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

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Chapter 7

APOLIPOPROTEIN CI INHIBITS SCAVENGER RECEPTOR BI AND INCREASES PLASMA HDL LEVELS *IN VIVO*

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Biochemical and Biophysical Research Communications
2008; 377:1294-1298

Abstract

Apolipoprotein CI (apoCI) has been suggested to influence HDL metabolism by activation of LCAT and inhibition of HL and CETP. However, the effect of apoCI on scavenger receptor BI (SR-BI)-mediated uptake of HDL-cholesteryl esters (CE), as well as the net effect of apoCI on HDL metabolism *in vivo* is unknown. Therefore, we evaluated the effect of apoCI on the SR-BI-mediated uptake of HDL-CE *in vitro* and determined the net effect of apoCI on HDL metabolism in mice. Enrichment of HDL with apoCI dose-dependently decreased the SR-BI-dependent association of [^{3}H]CE-labeled HDL with primary murine hepatocytes, similar to the established SR-BI-inhibitors apoCIII and oxLDL. ApoCI-deficiency in mice gene dose-dependently decreased HDL-cholesterol levels. Adenovirus-mediated expression of human apoCI in mice increased HDL levels at a low dose and increased the HDL particle size at higher doses. We conclude that apoCI is a novel inhibitor of SR-BI *in vitro* and increases HDL levels *in vivo*.

Introduction

Apolipoprotein CI (ApoCI) is a 6.6 kDa protein that is mainly synthesized by the liver, and also by other tissues such as lung, spleen, intestine, brain and adipose tissue. After secretion by the liver, apoCI associates with chylomicrons, VLDL and HDL and is exchangeable between these lipoproteins.[1] ApoCI is highly positively charged, and is present in a relatively high plasma concentration of about 10 mg/dL.[1]

Several functions of apoCI in lipoprotein metabolism have been described. ApoCI affects the metabolism of apoB-containing lipoproteins. Using apoCI-deficient and apoCI-overexpressing mice, it has been shown that apoCI attenuates the clearance of VLDL by inhibition of the lipolytic conversion of VLDL by lipoprotein lipase (LPL), either directly[2] or indirectly via product inhibition due to its fatty acid-binding properties.[3] In addition, apoCI decreases the clearance of VLDL by inhibition of the binding and uptake of VLDL by the classical apoE-recognizing receptors, including the LDL receptor (LDLr)[4] and LDLr-related protein (LRP)[5] on the liver, as well as the VLDL receptor (VLDLr)[6] that is mainly present on peripheral tissues. ApoCI has also been shown to increase the production of VLDL.[7] By these combined actions, apoCI thus increases the plasma levels of VLDL-associated triglyceride (TG) and cholesterol (C) in mice.[1,2]

ApoCI has also been suggested to be involved in HDL metabolism, although such a role has only been derived from *in vitro* observations. ApoCI is involved in HDL remodeling by activation of lecithin: cholesterol acyltransferase (LCAT) that esterifies cholesterol in HDL and, therefore, increases HDL-C levels and HDL particle size,[8,9] and by inhibition of hepatic lipase (HL) that lipolyzes TG and phospholipids (PL) in HDL.[10,11] However, the *in vivo* relevance of these actions of apoCI is unknown, but it is conceivable that apoCI increases the plasma levels and/or the particle size of HDL. ApoCI has also been identified as an inhibitor of the activity of cholesteryl ester (CE) transfer protein (CETP),[12] which may add to a potential HDL-raising effect of apoCI, at least in CETP-expressing species.

ApoCI may also affect the uptake of HDL-CE via SR-BI, as two homologues of apoCI (*i.e.* apoCII and apoCIII) have recently been demonstrated to inhibit SR-BI.[13] Although such an effect of apoCI has not been reported before, SR-BI inhibition would add to a potential HDL-raising effect of apoCI *in vivo*, since SR-BI is solely responsible for the selective hepatic uptake of HDL-CE in mice.[14]

Therefore, in the present study we evaluated whether apoCI would represent a novel modulator of SR-BI by evaluating the effect of apoCI on the uptake of HDL-CE by freshly isolated mouse hepatocytes. In addition, we examined the *in vivo* relevance of the combined effects of apoCI on HDL metabolism using

apoCI-deficient mice and mice that overexpress apoCI using adenoviral expression.

Methods

Mice
Heterozygous apoCI knockout (apoc1$^{-/-}$) mice[15] were crossbred to obtain wild-type (WT), apoc1$^{+/-}$ and apoc1$^{-/-}$ littermates (C57Bl/6 background). Overexpression of apoCI was achieved via injection of a recombinant adenovirus that expresses human apoCI as described.[16] MX1Cre:LRP$^{lox/lox}$.LDLr$^{-/-}$.VLDLr$^{-/-}$ mice (C57Bl/6 background), that are deficient for the LDLr, VLDLr and hepatic LRP after three intraperitoneal injections of polyinosinic: polycytidylic ribonucleic acid (pI:pC), have been generated as described previously.[17] Mice had access to regular chow and water *ad libitum*. When indicated, blood was drawn via the tail vein into paraoxon-coated capillaries after 4 h fasting at 13.00 h and plasma was collected after centrifugation.

Radiolabeling of HDL
HDL was isolated from human plasma by density gradient ultracentrifugation and labeled with [^{3}H]cholesteryl oleoyl ether (COEth) as described previously.[18]

In vitro hepatocyte studies
Hepatocytes were isolated from anesthetized WT and LRP^{-}.LDLr$^{-/-}$.VLDLr$^{-/-}$ mice by perfusion of the liver with collagenase.[19] Freshly isolated cells (1x10^6/mL) were incubated (3h at 37°C) in DMEM + 2% BSA with [^{3}H]COEth-labeled HDL (20 μg protein/mL) in the absence or presence of apoCI (Protein Chemistry Technology Center, Dallas TX, USA), apoCIII (Biodesign International, Saco, ME, USA), or oxidized (oxLDL) (100 μg protein/mL) under gentle shaking. After incubation, cells were pelleted by centrifugation and unbound label was removed by repeated washing with Tris-buffered saline. The pellet was lysed in 0.1 M NaOH and cell-associated radioactivity and protein content were measured. [^{3}H]COEth association was calculated as dpm/mg cell protein.

Biochemical analysis
Plasma cholesterol levels were measured with a commercially available enzymatic kit (236691, Roche Molecular Biochemicals, Indianapolis IN, USA). Plasma apoCI was measured by ELISA[2] and apoAI was measured by western blotting.[20] Lipoproteins were fractionated using fast performance liquid chromatography (FPLC). Hereto, plasma was pooled per group and 50 μL of each pool was injected onto a Superose 6 HR 10/30 column (Äkta System, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and eluted at a constant

flow rate of 50 µL/min in PBS, 1 mM EDTA, pH 7.4. Fractions of 50 µL were collected and assayed for cholesterol as described above.

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

Results

ApoCI inhibits the association of [^{3}H]COEth-labeled HDL with hepatocytes
To examine whether apoCI affects the SR-BI-mediated uptake of HDL-CE, we evaluated the effect of apoCI on the association [^{3}H]COEth-labeled HDL with primary hepatocytes from WT mice. Enrichment of HDL (20 µg protein/mL)

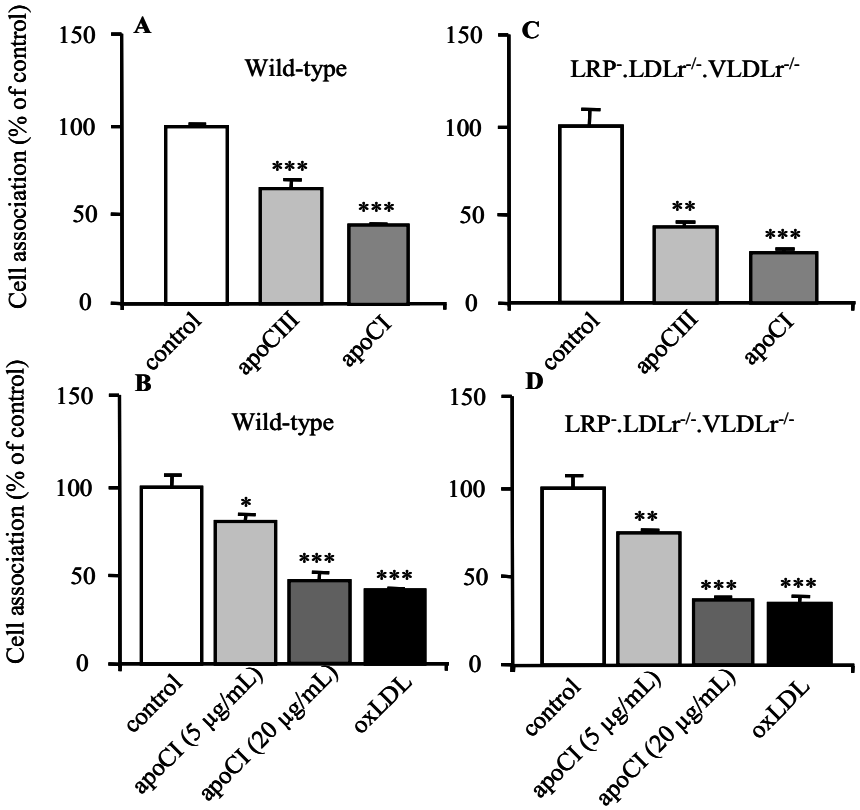

Figure 1. ApoCI dose dependently inhibits SR-BI *in vitro.* Primary hepatocytes were isolated from WT mice (A, B) and LRP$^-$.LDLr$^{-/-}$.VLDLr$^{-/-}$ mice (C, D) and incubated (3 h at 37°C) with [^{3}H]COEth-HDL (20 µg/mL) enriched with apoCIII (20 µg/mL) or apoCI (20 µg/mL) (A, C). In a separate experiment, cells were incubated with apoCI (5 and 20 µg/mL) or oxLDL (100 µg/mL) (B, D). Cell-associated radioactivity was expressed as dpm/mg cell protein. Values represent means ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001.

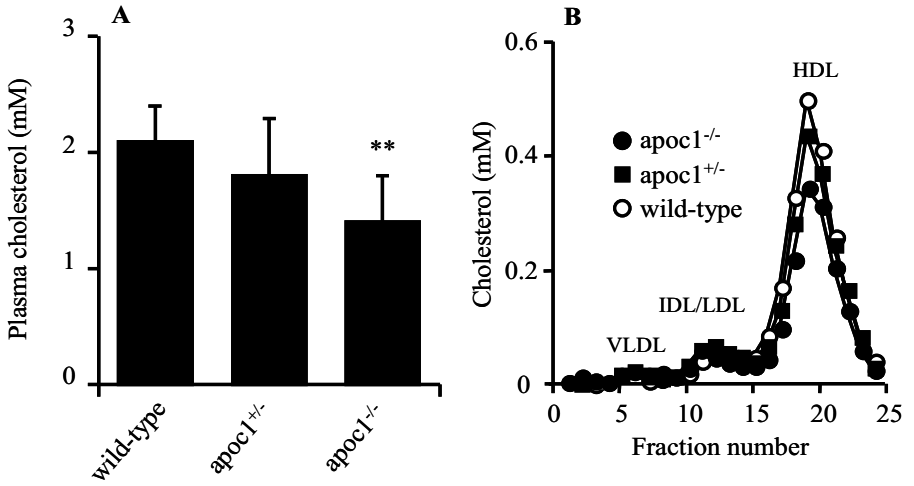

Figure 2. ApoCI deficiency in mice decreases HDL *in vivo.* Blood was drawn from WT, apoc1$^{+/-}$ and apoc1$^{-/-}$ littermate mice after 4 h fasting, and plasma was assayed for total cholesterol (A). Values represent means ± SD (n=12). **P<0.01. Plasma was pooled per group (n=12), lipoproteins were fractionated by FPLC and cholesterol in fractions was measured (B).

with the established SR-BI inhibitor apoCIII (20 μg/mL) inhibited HDL-CE association with WT hepatocytes (-37%; P<0.001), which is in line with the findings of Huard *et al.*[13] ApoCI, at the same concentration, inhibited HDL-CE association even more effic iently than apoCIII (-57%; P<0.001) (Fig. 1A). In a second experiment, we showed that the inhibition of HDL-CE association by apoCI is dose-dependent (-19% at 5 μg/mL; P<0.05 and -53% at 20 μg/mL; P<0.001), and that the highest apoCI concentration was equally effective as the established SR-BI inhibitor oxLDL (-57% at 100 μg/mL; P<0.001) (Fig. 1B). As apoCI is also an inhibitor of the classical apoE recognizing receptors (*i.e.* LRP, LDLr and VLDLr), we repeated this experiment with hepatocytes from LRP, LDLr and VLDLr triple-knockout mice. ApoCI affected the association of HDL-CE with LRP^{-}.LDLr$^{-/-}$.VLDLr$^{-/-}$ hepatocytes (Fig. 1C and D) similarly as with WT cells (Fig. 1A and B). This shows that the inhibitory effect of apoCI on HDL-CE association with hepatocytes is independent of the apoE-recognizing receptors, and confirms that HDL-CE association with hepatocytes is strictly dependent on SR-BI.[14]

ApoCI deficiency decreases plasma HDL levels in mice

Previous *in vitro* studies have demonstrated that apoCI activates LCAT[8,9] and inhibits HL [10,11] and we thus now show that apoCI additionally inhibits SR-BI. To evaluate the consequences of these combined effects for HDL-C metabolism *in vivo*, we first examined the effect of genetic apoCI-deficiency in mice on HDL-C levels. Hereto, blood was drawn from 4 hours fasted WT, apoc1$^{+/-}$ and apoc1$^{-/-}$ littermates. ApoCI-deficiency resulted in a gene dose-dependent

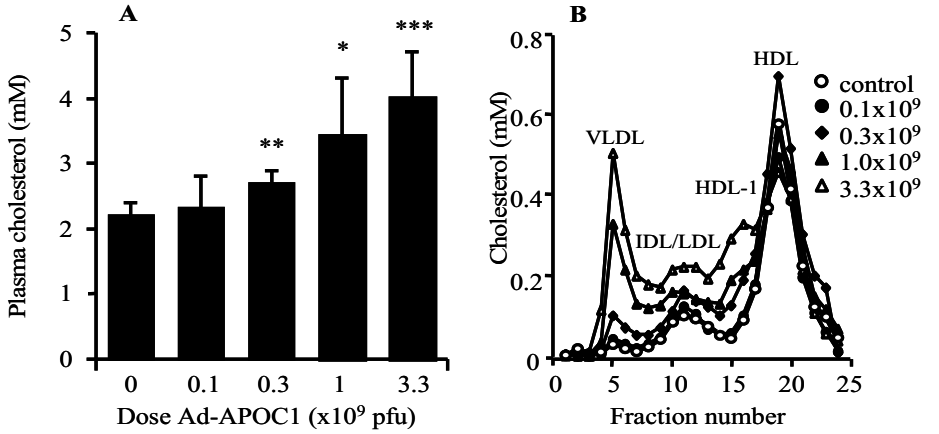

Figure 3. ApoCI overexpression in mice increases HDL *in vivo.* WT mice were injected with a recombinant adenovirus expressing human apoCI to dose-dependently increase apoCI plasma levels. After 5 days, blood was drawn after a 4 h fast, and plasma total cholesterol was measured (A). Values represent means ± SD (n=5). *P<0.05, **P<0.01, ***P<0.001. Plasma was pooled per group (n=5), lipoproteins were fractionated by FPLC and cholesterol in fractions was measured (B).

decrease in plasma cholesterol up to -32% (P<0.01) upon homozygous apoCI deficiency (Fig. 2A). Separation of the various lipoprotein fractions from plasma by FPLC showed that the decrease in plasma cholesterol was mainly confined to the HDL fraction. A gene dose-dependent decrease in HDL-C was observed up to -29% in apoc1$^{-/-}$ mice (Fig. 2B). In contrast, apoCI deficiency did not affect plasma apoAI levels (not shown).

ApoCI overexpression increases plasma HDL levels and enlarges HDL

To examine the effect of apoCI overexpression on HDL-C levels and HDL size, WT mice were injected with a recombinant adenovirus expressing human apoCI (0.1, 0.3, 1, and 3.3 x10^9 pfu/mouse).[16] This resulted in a virus dose-dependent increase in plasma levels of apoCI (0, 12, 23 and 33 mg/dL) and cholesterol (up to +48%; P<0.001) (Fig. 3A). Fractionation of lipoproteins by FPLC indicated that overexpression of apoCI led to a dose-dependent increase in VLDL, which is explained by the well-known attenuation of the catabolism of apoB-containing lipoproteins. Interestingly, overexpression of a low dose of apoCI led to an increase of the normal sized HDL (+15%), whereas higher doses rather led to an increase of HDL particle size as shown by the appearance of HDL-1 (Fig. 3B).

Discussion

The aim of our study was to determine the effect of apoCI on HDL metabolism, by addressing the effect of apoCI on the hepatic HDL receptor SR-BI *in vitro*

and by evaluating the net effect of apoCI on plasma HDL *in vivo*. We showed that apoCI inhibits the SR-BI-mediated association of HDL-CE with primary murine hepatocytes. Furthermore, we showed in mice that apoCI-deficiency reduces HDL levels and, conversely, that modest and high apoCI overexpression increases and enlarges HDL, respectively.

Previous *in vitro* studies have suggested that apoCI may be involved in HDL metabolism by stimulation of LCAT,[8,9] inhibition of HL[10,11] and inhibition of CETP,[12] which are all involved in the remodeling of HDL in the circulation. Based on the structural homology of apoCI with apoCII and apoCIII, which have been shown to inhibit the of SR-BI-mediated selective uptake of HDL-CE by HepG2 cells,[13] we postulated that apoCI may also inhibit SR-BI. Indeed, apoCI appeared even more effective in inhibiting the SR-BI-mediated uptake of HDL-CE by primary mouse hepatocytes than apoCIII.

It is interesting to speculate how apoCI affects SR-BI function. As compared to apoCII and apoCIII, apoCI is unusually rich in positively-charged lysine residues, which is important for both its lipopolysaccharide-binding[21] and CETP-inhibiting[22] properties. The fact that apoCI, apoCII and apoCIII all inhibit SR-BI suggests that the high positive charge of apoCI is not essential for this effect. The mechanism by which apoCII and apoCIII inhibit SR-BI, has not yet been resolved.[13] SR-BI binds HDL via multiple binding sites[23] and subsequently mediates selective CE uptake from this particle.[24] Therefore, apoCI, apoCII and apoCIII may interfere with this process by 1) modifying HDL particles in a way that binding of SR-BI to HDL is reduced, 2) stabilizing HDL particles in a way that CE can not easily be removed, and/or 3) interacting directly with SR-BI, thereby preventing binding to HDL.

Interestingly, the effects of apoCI on the various HDL-modulating proteins, including activation of LCAT, inhibition of HL, and inhibition of SR-BI, should theoretically all lead to an increase in HDL levels and/or particle size. First, overexpression of LCAT, an HDL-associated plasma enzyme that is responsible for cholesterol esterification in HDL,[25-27] increases both HDL-C and HDL size.[28] Second, homozygous deficiency for HL, a plasma enzyme that degrades TG and PL within HDL,[29] does not have a large impact on HDL-C, but does result in accumulation of large HDL-1 particles.[30] Third, heterozygous SR-BI deficiency primarily results in an increase in HDL-C, whereas homozygous SR-BI deficiency leads to accumulation of large HDL-1.[31] Therefore, we postulated that apoCI expression would positively correlate with HDL-C levels and HDL size. Indeed, we showed that apoCI deficiency reduced HDL-C. Conversely, overexpression of increasing amounts of apoCI increased HDL-C at moderate expression and gradually increased the formation of large HDL-1 at higher apoCI expression levels. These effects are thus all consistent with the expected effects of apoCI on LCAT, HL and SR-BI, albeit that it is not feasible to quantify the relative contribution of the individual pathways.

Regarding the current interest in raising HDL as a novel strategy to reduce cardiovascular risk, apoCI may be an exciting new lead. It is interesting to note that, in addition to its effect on LCAT, HL and SR-BI, apoCI is also the main endogenous protein inhibitor of CETP.[12] CETP is absent in mice but present in plasma of humans and may therefore also add to the HDL-raising effect of apoCI in humans. Although a causal effect of apoCI on determining HDL levels in humans would be difficult to study, more than 90% of plasma apoCI appears to be associated with HDL in normolipidemic subjects[32] and we recently demonstrated a positive correlation between plasma apoCI and HDL.[33] Of course, since levels of HDL do not always reflect the atheroprotective properties of HDL (*i.e.* its role in reverse cholesterol transport, its anti-inflammatory and antioxidative properties), studies on the effect of apoCI on HDL functionality are still warranted.

A drawback of apoCI as a lead in HDL-raising therapy would be that apoCI not only increases HDL levels, but also increases VLDL levels mainly by inhibition of LPL activity.[2] This appeared the predominant reason why apoCI overexpression on a hyperlipidemic apoE-deficient background aggravated atherosclerosis,[11] whereas apoCI-deficiency attenuated atherosclerosis.[34] Furthermore, SR-BI has recently been described to facilitate the hepatic uptake of VLDL and chylomicrons.[35,36] These effects may thus add to some extent to the VLDL-raising effect of apoCI. Therefore, it would be interesting to perform *in vitro* structure-and-function studies to determine the minimal domain of apoCI that targets subsets of HDL-modulating proteins, including preferentially LCAT, HL and CETP, without adversely affecting VLDL metabolism via LPL and potentially SR-BI.

In conclusion, we have demonstrated that apoCI is a novel inhibitor of the SR-BI-mediated uptake of HDL-CE by hepatocytes, and that apoCI is a determinant for the plasma levels and size of HDL *in vivo*.

Acknowledgments

This work was performed in the framework of the Leiden Center for Cardiovascular Research LUMC-TNO, and supported by the Netherlands Heart Foundation (NHS grant 2003B136 and 2005B226 to P.C.N.R.), the Netherlands Organization for Scientific Research (NWO grant 908-02-097 and NWO VIDI grant 917.36.351 to P.C.N.R.) J.W.J. is an established clinical investigator of the Netherlands Heart Foundation (2001D032).

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