Disruption of the murine procollagen C-proteinase enhancer 2 gene causes accumulation of pro-apoA-I and increased HDL levels

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Abstract Given the increased prevalence of cardiovascular disease in the world, the search for genetic variations that impact risk factors associated with the development of this disease continues. Multiple genetic association studies demonstrate that procollagen C-proteinase enhancer 2 (PCPE2) modulates HDL levels. Recent studies revealed an unexpected role for this protein in the proteolytic processing of pro-apolipoprotein (apo) A-I by enhancing the cleavage of the hexapeptide extension present at the N-terminus of apoA-I. To investigate the role of the PCPE2 protein in an in vivo model, PCPE2-deficient (PCPE2 KO) mice were examined, and a detailed characterization of plasma lipid profiles, apoA-I, HDL speciation, and function was done. Results of isoelectric focusing (IEF) electrophoresis together with the identification of the amino terminal peptides DEPQSQWDK and WHVWQQDEPQSQWDVK, representing mature apoA-I and pro-apoA-I, respectively, in serum from PCPE2 KO mice confirmed that PCPE2 has a role in apoA-I maturation. Lipid profiles showed a marked increase in plasma apoA-I and HDL-cholesterol (HDL-C) levels in PCPE2 KO mice compared with wild-type littermates, regardless of gender or diet. Changes in HDL particle size and electrophoretic mobility observed in PCPE2 KO mice suggest that the presence of pro-apoA-I impairs the maturation of HDL. ABCA1-dependent cholesterol efflux is defective in PCPE2 KO mice, suggesting that the functionality of HDL is altered.—Francone, O. L., B. Y. Ishida, M. de la Llera-Moya, L. Royer, C. Happe, J. Zhu, R. J. Chalkey, P. Schaefer, C. Cox, A. Burlingame, J. P. Kane, and G. H. Rothblat. **Disruption of the murine procollagen C-proteinase enhancer 2 gene causes accumulation of pro-apoA-I and increased HDL levels. *J. Lipid Res.* 2011.** 52: **1974–1983.**

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Epidemiological studies from the past five decades have consistently shown that levels of HDL-cholesterol (HDL-C) are inversely correlated with clinical events resulting from atherosclerosis (1, 2), whereas LDL-cholesterol (LDL-C) levels are directly related to the incidence of these events. HDL protects against cardiovascular disease by regulating cholesterol efflux from peripheral tissues and modulating inflammation (3). Other antiatherogenic properties of HDL include its antioxidant and vasoprotective properties (4). The importance of HDL in disease together with the accuracy with which it can be measured in plasma specimens makes it amenable for genetic studies. Twin studies have indicated that 50% of the variation in HDL-C levels is genetically determined (5) and that at least 50 different genes are reported to be associated with HDL-C. Large population studies (6–8) have identified single-nucleotide polymorphisms at multiple loci associated with various HDL-cholesterol levels. Recently, studies have started to address the extent to which genetics impacts HDL. However, the genetic component

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Abbreviations: BMP-1, bone morphogenetic protein-1; FC, free cholesterol; FPLC, fast-protein liquid chromatography; HDL-C, HDL-cholesterol; HMGCR, HMG-CoA reductase; IEF, isoelectric focusing; KO, knockout (deficient); LDL-C, LDL-cholesterol; MRM, multiple reaction monitoring; PCPE2, procollagen C-proteinase enhancer 2; pI, isoelectric point; SR-BI, scavenger receptor class B type I; TC, total cholesterol; TG, triglyceride; V+LDL, very low density lipoproteins + low density lipoproteins; WT, wild-type.

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of variation of HDL-C is not fully explained by known genes, indicating that many more genes exist that have weak effects or that are only important in certain populations. With some of these genes, the genetic effects may never be strong enough to be absolutely convincing, due either to their small direct impact on HDL-C or to the lack of common high-effect variants. Moreover, genes with unknown functions pose an additional complication as their significance and relevance are unclear. Thus, further proof that such genes are important in the modulation of HDL-C and cardiovascular disease requires direct functional studies driven by suggestive genetic data.

One gene previously examined is procollagen C-proteinase enhancer-2 (originally named PCOLCE2 and now known as PCPE2) (9). PCPE2 was found to be associated with HDL in three independent population cohorts (10, 11). PCPE2 was initially identified by expressed sequence tag (EST) sequencing (9) in the glaucoma candidate gene region on 3q21-q24 in an effort to identify the gene responsible for primary open-angle glaucoma. No coding sequence mutations were detected, and PCPE2 is no longer considered as the primary open-angle glaucoma candidate gene, leaving its biological function unknown. PCPE2, a 52 KDa protein composed of 415 amino acids, shares 43% identity to the type I procollagen C-proteinase enhancer protein (PCPE1) (12). PCPE1 and PCPE2 are extracellular glycoproteins that can stimulate the C-terminal processing of fibrillar procollagens by tolloid proteinases such as bone morphogenetic protein-1 (BMP-1). PCPE2, like PCPE1, consists of two cubulin (CUB) domains (CUB1 and CUB2) involved in protein-protein interaction. While several studies have been published addressing the physiological role of PCPE1 (13, 14), the function of PCPE2 remains unclear. PCPE2-deficient (PCPE2 KO) mice are viable and fertile, and they do not show gross developmental abnormalities (15). Very recently, a series of biochemical experiments (10) sparked interest in PCPE2 when an important role for this protein in apoA-I synthesis and HDL metabolism was described. Consistent with the proposed relationship to HDL levels observed in genetic studies, PCPE2 is present and resides on HDL in plasma from fasted normolipidemic humans. A series of biochemical and mechanistic experiments further confirmed this genetic-functional relationship by demonstrating that PCPE2 participates in the regulation of apoA-I synthesis by accelerating the proteolytic processing of pro-apolipoprotein A-I (pro-apoA-I) by BMP-1 (16). Biophysical and biochemical studies indicate that PCPE2 interacts with BMP-1 and pro-apoA-I to form a ternary pro-apoAI/BMP-1/PCPE2 complex and causes the cleavage of the hexapeptide extension present at the N-terminus of apoA-I.

In an attempt to further define the role of PCPE2 on HDL metabolism and gain insight into the genetic-functional relationship suggested by in vitro studies, we examined PCPE2 KO mice and characterized the effect of PCPE2 deletion on plasma lipids, apoA-I, and HDL properties and function.

METHODS

Mice

The PCPE2 gene-targeted mice were created (15) and maintained at the Max Planck Institute of Immunology and Epigenetics (Freiburg, Germany) according to protocols approved by the Institutional Animal Care and Use Committee of the Max Planck Institute of Immunobiology and Epigenetics. Mice were maintained on a 12 h light/dark cycle and fed either a rodent chow or high-fat diet (Harlan Teklab). Tissue and plasma samples from PCPE2 KO and age-matched wild-type control littermates were shipped and processed at Pfizer Central Research (Groton, CT).

Plasma lipids and lipoprotein analysis

Plasma samples were isolated from blood collected either retro-orbitally or by terminal bleeds. Cholesterol, triglyceride (TG), and phospholipid levels were determined by using enzymatic colorimetric assays (Wako Biochemicals). ApoA-I and apoB were determined by using ELISA as previously described (17). Lipoproteins were isolated from pooled plasma from PCPE2 KO and wild-type littermates fed either a chow or high-fat, high-cholesterol diet by fast-protein liquid chromatography (FPLC) as previously described (17). To determine lipid composition and apoA-I concentrations in HDL, tubes containing the HDL lipoprotein fractions were pooled and concentrated in Macrosep centrifugal concentrators (Filtron Technology, Northborough, MA).

Identification of mature and pro-apoA-I forms

Mass spectrometric analysis of apoA-I bands resolved by isoelectric focusing (IEF) and SDS-PAGE were carried out after in-gel tryptic digestion. Initial sample analysis was by LC-MS using a NanoAcquity (Waters) HPLC system and a QSTAR Elite (AB-Sciex) mass spectrometer, cycling between acquiring a survey scan of peptide masses followed by automated CID fragmentation analysis of the three most abundant eluting multiply-charged peptides. Peptide and protein identification was performed using Protein Prospector (version 5.7), searching against SwissProt (version downloaded on August 10, 2010) mammalian entries (67,107 entries). As the entry for apoA-I in this database contains the signal peptide, the data was searched considering peptides with semitryptic specificity (i.e., only one of the peptide termini was required to meet trypsin specificity) to allow identification of the protein N-terminus of preprotein and mature protein. ApoA-I was the major component of each band, with over 90% of the protein sequence observed in each sample. In these analyses, the N-terminus corresponding to both the preprotein and mature protein was detected. There was evidence of a different ratio of pre-to-mature protein between the two bands, but for more accurate quantification, multiple reaction monitoring (MRM) transitions were chosen based on the more intense ions observed in the fragmentation spectra acquired of the relevant protein N-terminal peptides in the QSTAR analysis. Five or six transitions were monitored for each precursor, and analysis was performed using a NanoAcquity interfaced to a QTRAP5500 (AB-Sciex) mass spectrometer. The sum of the signal for all transitions for a given peptide was used for quantitative comparisons of peptide abundance.

Characterization of HDL and apoA-I in wild-type and PCPE2 KO mice

ApoA-I was characterized for charge isoforms by IEF. The plasma lipoprotein fraction containing HDL ($d < 1.21$ g/ml) was isolated by ultracentrifugation in a potassium bromide solution ($d < 1.21$ g/ml) at 35,000 rpm, 10°C for 18 h (Beckman 42.2 rotor)

and buffer was exchanged (PD-10, GE Healthcare) to 0.15 M NaCl, 1 mM EDTA (pH 7.4), and 0.025% NaN_3. Lipoproteins were treated with equal volumes of sample buffer (8 M urea, 4% ampholines mixture, w/v (pH 3/10 + pH 5/7, 1:2) and applied to 1% agarose gels (Isogel, Lonza-Rockland, Rockland, ME) previously equilibrated in 6 M urea + 2% ampholine mixture. Gels were electrophoresed using 0.5 N acetic acid (anode) and 1 N NaOH (cathode) solutions at 500 V for 45 min at 10°C. pH measurements were made by equilibrating gel slices in deionized water overnight at room temperature. Gels were Coomassie stained for protein visualization.

HDL was examined by anti-apoA-I immunoblotting of plasma separated by charge and particle size by nondenaturing agarose and polyacrylamide gradient gel (PAG) electrophoresis, respectively. Flatbed agarose electrophoresis was carried out in 0.8% agarose (Bio-Rad, low mr) for 40 min at 250V, 10°C in a continuous buffer system consisting of 0.062 M tris, 0.025 M tricine, and 0.01 M Ca-lactate, pH 8.0. Resolved proteins were transferred to nitrocellulose membranes by capillary blotting. Membranes were reacted with monospecific rabbit anti-mouse apoA-I antibody (BioDesign), biotinylated donkey anti-rabbit (IgG), and avidin-biotin-HRP for chemilumenescent detection (West Femto kit, Pierce; Chemidoc, Bio-Rad). HDL particle size characterization was determined on 0.5-30% PAGE employing biotinylated calibrators (Pharmacia HMW kit, FPLC-purified ovalbumin and human LDL [1.030 < d < 1.050]). Gels were run to equilibrium (3,000 V h, 10°C) and transferred to nitrocellulose membranes (0.2 μm, Bio-Rad) at 55 V, 10°C for 18 h. ApoA-I was detected as above.

Bidirectional cholesterol flux

The configuration for the assay was an adaptation of a published assay using Fu5AH cells (18), where flux is largely via scavenger receptor class B type I (SR-BI) to J774 macrophages expressing several efflux pathways. J774 cells, maintained in RPMI plus 10% fetal bovine serum and antibiotics, were plated in 24 multiwell plates (70,000 cells/well). Cells were incubated for 24 h with RPMI medium containing 2 μCi ^{3}H-cholesterol (PerkinElmer) in the presence of an ACAT inhibitor (2 μg/ml CP113 818) to have a single pool of radioactive, free cholesterol (FC) and 50 μg/ml AcLDL to enrich the cells in FC. Cells were then incubated for 16 h with MEM-HEPES containing 0.2% BSA and 0.3 mM Cpt-cAMP (Sigma) to upregulate ABCA1 expression. To measure flux, cells were incubated for 8 h with MEM-HEPES containing 2.8% (equivalent to 2% serum) apoB-depleted mouse serum prepared using polyethylene glycol (MW 8000, Sigma) as previously described (19). After 8 h, radioactive cholesterol released to the medium was measured using liquid-solid chromatography, and the fractional release of radioactive cholesterol (i.e., the fraction of the total radioactivity released per well) was calculated as previously published (18). Cellular cholesterol mass was measured by gas liquid chromatography to establish the specific activity of cellular cholesterol, which was then used to obtain estimates of cholesterol mass flux (i.e., efflux, influx, and net efflux [efflux minus influx]). In every experiment, cells were incubated with MEM-HEPES alone to establish the background efflux and with a standard mouse serum pool to monitor potential variability. All efflux measurements were done in triplicate.

Pathway-specific efflux

For these experiments, the various cell models were radiolabeled with ^{3}H-cholesterol in the presence of an ACAT inhibitor and incubated with 2.8% apoB-depleted mouse serum to measure efflux as described above. ABCA1-specific efflux was calculated as the difference in efflux between c-AMP upregulated J774 cells and untreated controls. Fu5AH cells were used to measure SR-BI-specific efflux as the BLT-1-sensitive efflux. To inhibit SR-BI, the cells were preincubated 2 h with BLT-1 at a final concentration of 20 μM as previously described (20). The BLT-1-resistant efflux in Fu5AH cells is the efflux occurring via aqueous diffusion. To assay cellular cholesterol efflux, radioactive cholesterol released was measured by liquid-solid chromatography after 4 h incubation with apoB-depleted mouse serum. Fractional efflux (i.e., the fraction of the total radioactivity released per well) was calculated as usual (19). ApoA-I (20 μg /ml) was used to monitor efflux via ABCA1, and HDL (20 μg /ml) was used to monitor efflux via SR-BI. In every experiment, cells were incubated with MEM-HEPES alone to establish the background efflux and with a standard mouse serum pool. All efflux measurements were done in triplicate.

RESULTS

Identification of mature and pro-apoA-I forms in wild-type and PCPE2-KO mice

We studied PCPE2 KO mice to assess whether the conclusions drawn from in vitro experiments on the proposed role of PCPE2 in the proteolytic conversion of pro-apoA-I to apoA-I occurs in vivo. A series of experiments were conducted to determine whether in the absence of PCPE2 (PCPE2 KO mice) there was an increase in the amount of pro-apoA-I in plasma compared with wild-type control littermates. For this purpose, lipoproteins were isolated from wild-type and PCPE2 KO plasma by ultracentrifugation. Desalted, delipidated lipoproteins (d < 1.21 g/ml) were analyzed by IEF and stained with Coomassie blue as indicated in Methods. For comparison, a mixture of HDL isolated from various mouse models was included. As shown in **Fig. 1A**, within the isoelectric point (pI) range for apoA-I, there was an approximately 0.1 pH unit increase for the apoA-I present in PCPE2 KO mouse plasma compared with controls. Apparent calculated pI were 6.09 and 6.18 for wild-type and PCPE2 KO mice, respectively. The pH unit differences and apparent pI obtained experimentally were consistent with the predicted pI of 5.42 and 5.52 for mature and pro-apoA-I using computer software. These findings were expected from an increase in basic residues resulting from the presence of the hexapeptide WHVWQQ corresponding to pro-apoA-I. No detectable band with the migration characteristics of apoA-I isolated from wild-type HDL was observed in PCPE2 KO mice. To confirm whether the changes in pI were due to the presence of pro-apoA-I, HDL from wild-type and PCPE2 KO mice was isolated by ultracentrifugation, subjected to trypsin digestion, and resolved by reverse-phase HPLC as described in Methods. N-terminal peptides DEPQSQWDK and WHVWQQDEPQSQWDVK, representing the mature and propeptide of apoA-I, were identified by mass spectrometry in HDL isolated from wild-type and PCPE2KO mice (Fig. 1A), respectively, confirming the presence of pro-apoA-I in HDL isolated from PCPE2 KO. An additional study was performed in which apoA-I was isolated from HDL by SDS-PAGE and subjected to tryptic digestion. Peptides were resolved by reverse-phase HPLC, and the amino terminal peptides representing the pro and mature forms of apoA-I were quantified by MRM mass spectrometry. As shown in

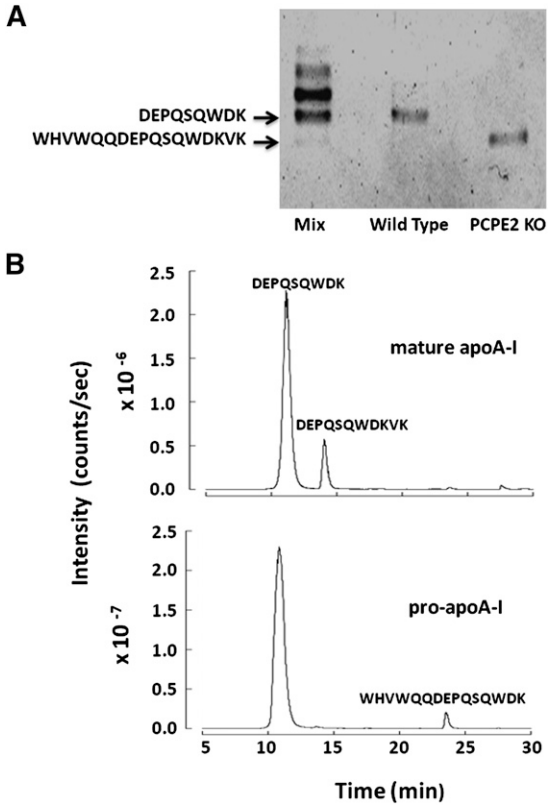

Fig. 1. Identification of mature and pro-apoAI forms in wild-type and PCPE2 KO mice. A: Wild-type and PCPE2 KO mouse HDL was analyzed by IEF electrophoresis and stained with Coomassie blue as indicated in Methods. For comparison, a mixture of HDL isolated from various mouse models was included. B: Wild-type and PCPE2$^{-/-}$ HDL was isolated by ultracentrifugation, subjected to tryptic digestion, and resolved by reverse phase HPLC as described in Methods. Amino terminal peptides DEPQQWDK and DEPQQWDKVK, representing mature apoA-I, and WHVWQQDEPQSQWDVK, representing the propeptide of apoA-I, were quantified by MRM mass spectrometry.

Fig. 1B, the N-terminal peptide WHVWQQDEPQSQWDVK was clearly present in PCPE2 KO mice, confirming the presence of pro-apoA-I. The magnitude of the peaks was not indicative of the proportion of pro and mature apoA-I present in plasma of PCPE2 KO mice due to different ionization efficiencies in the mass spectrometer, but the change in ratio of signal for pro and mature peptides in the two samples quantified the difference in the amount of pro-apoA-I present.

Plasma lipoproteins in wild-type and PCPE2 KO mice

To assess the consequences of the deletion of PCPE2 and increased pro-apoA-I on plasma lipids, PCPE2 KO and wild-type control littermates fed a chow or high-fat diet were studied. As shown in **Table 1**, male and female PCPE2 KO mice had a 2-fold increase in apoA-I levels compared with wild-type mice. ApoA-I mRNA levels were determined in intestines and livers of wild-type and PCPE2 KO mice as a biomarker for apoA-I synthesis. No changes were observed in PCPE2 KO mice compared with wild-type littermates, suggesting that the increase in plasma levels of apoA-I observed in PCPE2 KO mice was not the consequence of increased synthesis (data not shown). HDL-C was also increased in male and female PCPE2 KO mice, albeit not to the same extent as apoA-I. The absence of PCPE2 did not alter apoB-containing lipoproteins, as attested to by the plasma levels of very low density lipoproteins + low density lipoproteins (V+LDL) cholesterol and apoB. To confirm that the impact of PCPE2 on plasma apoA-I and HDL occurs irrespective of dietary conditions, wild-type and PCPE2 KO mice were metabolically stressed by being fed a high-fat, high-cholesterol diet for four weeks. As shown in **Table 2**, plasma levels of apoA-I were increased by ~50% in male and female PCPE2 KO mice. HDL-cholesterol was marginally increased in males and significantly increased in female PCPE2 KO mice. While V+LDL cholesterol and apoB levels were not impacted by diet, there was a significant increase in plasma triglycerides in PCPE2 KO mice compared with wild-type littermates.

Size fractionation and characterization of HDL in wild-type and PCPE2 KO mice

To further explore the impact of the absence of PCPE2 and increased pro-apoA-I on HDL speciation, pooled plasmas from PCPE2 KO and wild-type control mice were fed a chow diet and fractionated by FPLC as described (17). As shown in **Fig. 2**, the increased apoA-I observed in PCPE2 KO mice was present in the HDL fraction. No redistribution of apoA-I onto the VLDL or LDL fractions was observed. Consistent with the lipid profiles (Table 1), a small increase in cholesterol was observed in HDL. The disproportionate increase in apoA-I and HDL-C observed in PCPE2 KO mice suggests that the composition of HDL in PCPE2 KO mice was altered. To assess the lipid composition of wild-type and PCPE2 KO HDL, FPLC fractions corresponding to HDL were pooled and concentrated, and then phospholipids, triglycerides, and cholesterol were

TABLE 1. Plasma lipids, lipoproteins, apoA-I and apoB levels in wild-type and PCPE2 KO

Mice	TC	TG	V+LDL	HDL	ApoA-I	ApoB
WT males (n = 4)	97 ± 3	68 ± 14	20 ± 4	70 ± 15	0.6 ± 0.1	0.07 ± 0.03
KO males (n = 6)	109 ± 1	76 ± 2	15 ± 2	94 ± 11[a]	1.2 ± 0.4[b]	0.05 ± 0.01
WT females (n = 4)	70 ± 6	55 ± 3	13 ± 1	56 ± 6	0.4 ± 0.1	0.08 ± 0.01
KO females (n = 5)	82 ± 5	73 ± 19	13 ± 3	70 ± 5[b]	0.7 ± 0.2[b]	0.07 ± 0.01

Animals were age-matched and fed a chow diet. Total cholesterol (TC), triglycerides (TG), V+LDL, and HDL are expressed as milligrams per deciliter. ApoA-I and apoB concentrations are expressed as milligrams per milliliter.

[a] $P < 0.05$.

[b] $P < 0.005$.

TABLE 2. Plasma lipids, lipoproteins, apoA-I, and apoB levels in wild-type and PCPE2 KO mice

Mice	TC	TG	V+LDL	HDL	ApoA-I	ApoB
WT males (n = 4)	199 ± 34	17 ± 1	69 ± 33	129 ± 12	1.1 ± 0.2	0.08 ± 0.03
KO males (n = 6)	228 ± 47	46 ± 25[a]	80 ± 29	147 ± 27	1.5 ± 0.2[b]	0.09 ± 0.02
WT females (n = 4)	122 ± 25	7 ± 2	38 ± 10	84 ± 18	0.9 ± 0.2	0.06 ± 0.01
KO females (n = 5)	180 ± 39	28 ± 21[b]	59 ± 23	121 ± 25[a]	1.4 ± 0.3[b]	0.08 ± 0.01

Age-matched wild-type and PCPE2 KO mice were fed a high-fat, high-cholesterol diet for four weeks as described in Methods. Total cholesterol (TC), triglycerides (TG), V+LDL, and HDL are expressed as milligrams per deciliter. Plasma concentrations of apoA-I and apoB are expressed as milligrams per milliliter.

[a] $P < 0.05$.

[b] $P < 0.005$.

determined by commercially available kits. While the percentage composition of the major lipid classes was not altered, there was a 2-fold increase in apoA-I in HDL isolated from PCPE2 KO mice (**Table 3**). The increased apoA-I in PCPE2 KO mice relative to any of the lipids assayed on HDL suggests that there was a defect in the degree of lipidation and maturation of HDL in PCPE2 KO mice. As an example (Table 3), the apoA-I to cholesterol ratio is increased in PCPE2 KO compared with wild-type littermates (1 and 0.7 for PCPE2 KO and wild-type mice, respectively). Cholesterol to cholesteryl ester ratio was not altered, suggesting that the LCAT-mediated esterification of plasma cholesterol was not impaired in PCPE2 KO mice (data not shown).

Further characterization of HDL in PCPE2 KO mice was accomplished by running agarose gel electrophoresis,

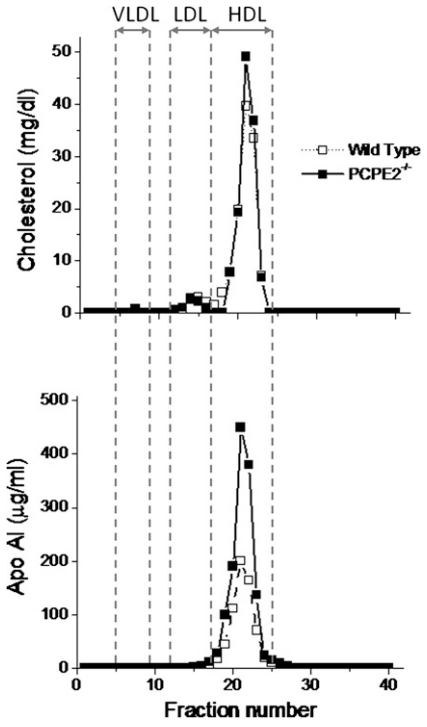

Fig. 2. Plasma lipoprotein cholesterol and apoA-I distribution in mice of different phenotypes. Two-hundred microliters of pooled plasma from fasted wild-type and PCPE2 KO mice were fractionated by FPLC as described in Methods. Cholesterol and apoA-I were determined and plotted as a function of FPLC fractions. The position at which known lipoproteins eluted from the column is indicated.

which separates HDL by charge into preß-HDL and α-HDL species. ApoA-I-containing HDL was visualized using an anti-apoA-I antibody. While α-migrating HDL was not different between wild-type and PCPE2 KO mice (**Fig. 3A**), the migration of preß-HDL was significantly slower than wild-type HDL. Because preß-HDL contains only apoA-I, which dictates its migration by agarose gel electrophoresis, these findings suggest that the increased presence of pro-apoA-I in PCPE2 KO mice markedly altered the physicochemical properties of these particles. Consistent with this hypothesis, resolution of HDL species by one-dimensional gradient gel electrophoresis identified a slightly larger HDL species within the preß-HDL size range compared with wild-type littermates (Fig. 3B). Changes in α-migrating HDL were also apparent when plasma samples from wild-type and PCPE2 KO mice were fractionated by two-dimensional gradient gel electrophoresis. As shown in Fig. 3C, there was an increased proportion of α-HDL present in a larger HDL population. This larger population of HDL observed in PCPE2 KO mice may have accommodated additional apoA-I molecules and may have accounted, at least in part, for the increased apoA-I levels observed in plasma from those mice.

Cholesterol efflux

A series of experiments were undertaken to evaluate whether the changes in HDL size and physicochemical properties observed in PCPE2 KO mice have an impact on the ability of HDL to move cholesterol in and out of macrophages. ApoB-containing lipoproteins were removed from plasma by precipitation, and the remaining supernatants containing HDL were incubated with FC-enriched J774 macrophages according to the protocol described in Methods. While the movement of cholesterol into (influx) and out of (efflux) macrophages was significantly increased by HDL from PCPE2 KO mice (**Fig. 4A**, B), the overall net loss of cellular cholesterol (Fig. 4C) did not change despite increases in apoA-I and HDL-C observed in PCPE2 KO mice, suggesting that HDL from PCPE2 KO mice is not as efficient as wild-type HDL in releasing cholesterol from macrophages. To further address these findings, cholesterol influx, efflux, and net loss were normalized to the apoA-I and HDL-C levels in the mouse serum, which are well-known indicators of HDL function and efflux potential. As shown in **Fig. 5C**, normalizing flux to the serum concentration of apoA-I resulted in a significant and prominent reduction in the net amount of cholesterol

TABLE 3. HDL lipid composition in wild-type and PCPE2 KO mice

Mice	PL	TG	TC	ApoA-I	ApoA-I/TC
Wild-type	30.1 (56%)	1.2 (2%)	22.8 (42%)	16.4	0.7
PCPE2 KO	39.3 (55%)	1.5 (2%)	30.7 (43%)	30.6	1.0

Two hundred microliters of pooled plasma from control and PCPE2 KO mice were used to isolate HDL by FPLC as described in Methods. Tubes containing the HDL lipoprotein fractions were pooled and concentrated. Phospholipids (PL), triglycerides (TG), total cholesterol (TC), and apoA-I were determined as described in Methods. Lipids and apoA-I are expressed in milligrams per deciliter. Shown in parentheses is the percentage composition relative to the sum of TG, PL, and TC.

released from macrophages incubated with PCPE2 KO HDL. The decrease in net loss of cholesterol to HDL from PCPE2 KO compared with wild-type HDL was mainly driven by the decrease in the efflux of cholesterol (Fig. 5). On the contrary, no significant changes were observed when the movement of cholesterol in and out of macrophages was normalized to levels of cholesterol present in the HDL fraction (data not shown). The serum concentration of apoA-I could be taken as an estimate of the number of HDL particles present. Thus the results shown in Fig. 5C indicated that, although more numerous, HDL particles in serum from KO mice had a reduced capacity to promote cellular cholesterol efflux.

In an effort to understand the mechanism implicated in the decreased ability of PCPE2 KO mice HDL to efflux cholesterol from macrophages, an experiment was designed to measure ABCA1 and SR-BI-dependent efflux as well as the remaining cholesterol efflux via aqueous diffusion (**Fig. 6**). As shown in Fig. 6B, C, E, and F, no changes in efflux of cholesterol driven by either SR-BI or aqueous diffusion were observed when PCPE2 KO mouse HDL was used. On the contrary, when normalized to apoA-I content, HDL from PCPE2 KO mice showed a significant decrease in the ability to efflux cholesterol through the ABCA1 pathway (Fig. 6A, D). Taken together, these results suggest that, in the absence of PCPE2, the population of HDL particles in serum are less efficient in promoting efflux via ABCA1, resulting in a decreased net loss of macrophage cholesterol mass.

DISCUSSION

ApoA-I is the major protein component of HDL. The liver and small intestine are the main organs of synthesis in both mammals and birds (21, 22). ApoA-I appears intracellularly as an intermediate precursor (pro-apoA-I) with a hexapeptide extension (RHFWQQ) at its amino (N-) terminus (23). Proteolytic processing of pro-apoA-I to apoA-I occurs in extracellular fluids and plasma via BMP-1 (16). Cleavage of the N-terminal hexapeptide adjacent to two acidic amino acids (RHFWQQ↓DEPP) generates the mature 243 amino acids protein responsible for the assembly and biogenesis of HDL (24). ApoA-II, the second major apolipoprotein of HDL, is also synthesized and secreted as pro-apolipoprotein (25). Recently in a series of biochemical experiments, we demonstrated that, in addition to BMP-1, there is another player in the process of apoA-I maturation, procollagen C-proteinase enhancer-2 (PCPE2) (10). Immunoprecipitation and surface plasmon resonance studies have established the molecular mechanisms of the interaction of apoA-I, BMP-1, and PCPE2. BMP-1 binds to pro-apoA-I first, followed by PCPE2 forming the ternary complex pro-apoA-I/BMP-1/PCPE2, which doubles the rate of pro-peptide cleavage and the formation of mature apoA-I (10). Sequence similarities between BMP-1 and PCPE2 in humans and mice are over 80%. The association between genetic polymorphisms within the PCPE2 gene and HDL levels (9, 10) together with current biochemical studies (10) provide compelling evidence for a role of PCPE2 in plasma HDL biogenesis. Its physiological impact, however, remains unclear as no rare mutations are known to occur in humans or rodents. Mice, like humans,

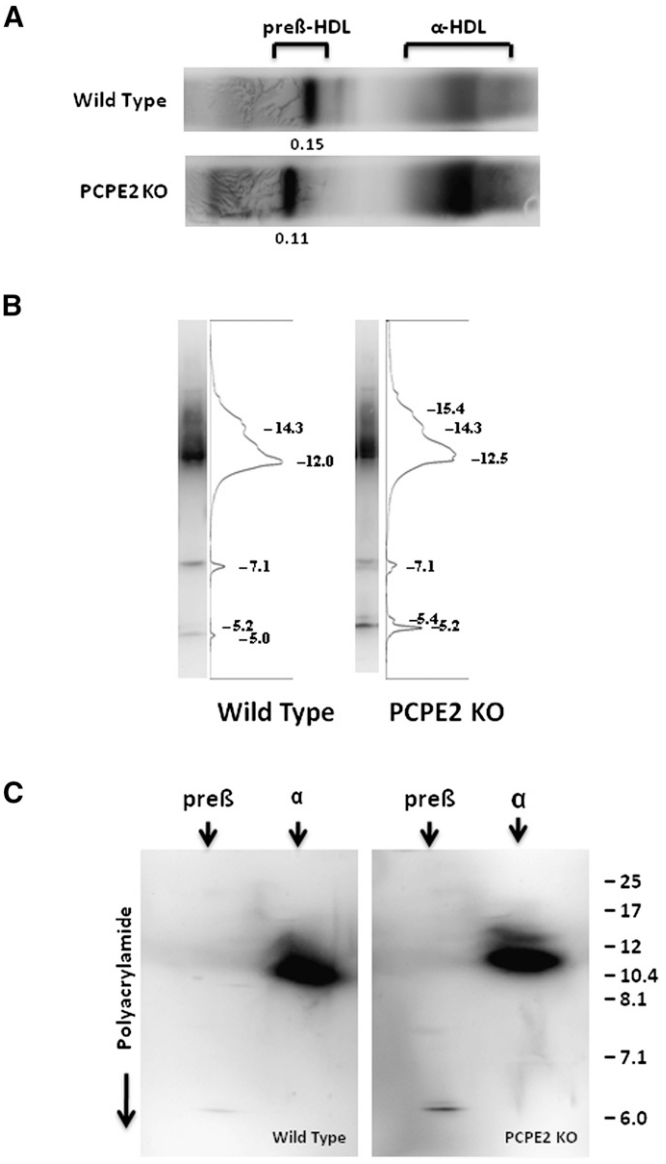

Fig. 3. Characterization of HDL in wild-type and PCPE2 KO mice. Plasma from wild-type and PCPE2 KO mice was used to assess their migration properties in agarose gel and nondenaturing polyacrylamide electrophoresis as described in Methods.

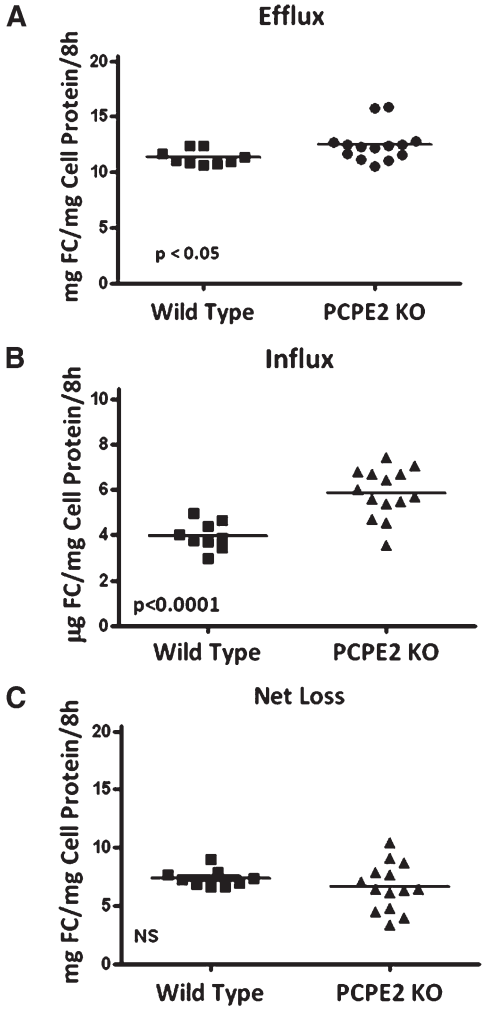

Fig. 4. Cholesterol flux between FC-enriched, upregulated J774 macrophages and apoB-depleted serum from wild-type and PCPE2 KO mice. J774 cells were enriched in free cholesterol (FC) and radiolabeled by 24 h incubation in medium containing 50 µg/ml Ac LDL and 2 µCi/ml ^{3}H-cholesterol in the presence of an ACAT inhibitor and then treated with c-AMP for an additional 16 h to upregulate cholesterol transporters. Estimates of cholesterol mass flux were obtained after 8 h incubation with 2.8% apoB-depleted mouse serum from the change in specific activity of cellular cholesterol measured as described in Methods. The cellular FC content of cells before incubation with the various apoB-depleted serum was 48.7 µg FC/mg cellular protein. Each data point represents average flux to apoB-depleted serum from a single mouse. Significant differences ($P < 0.05$) were obtained by unpaired, two-tailed t test, and cholesterol mass is expressed as micrograms of FC per milligrams of cellular protein. A: Efflux of cellular cholesterol to 2.8% (equivalent to 2% serum) apoB-depleted serum from wild-type and PCPE2 KO mice. B: Influx of HDL-cholesterol into J774 macrophages. C: Net loss of cellular cholesterol calculated as efflux minus influx.

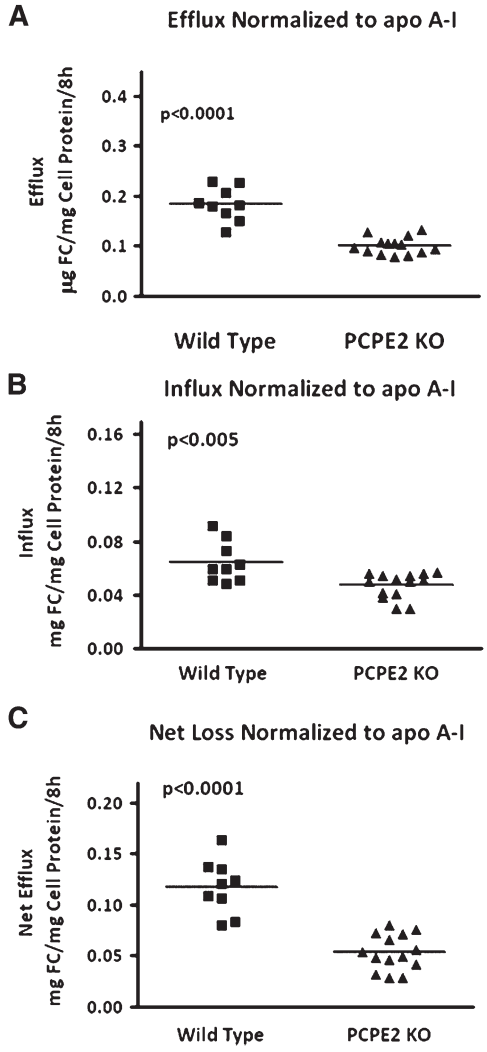

Fig. 5. FC flux normalized to serum apoA-I and HDL-C values. Estimates of cellular FC flux to 2.8% apoB-depleted serum shown in Fig. 4 were normalized to (i.e., divided by) either the serum concentration of apoA-I (panels A–C). The average concentrations in serum from wild-type mice were apoA-I = 60 mg/dl and HDL-C = 37 mg/dl. The average concentrations in serum from PCPE2 KO mice were apoA-I = 130 mg/dl and HDL-C = 51 mg/dl. Statistical significance was established by unpaired, two-tailed t-test.

secrete apoA-I in the form of pro-apoA-I containing a hexapeptide extension at the N-terminal that is processed extracellularly, suggesting that a process described for humans occurs in rodents. In addition, the murine hexapeptide extension and cleavage site WHVWQQ↓DEPP is identical to that in humans, with the exception of the tryptophan residue at position -6 in place of an arginine in the human sequence and a valine replacing a phenylalanine at the -4 position (26).

The findings from our experiments using PCPE2 KO mice confirm the role of PCPE2 in the processing of pro-apoA-I. While almost undetectable levels of pro-apoA-I were observed in the plasma of wild-type mice, the presence of the N-terminal WHVWQQ↓DEPPSQWDK peptide containing the propeptide WHVWQQ in apoA-I isolated from PCPE2 KO mice demonstrated that in mice, like humans, PCPE2 participated in the processing and maturation of apoA-I. Unfortunately, we could not determine the exact proportion of unprocessed pro-apoA-I circulating in the plasma of PCPE2 KO mice because there are no commercially available antibodies. In addition, the peak intensities of the N-terminal peptides from mature and pro-apoA-I analyzed by MRM mass spectrometry are misleading, as they are affected not only by amount but also by their ionization properties. Nevertheless, several lines

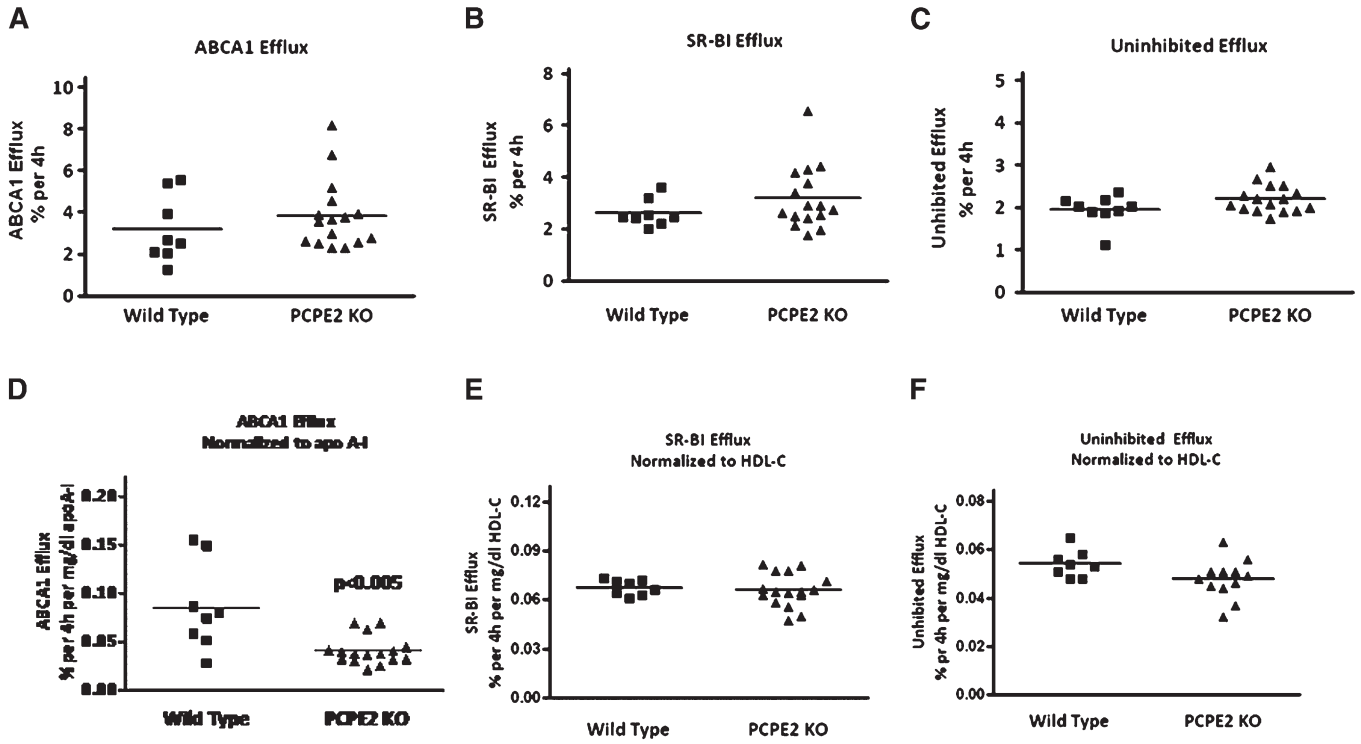

Fig. 6. Efflux of cellular FC via different pathways. Pathway-specific efflux of radiolabeled cholesterol to 2.8% apoB-depleted serum from WT and KO mice was measured as described in Methods. Each point represents average efflux to apoB-depleted serum from a single mouse. Specifically, ABCA1 efflux (A, D) was the difference in efflux between upregulated J774 cells and control cells, and SR-BI (B, E) was the BLT-1-sensitive efflux from Fu5AH cells. Uninhibited efflux (C, F) was the BLT-I-resistant efflux from Fu5AH cells and is equivalent to aqueous diffusion. Panels D–F show the pathway-specific efflux normalized to (i.e., divided by) apoA-I (D) or HDL-C (E, F) serum concentrations. The average concentrations in apoB-depleted serum from wild-type mice were apoA-I = 48 mg/dl and HDL-C = 38 mg/dl. The average concentrations in PCPCE2 KO mice were apoA-I = 95 mg/dl and HDL-C = 48 mg/dl. Statistical significance was estimated by unpaired, two-tailed *t*-tests.

of evidence suggest that a significant amount of the apoA-I in plasma of mice deficient in PCPE2 was present as a proform compared with wild-type littermates. IEF electrophoresis of apoA-I from wild-type and PCPE2 KO mice showed a shift in the isoelectric point, supporting the presence of a more basic isoform of apoA-I in the PCPE2 KO mice. Trypsin digestion, HPLC, and identification of amino terminal peptides by mass spectrometry demonstrated the presence of pro-apoA-I in PCPE2 KO plasma. In addition, the electrophoretic migration of PCPE2 KO preß-HDL in agarose gels was significantly slower than those present in wild-type mouse plasma. As preß-HDL contained only apoA-I (27), these findings were consistent with the predicted increase in negative charges in pro-apoA-I at basic pH. Interestingly, neither IEF of apoA-I nor agarose gel electrophoresis of plasma HDL showed the presence of apoA-I and preß-HDL with the physical chemical properties of native, wild-type species, suggesting that the majority of apoA-I present in PCPE2 KO mice was in the pro form. Although unlikely, it is also possible that the presence of one pro-apoA-I molecule in preß-HDL was sufficient to abrogate the physical chemical properties of wild-type preß-HDL.

Our findings in PCPE2 KO mice confirm the relationship between polymorphisms in the human PCPE2 gene and HDL levels. Upon investigation of three distinct populations (8, 9), only a weak and suggestive association between polymorphisms located 5′ and within introns 5 and 6 of the PCPE2 gene and HDL levels was shown. It is important to acknowledge that a strong association of polymorphisms and HDL levels is only phenotypically manifested for a few genes with either high penetrance or studied in large populations (28). A remarkable example is the gene coding for HMG-CoA reductase (HMGCR). HMGCR; the target enzyme of the most widely used class of drugs for the treatment of cardiovascular disease; shows weak and sporadic association with LDL-C and statin response (29–32). Nonetheless, biochemical and other data convincingly show the major influence that HMGCR plays on the regulation of LDL-C levels. Thus, in what is likely to become a more common paradigm, biochemical and functional studies on a gene will have to be undertaken to complement the genetic findings to determine whether weak genetic effects have any functional relevance. To date, the extent to which it would be possible to explain a low or high HDL phenotype in a person by studying the genetic makeup is not known. On the basis of the present literature, Holleboom et al. (28) denoted 10 genes with proven association to HDL-C levels (ApoA-I, LCAT, LIPC, CETP, SCARB1, ABCG1, ATP5B, PLTP, LIPG, and APOM). Recently, several genome-wide association studies of circulating lipid levels have identified numerous loci

(7, 8). Nevertheless, a substantial portion of the heritability of these traits remains unexplained (33). Our biochemical studies together with the examination of the PCPE2 KO mice provide compelling evidence in support of an association between PCPE2 and plasma HDL levels, highlighting the need for biochemical and animal studies to bring mechanistic and physiological relevance to those genetic associations observed in genes with unprecedented function and unknown molecular mechanisms.

While the anti-atherogenic role of HDL has been extensively studied and demonstrated for many decades, only recently it has been acknowledged that HDL is highly heterogeneous in its lipid and protein composition (34, 35), leading to multiple distinct species with different functions and metabolic fates. Very recently (36), an association between HDL-C efflux and carotid intima media thickness has been shown, suggesting that measuring cholesterol efflux can add a prognostic value as a clinical marker for HDL functionality. Our studies using PCPE2 KO mice demonstrate that changes in apoA-I processing and maturation impaired the ability of HDL to efflux cholesterol. Most importantly, our studies indicate that the net loss of cholesterol from cells was not increased in PCPE2 KO mice, despite the marked increase in plasma apoA-I and HDL-C levels. Further examination of the various pathways involved in cholesterol efflux demonstrated a significant reduction in the ability to efflux cholesterol through the ABCA1 pathway. As the ABCA1 transporter is responsible for the efflux of cholesterol and initial lipidation of apoA-I to initiate the biogenesis of HDL, these findings suggest that the presence of pro-apoA-I could impair the natural ability of nascent, preß-HDL to efflux cholesterol. The biological properties of pro-apoA-I remain unclear as the published reports used reconstituted particles that mimic in vivo intermediates between free apoA-I and mature spherical HDL. Whereas several studies have shown that pro-apoA-I is biologically active with lipid binding properties (37), LCAT activation (38), and cholesterol efflux essentially identical to those for mature apoA-I, two independent reports (16, 39) demonstrated that the efflux of cholesterol and phospholipids to pro-apoA-I was about half that of mature apoA-I. More refined structural data using HDL isolated from PCPE2 KO mouse plasma will be needed to assess the structural impact and elucidate the mechanism whereby the presence of the WHVWQQ sequence in pro-apoA-I disturbs the interaction with ABCA1 and subsequent lipidation of apoA-I. Nevertheless, these findings further support the current hypothesis that assessing the functionality of HDL may be more important than measuring its levels. Further studies will have to be conducted to ascertain whether these findings have an impact on the development of atherosclerosis.

The mechanism responsible for the increased plasma levels of apoA-I and HDL-C observed in PCPE2 KO mice is unclear. Lack of changes in the expression of apoA-I in intestines and livers of PCPE2 KO mice strongly suggest that the increase in apoA-I is not due to changes in the production rates of apoA-I. Similarly, no changes in the expression levels of HMGCR or ABCA1 were observed in livers and intestines from PCPE2 KO mice. The changes in particle sizes shown in PCPE2 KO mice, although minimal; together with the presence of a larger α-migrating HDL species shown by two-dimensional gradient gel electrophoresis suggest that the intravascular remodeling of HDL might be altered. Interestingly, the discordant increase in lipids (approximately 30%) and apoA-I (2-fold) suggests, at least in part, an increase in the number of apoA-I molecules in PCPE2 KO mouse HDL compared with wild-type mice. Changes in the number of apoA-I molecules per HDL particle have been previously observed in human apoA-I transgenic mice and have been associated with an increased particle size and decreased catabolic rate, resulting in the buildup of HDL in the plasma compartment. Kinetic studies will have to be performed in subsequent experiments to address whether a decreased clearance is responsible for the accumulation of apoAI-enriched HDL in PCPE2 KO mice. In addition, changes in the metabolism of triglyceride-rich lipoproteins could also contribute to the increased levels of apoA-I and HDL-C observed in PCPE2 KO mice.

Taken together, the findings from these studies implicate a new player in the regulation of apoA-I posttranslational processing. The combination of genetic association data, biochemical experiments (11), and characterization of PCPE2 KO mice unraveled a functional role for a previously poorly characterized gene with no known role in lipids. This study exemplifies what will have to be done in the future to generate practical information from weak genetic data or genes with unknown function.

REFERENCES

1. Robins, S. J., D. Collins, J. T. Wittes, V. Papademetriou, P. C. Deedwania, E. J. Schaefer, J. R. McNamara, M. L. Kashyap, J. M. Hershman, L. F. Wexler, et al. 2001. Relation of gemfibrozil treatment and lipid levels with major coronary events. VA-HIT: a randomized controlled trial. *JAMA.* **285:** 1585–1591.
2. Manninen, V., M. O. Elo, M. H. Frick, K. Haapa, O. P. Heinonen, P. Heinsalmi, P. Helo, J. K. Huttunen, P. Kaitaniemi, P. Koskinen, et al. 1988. Lipid alterations and decline in the incidence of coronary heart disease in the Helsinki Heart Study. *JAMA.* **260:** 641–651.
3. Tall, A. R., L. Yvan-Charvet, N. Terasaka, T. Pagler, and N. Wang. 2008. ABC transporters and cholesterol efflux: implications for the treatment of atherosclerosis. *Cell Metab.* **7:** 365–375.
4. Navab, M., S. T. Reddy, B. J. Van Lenten, and A. M. Fogelman. 2011. HDL and cardiovascular disease: atherogenic and atheroprotective mechanisms. *Nat. Rev. Cardiol.* **8:** 222–232.
5. Goode, E. L., S. S. Cherny, J. C. Christian, G. P. Jarvik, and M. de Andrade. 2007. Heritability of longitudinal measures of body mass index and lipid and lipoprotein levels in aging twins. *Twin Res. Hum. Genet.* **10:** 703–711.
6. Spirin, V., S. Schmidt, A. Pertsemlidis, R. S. Cooper, J. C. Cohen, and S. R. Sunyaev. 2007. Common single-nucleotide polymorphisms act in concert to affect plasma levels of high-density lipoprotein cholesterol. *Am. J. Hum. Genet.* **81:** 1298–1303.
7. Teslovich, T. M., K. Musunuru, A. V. Smith, A. C. Edmondson, I. M. Stylianou, M. Koseki, J. P. Pirruccello, S. Ripatti, D. I. Chasman, C. J. Willer, et al. 2010. Biological, clinical, and population relevance of 95 loci for blood lipids. *Nature.* **466:** 707–713.
8. Willer, C. J., S. Sanna, A. U. Jackson, A. Scuteri, L. L. Bonnycastle, R. Clarke, S. C. Heath, N. J. Timpson, S. S. Najjar, H. M. Stringhman, et al. 2008. Newly identified loci that influence lipid concentrations and risk of coronary artery disease. *Nat. Genet.* **40:** 161–169.
9. Xu, H., T. A. Acott, and M. K. Wirtz. 2000. Identification and expression of a novel type I procollagen C-proteinase enhancer

protein gene from the glaucoma candidate region on 3q21-q24. *Genomics.* **66:** 264–273.
10. Hinds, D. A., A. B. Seymour, L. K. Durham, P. Banerjee, P. G. Milos, D. R. Cox, J. F. Thompson, and K. A. Frazer. 2004. Application of pooled genotyping to scan candidate regions for association with HDL cholesterol levels. *Hum. Genomics.* **1:** 421–434.
11. Zhu, J., J. Gardner, C. R. Pullinger, J. P. Kane, J. F. Thompson, and O. L. Francone. 2009. Regulation of apoAI processing by procollagen C-proteinase enhancer-2 and bone morphogenetic protein-1. *J. Lipid Res.* **50:** 1330–1339.
12. Steiglitz, B. M., D. R. Keene, and D. S. Greenspan. 2002. PCOLCE2 encodes a functional procollagen C-proteinase enhancer (PCPE2) that is a collagen-binding protein differing in distribution of expression and post-translational modification from the previously described PCPE1. *J. Biol. Chem.* **277:** 49820–49830.
13. Weiss, T., S. Ricard-Blum, L. Moschcovich, E. Wineman, S. Mesilaty, and E. Kessler. 2010. Binding of procollagen C-proteinase enhancer-1 (PCPE-1) to heparin/heparin sulfate. Properties and role in pcpe-1 interaction with cells. *J. Biol. Chem.* **285:** 33867–33874.
14. Bekhouche, M., D. Kronenberg, S. Vadon-Le Goff, C. Bijakowski, N. H. Lim, B. Font, E. Kessler, A. Colige, H. Nagase, G. Murphy, et al. 2010. Role of the netrin-like domain of procollagen C-proteinase enhancer-1 in the control of metalloproteinase activity. *J. Biol. Chem.* **285:** 15950–15959.
15. Heinzel, K., and C. C. Bleul. 2007. The Foxn1-dependent transcripts PCOLCE2 and mPPP1R6B are not required for normal thymopoiesis. *Eur. J. Immunol.* **37:** 2562–2571.
16. Chau, P., P. E. Fielding, and C. J. Fielding. 2007. Bone morphogenetic protein-1 (BMP-1) cleaves human proapolipoprotein A1 and regulates its activation for lipid binding. *Biochemistry.* **46:** 8445–8450.
17. Aiello, R. J., D. Brees, P-A. Bourassa, L. Royer, S. Lindsey, R. Coskran, M. Haghpassand, and O. L. Francone. 2002. Increased atherosclerosis in hyperlipidemic mice with inactivation of ABCA1 in macrophages. *Arterioscler. Thromb. Vasc. Biol.* **22:** 630–637.
18. Zimetti, F., G. K. Weibel, M. Duong, and G. H. Rothblat. 2006. Measurment of cholesterol bidirectional flux between cells and lipoproteins. *J. Lipid Res.* **47:** 605–613.
19. Asztalos, B. F., M. de la Llera-Moya, G. E. Dallal, K. V. Horvath, E. J. Schaefer, and G. H. Rothblat. 2005. Differential effects of HDL subpopulations on cellular ABCA1- and SR-BI-mediatd cholesterol efflux. *J. Lipid Res.* **46:** 2246–2253.
20. Adorni, M. P., F. Zimetti, J. T. Billheimer, N. Wang, D. J. Rader, M. C. Phillips, and G. H. Rothblat. 2007. The roles of different pathways in the release of cholesterol from macrophages. *J. Lipid Res.* **48:** 2453–2462.
21. Sliwkowski, M. B., and H. G. Windmueller. 1984. Rat liver and small intestine produce proapolipoprotein A-I which is slowly processed to apolipoprotein A-I in the circulation. *J. Biol. Chem.* **259:** 6459–6465.
22. Rajavashisth, T. B., P. A. Dawson, D. L. Williams, J. E. Shackelford, H. Lebherz, and A. J. Lusis. 1987. Structure, evolution and regulation of chicken apolipoprotein A-I. *J. Biol. Chem.* **262:** 7058–7065.
23. Zannis, V. I., S. K. Karathanasis, H. T. Keutmann, G. Goldberger, and J. L. Breslow. 1983. Intracellular and extracellular processing of human apolipoprotein A-I: secreted apolipoprotein A-I isoprotein2 is a propeptide. *Proc. Natl. Acad. Sci. USA.* **80:** 2574–2578.
24. Zannis, V. I., A. Chroni, and M. Krieger. 2006. Role of apoAI, ABCA1, LCAT, and SR-BI in the biogenesis of HDL. *J. Mol. Med.* **84:** 276–294.
25. Stoffel, W. 1984. Synthesis, transport, and processing of apolipoproteins of high density lipoproteins. *J. Lipid Res.* **25:** 1586–1592.
26. Januzzi, J. L., N. Azrolan, A. O'Connell, K. Aalto-Setala, and J. L. Breslow. 1992. Characterization of the mouse apolipoprotein Apoa-1/Apoc-3 gene locus: genomic, mRNA, and protein sequences with comparisons to other species. *Genomics.* **14:** 1081–1088.
27. Castro, G. R., and C. J. Fielding. 1988. Early incorporation of cell-derived cholesterol into pre-beta migrating high-density lipoproteins. *Biochemistry.* **27:** 25–29.
28. Holleboom, A. G., M. Vergeer, G. Kees Hovingh, J. J. P. Kastelein, and J. A. Kuivenhoven. 2008. The value of HDL genetics. *Curr. Opin. Lipidol.* **19:** 385–394.
29. Thompson, J. F., C. L. Hyde, L. S. Wood, S. A. Paciga, D. A. Hinds, D. R. Cox, G. K. Hovingh, and J. J. P. Kastelein. 2009. Comprehensive whole genome and candidate gene analysis for response to statin therapy in TNT cohort. *Circ. Cardiovasc. Genet.* **2:** 173–181.
30. Chasman, D. I., D. Posada, L. Subrahmanyan, N. R. Cook, V. P. Stanton, and P. M. Ridker. 2004. Pharmacogenomic study of statin therapy and cholesterol reduction. *JAMA.* **291:** 2821–2827.
31. Thompson, J. F., M. Man, K. J. Johnson, L. S. Wood, M. E. Lira, D. B. Lloyd, P. Banerjee, P. M. Milos, S. P. Myrand, J. Pauluaskis, et al. 2005. An association study of 43 SNPs in 16 candidate genes with atorvastatin response. *Pharmacogenomics J.* **5:** 352–358.
32. Krauss, R. M., L. M. Mangravite, J. D. Smith, M. W. Medina, D. Wang, X. Guo, M. J. Rieder, J. A. Simon, S. B. Hulley, D. Waters, et al. 2008. Variation in the 3-hydroxyl-3-methylglutaryl coenzyme a reductase gene with racial differences in low-density lipoprotein cholesterol response to simvastatin treatment. *Circulation.* **117:** 1537–1544.
33. Demirkan, A., N. Amin, A. Isaacs, M-R. Jarvelin, J. B. Whitfield, H. E. Wichmann, K. O. Kyvik, I. Rudan, C. Gieger, A. A. Hicks, et al. 2011. Genetic architecture of circulating lipid levels. *Eur. J. Hum. Genet.* **19:** 813–819.
34. Rye, K-A., C. A. Bursill, G. Lambert, F. Tabet, and P. J. Barter. 2009. The metabolism and anti-atherogenic properties of HDL. *J. Lipid Res.* **50(Suppl.):** S195–S200.
35. Rothblat, G. H., and M. C. Phillips. 2010. High-density lipoprotein heterogeneity and function in reverse cholesterol transport. *Curr. Opin. Lipidol.* **21:** 229–238.
36. Khera, A. V., M. Cuchel, M. de la Llera-Moya, A. Rodrigues, M. F. Burke, K. Jafri, B. C. French, J. A. Phillips, M. L. Mucksavage, R. L. Wilensky, et al. 2011. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N. Engl. J. Med.* **364:** 127–135.
37. McGuire, K. A., W. S. Davidson, and A. Jonas. 1996. High yield oer-expression and characterization of human recombinant proapolipoprotein A-I. *J. Lipid Res.* **37:** 1519–1528.
38. Huang, W., A. Matsunaga, W. Li, H. Han, A. Hoang, M. Kugi, T. Koga, D. Sviridov, N. Fidge, and J. Sasaki. 2001. Recombinant proapoA-I (Lys107del) shows impaired lipid binding associated with reduced binding to plasma high density lipoprotein. *Atherosclerosis.* **159:** 85–91.
39. Sviridov, D., L. Pyle, and N. Fidge. 1996. Efflux of cellular cholesterol and phospholipid to apolipoprotein A-I mutants. *J. Biol. Chem.* **271:** 33277–33283.