL-like emulsion particles (0.5 mg TG/mL), pre-incubated with apoCI (30 min at 37°C), were added in the presence of essentially fatty acid-free BSA (60 mg/mL, Sigma) and the LPL-cofactor apoCII (2.5 µg/mL) in 0.1 M Tris.HCl, pH 8.5. After incubation (20 min at 37°C) the reaction was stopped by placing plates on ice and 20 µL sample was added to 1.5 mL of methanol: chloroform: hexane: oleic acid (1,410: 1,250: 1,000: 1, v/v/v/v) and 0.5 mL of 0.1 N NaOH was added. To quantify the [³H]oleate generated, 0.5 mL of the aqueous phase obtained after vigorous mixing (15 sec) and centrifugation (15 min at 3,600 rpm) was counted in Ultima Gold.

Statistical analysis. Data were analyzed using the Mann-Whitney nonparametric test. Analyses were performed with SPSS 14.0 (SPSS Inc, Chicago, USA).

Results

ApoCI dose-dependently inhibits CETP activity.

To confirm the previously reported CETP-inhibitory effect of full-length human apoCI, we determined the dose-dependent effect of apoCI₁₋₅₇ on the transfer of [³H]CO from human LDL to human HDL in the presence of LPDS as source of CETP (Fig. 1). Indeed, apoCI dose-dependently inhibited CETP activity, with an IC₅₀ of approx. 4 μM, and nearly completely inhibited CETP activity at 16 μM (-91%; P<0.05).

The CETP-inhibitory property of apoCI is confined to its C-terminal domain.

Since the CETP-inhibitory effect of apoCI has been attributed to either its N-terminus^{13,14} or C-terminus,³ we generated an array of N-terminal and C-terminal apoCI-derived peptides by solid-phase synthesis (shown in Table 1), and determined their effect on CETP activity (Fig. 2). The N-terminal peptides apoCI₁₋₂₀ to apoCI₁₋₃₈ did not affect CETP activity at 16 μM, and only marginally decreased CETP activity at 64 μM in a length-dependent way (-19% for apoCI₁₋₃₈; P<0.05) (Fig. 2A). In contrast, the C-terminal peptides apoCI₃₅₋₅₇ and apoCI₃₂₋₅₇ already affected CETP at 16 μM (-37% for apoCI₃₂₋₅₇; P<0.05), and largely decreased CETP activity at 64 μM, again in a length-dependent way (-100% for apoCI₃₂₋₅₇; P<0.05) (Fig. 2B).

The CETP-inhibitory property of apoCI₃₂₋₅₇ depends on positively charged amino acids.

It has been shown that the ability of apoCI to inhibit CETP activity could be explained by reduction of the electronegative surface charge of HDL.³ Since apoCI is unusually rich in electropositive K and R residues, we thus determined the contribution of these residues to the CETP-inhibitory effect of apoCI₃₂₋₅₇ by site-specific replacement into A residues that are electroneutral and do not affect the overall peptide structure (Fig. 3). At a concentration of 16 μM,

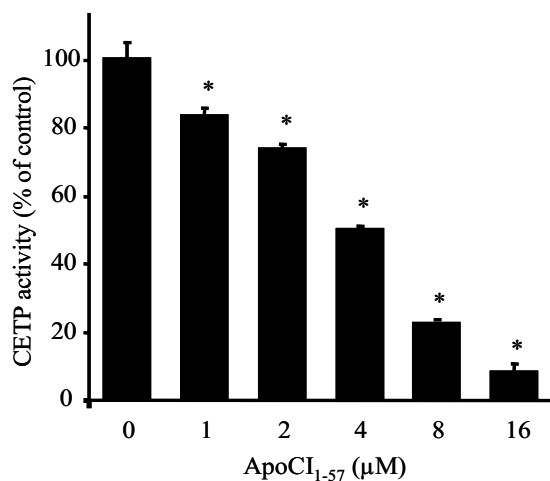


Figure 1. ApoCI dose-dependently inhibits CETP activity. Full-length apoCI (apoCI₁₋₅₇; 0-16 μM) was incubated (6 h at 37°C) with [³H]CO-LDL and HDL in PBS in the presence of LPDS as source of CETP. The amount of [³H]CO transferred from LDL to HDL was determined by counting [³H]CO-HDL after precipitation of LDL. CETP activity was calculated relatively to control incubations without apoCI. Values are means ±SD, n=3, * P<0.05, as compared to control without peptides added.

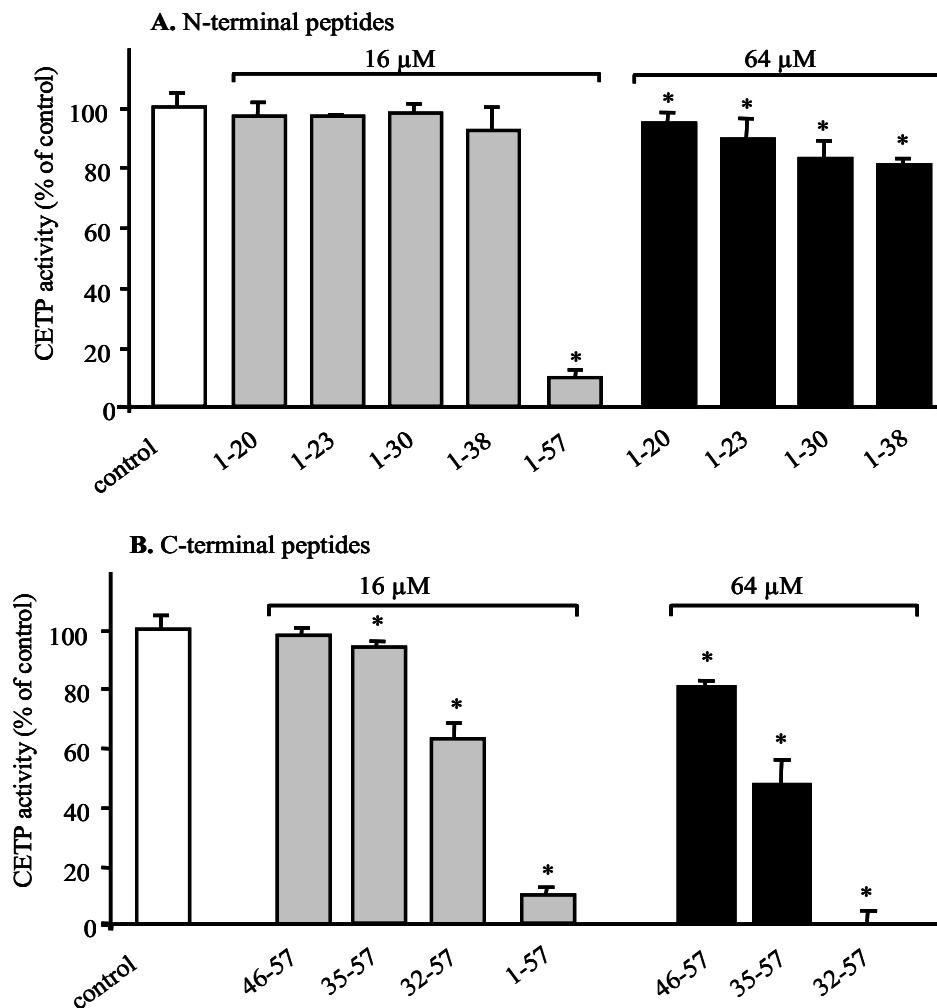


Figure 2. The CETP-inhibitory property of apoCI is confined to its C-terminal domain. ApoCI peptides derived from the N-terminal domain (A) or C-terminal domain (B) were incubated (6 h at 37°C) at concentrations of 16 μM and 64 μM with [^3H]CO-LDL and HDL in PBS in the presence of LPDS as source of CETP. CETP activity was calculated relatively to control incubations without apoCI. Values are means \pm SD, n=3, * P<0.05, as compared to control without peptides added.

wild-type apoCI₃₂₋₅₇ decreased CETP activity appreciably (-37%; P<0.05). Neutralization of positively charged residues, within either the KMR cluster at the N-terminal end or within the KVKEKLLK cluster at the C-terminal end, reduced CETP inhibition partially (-22% and -21%; P<0.05), whereas neutralization of all positively charged amino acids completely abolished the CETP-inhibitory property of apoCI₃₂₋₅₇. A similar pattern was observed at 64 μM , albeit that the peptide in which all K and R residues have been replaced by A still inhibit CETP activity to some extent (-31%; P<0.05).

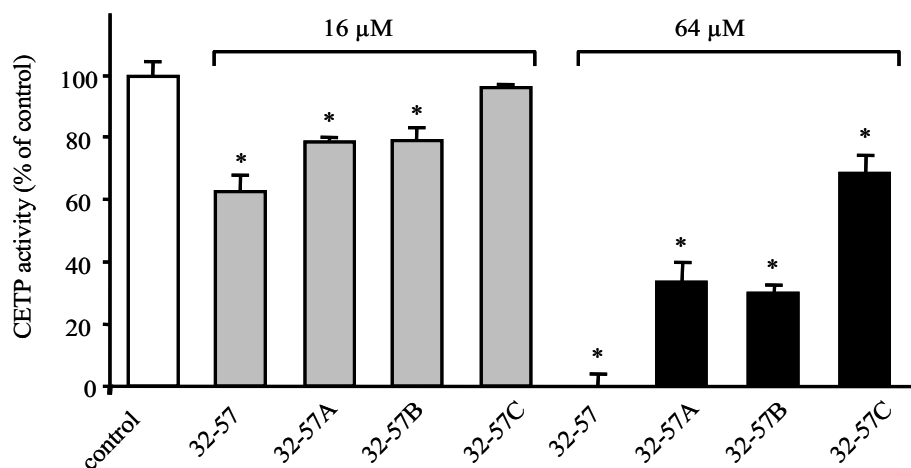


Figure 3. The CETP-inhibitory property of apoCI₃₂₋₅₇ depends on positively charged amino acids. ApoCI₃₂₋₅₇ or apoCI₃₂₋₅₇ in which positively charged K and R are replaced at the N-terminal end (A), C-terminal end (B), or both ends (C) were incubated (6 h at 37°C) at concentrations of 16 μM and 64 μM with [³H]CO-LDL and HDL in PBS in the presence of LPDS as source of CETP. CETP activity was calculated relatively to control incubations without apoCI. Values are means ±SD, n=3, * P<0.05, as compared to control without peptides added.

ApoCI₃₂₋₅₇ does not inhibit LPL activity as compared to full-length apoCI₁₋₅₇. ApoCI₃₂₋₅₇, which is the minimal apoCI peptide that results in maximum CETP inhibition, may have therapeutic value provided that it selectively inhibits the activity of CETP as compared to LPL. Therefore, we next determined the effect of full-length apoCI₁₋₅₇ and of apoCI₃₂₋₅₇ on LPL activity by incubation with glycerol tri[³H]oleate-labeled VLDL-like emulsion particles and HSPG-bound LPL (Fig. 4). Indeed, whereas apoCI₁₋₅₇ dose-dependently decreased LPL activity (-90% at 16 μM; P<0.05), LPL activity was not significantly affected by apoCI₃₂₋₅₇.

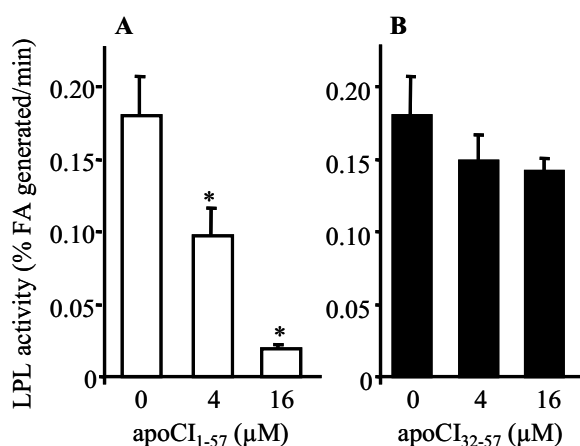


Figure 4. Full-length apoCI, but not apoCI₃₂₋₅₇, inhibits LPL activity. ApoCI₁₋₅₇ (A) and apoCI₃₂₋₅₇ (B) were incubated (20 min at 37°C) at concentrations of 4 μM and 16 μM with glycerol tri[³H]oleate-labeled VLDL-like emulsion particles and HSPG-bound LPL. Generated [³H]oleate was separated from glycerol tri[³H]oleate by extraction and quantified. LPL activity was determined as the percentage of fatty acids (FA) generated per minute. Values are means ±SD, n=3, * P<0.05, as compared to control without peptides added.

Discussion

Using an array of apoCI-derived peptides, we demonstrated that the CETP-inhibitory property of apoCI is restricted to the C-terminal domain of apoCI. We identified apoCI₃₂₋₅₇ as a minimal CETP inhibitory sequence that does not inhibit LPL activity, and showed that the positively charged amino acids K and R are largely responsible for the CETP-inhibitory effect.

First, we thus demonstrated that the CETP-inhibitory property of apoCI resides in its C-terminal domain. This observation is consistent with a previous study comparing the CETP-inhibitory effect of apoCI₃₄₋₅₄ and apoCI₄₋₂₅.³ By direct comparison, we showed that apoCI₃₂₋₅₇ is more effective in CETP inhibition than apoCI₃₄₋₅₄ (not shown). However, our data contradict earlier findings in a strain of baboons with high HDL in which apoCI₁₋₃₈ was identified as the naturally occurring CETP inhibitor in plasma.¹³ Notably, human apoCI₁₋₃₈ also inhibits baboon CETP.¹⁴ It is therefore likely that the discrepancy between the various studies is explained by species differences on the level of CETP: the N-terminus of apoCI affects baboon CETP whereas the C-terminus of apoCI affects human CETP. It should be noted that we did show a modest effect of apoCI₁₋₃₈ on CETP activity (Fig. 2A), and the studies on baboon CETP did not compare apoCI₁₋₃₈ with a C-terminal peptide.¹⁴ Therefore, it is still possible that the baboon CETP activity assay is more sensitive than the human CETP activity assay, and thus detects a larger CETP-inhibitory effect of apoCI₁₋₃₈, without ruling out that apoCI₃₂₋₅₇ is even more effective.

It has been shown that the ability of apoCI to inhibit CETP activity could be explained by reduction of the electronegative surface charge of HDL,³ thereby inhibiting the physical association of CETP with HDL. Therefore, we evaluated the contribution of the positively charged amino acids within apoCI₃₂₋₅₇ to its CETP-inhibitory effect by replacement of K and R residues in the KMR cluster at the N-terminal end or within the KVKEKLLK cluster at the C-terminal end by electroneutral A. This is a small amino acid that does not affect the overall peptide structure as predicted from secondary structure analysis using Peptide Companion software (not shown). We demonstrated that replacement of positively charged amino acids in either the KMR or KVKEKLLK motif both reduced the CETP inhibitory effect of apoCI₃₂₋₅₇. In addition, despite the presence of positively charged amino acids in both the N-terminus and C-terminus of apoCI, the C-terminal peptide apoCI₃₂₋₅₇ had a higher isoelectric point (pI = 8.11) than apoCI₁₋₃₈ (pI = 6.02). These observations thus confirm the hypothesis that the CETP-inhibitory property of apoCI is dependent on its overall positive charge rather than on specific electropositive residues.

In addition to electropositive charge, other structural properties of apoCI likely contribute to inhibition of CETP activity. First, apoCI₃₂₋₅₇ appeared to be a stronger CETP inhibitor than apoCI₃₅₋₅₇ whereas there is no difference in the amount of positively charged residues. Second, apoCI₄₆₋₅₇ has a higher isoelectric point (pI = 9.53) as compared to apoCI₃₂₋₅₇ (pI = 8.11), but is a less

efficient CETP inhibitor. Third, replacement of all K and R residues in apoCI₃₂₋₅₇ did not completely abrogate its CETP-inhibitory effect. It is thus conceivable that, in addition to the importance of the peptide charge, the peptide length positively influences the α -helical conformation of the peptides and the binding affinity of the peptide for HDL.

Whereas full-length apoCI is an effective inhibitor of LPL activity, apoCI₃₂₋₅₇ did not affect LPL activity. In fact, none of the applied apoCI-derived peptides were able to inhibit LPL activity (not shown). The mechanism by which apoCI inhibits LPL activity has not been fully elucidated. ApoCI may directly interact with LPL and/or interfere with other apolipoproteins that modulate LPL activity such as the LPL co-activator apoCII or apoCIII or apoAV.⁸ Alternatively, apoCI may affect LPL activity by product inhibition, since apoCI effectively binds free fatty acids.¹⁸ The lack of an LPL-inhibiting effect of the truncated apoCI peptides could thus result from reduced interference with any of these three processes. The fact that C-terminal peptides inhibit the activity of CETP, but not that of LPL, suggests that these peptides may still bind avidly with HDL (thereby interfering with CETP activity), but weakly with VLDL (thereby precluding interference with LPL activity). Since the anti-apoCI antibody that we used in our ELISA does not react with apoCI-peptides even after minimal truncation of apoCI, such a hypothesis is difficult to test by routine techniques.

In our study, we focused on the most prominent features of apoCI in lipid metabolism, *i.e.* CETP inhibition and LPL inhibition. ApoCI has also been described to stimulate LCAT,^{19,20} inhibit HL^{11,12} and inhibit the HDL receptor scavenger receptor class B type I (SR-BI),²¹ which may contribute to HDL increase. In addition, apoCI inhibits (V)LDL clearance via apoE-recognizing receptors,^{22,23} which may contribute to hyperlipidemia. Therefore, *in vivo* studies using CETP transgenic mice and wild-type mice are needed to evaluate whether apoCI₃₂₋₅₇ is capable to increase HDL without inducing hyperlipidemia and whether such an effect can be ascribed to CETP inhibition only.

The fact that apoCI₃₂₋₅₇ inhibits CETP activity without affecting LPL activity suggests that apoCI₃₂₋₅₇ is a valuable lead for a new anti-atherogenic therapy, as raising HDL by inhibiting CETP is generally seen as a protective lipoprotein in atherosclerosis development. This is especially relevant considering the recent clinical failure of the CETP inhibitor torcetrapib.²⁴ Despite evoking a large increase in HDL-cholesterol levels, torcetrapib did not potentiate the anti-atherogenic potency of atorvastatin as judged from coronary intima-media thickness (IMT) and intravascular ultrasonography (IVUS) measurements.²⁵⁻²⁷

In fact, torcetrapib increased overall mortality and the amount of non fatal cardiovascular events.²⁴ These disappointing results are probably explained by compound-specific off target toxic effects of torcetrapib, including hypertension and hyperaldosteronism.²⁸ Being derived from an endogenous protein, apoCI₃₂₋₅₇ is not expected to induce such side effects. Also, whereas torcetrapib forms an inactive complex between HDL and CETP, thereby resulting in an accumulation of CETP protein in plasma,²⁹ apoCI is not expected to result in

accumulated CETP in plasma as its proposed working mechanism (*i.e.* reduction of the interaction between HDL and CETP) is different from that of torcetrapib.

In conclusion, we identified apoCI₃₂₋₅₇ as the minimal domain in apoCI that inhibits CETP activity without affecting LPL activity. Therefore, future studies are warranted to evaluate whether apoCI₃₂₋₅₇ will raise HDL without inducing hyperlipidemia and may be a valuable lead in the search for new CETP inhibitors that aim at raising HDL and reducing atherosclerosis.

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