The N-terminal Globular Domain and the First Class A Amphipathic Helix of Apolipoprotein A-I Are Important for Lecithin:Cholesterol Acyltransferase Activation and the Maturation of High Density Lipoprotein in Vivo*

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Brian R. Scott‡‡§§, Dan C. McManus‡§§, Vivian Franklin‡, Andrea G. McKenzie§§, Tracey Neville‡, Daniel L. Sparks§§**, and Yves L. Marcel§§‡‡

From the Liprotein and Atherosclerosis Research Group, the Department of Pathology & Laboratory Medicine and the Department of Biochemistry, Microbiology and Immunology, University of Ottawa Heart Institute, Ottawa, Ontario K1Y 4W7, Canada.

To investigate the role of the N terminus of apolipoprotein A-I (apoA-I) in the maturation of high density lipoproteins (HDL), two N-terminal mutants with deletions of residues 1–43 and 1–65 (referred to as Δ1–43 and Δ1–65 apoA-I) were studied. In vitro, these deletions had little effect on cellular cholesterol efflux from macrophages, but LCAT activation was reduced by 50 and 70% for the Δ1–43 and Δ1–65 apoA-I mutants, respectively, relative to wild-type (Wt) apoA-I. To further define the role of the N terminus of apoA-I in HDL maturation, we constructed recombinant adenoviruses containing Wt apoA-I and two similar mutants with deletions of residues 7–43 and 7–65 (referred to as Δ7–43 and Δ7–65 apoA-I, respectively). Residues 1–6 were not removed in these mutants to allow proper cleavage of the pro-sequence in vivo. Following injection of these adenoviruses into apoA-I-deficient mice, plasma concentrations of both Δ7–43 and Δ7–65 apoA-I were reduced 4-fold relative to Wt apoA-I. The N-terminal deletion mutants, in particular Δ7–65 apoA-I, were associated with greater proportions of preβ-HDL and accumulated fewer HDL cholesteryl esters relative to Wt apoA-I. Wt and Δ7–43 apoA-I formed predominantly α-migrating and spherical HDL, whereas Δ7–65 apoA-I formed only preβ-HDL of discoidal morphology. This demonstrates that deletion of the first class A amphipathic α-helix has a profound additive effect in vivo over the deletion of the globular domain alone (amino acids 1–43) indicating its important role in the production of mature α-migrating HDL. In summary, the combined in vitro and in vivo studies demonstrate a role for the N terminus of apoA-I in lecithin:cholesterol acyltransferase activation and the requirement of the first class A amphipathic α-helix for the maturation of HDL in vivo.

High density lipoproteins (HDL) transport cholesterol from peripheral tissues to the liver in a process known as reverse cholesterol transport (1). This pathway is accepted as a primary mechanism by which HDL exert their anti-atherogenic effects. Nascent HDL are secreted by hepatocytes, liberated from chylomicrons during triglyceride lipolysis, and are derived from HDL remodeling by hepatic lipase and cholesteryl ester transfer protein. The importance of phospholipid transfer protein (PLTP) in the lipidation of this nascent HDL pool has recently emerged, because PLTP-deficient mice exhibit defective phospholipid transfer from triglyceride-rich lipoproteins to HDL, reduced HDL levels, and increased HDL catabolism (2). Efflux of cholesterol and phospholipids from cells provide nascent HDL with lipid constituents. This step is important for steady-state concentrations of HDL, because efflux is a rate-limiting step in HDL maturation as heterozygous mutations in the ATP binding cassette transporter A1 (ABCA1) can cause familial HDL deficiency (3). The combined actions of PLTP and ABCA1 generate larger discoidal preβα and preββ-HDL from the nascent HDL pool, which are converted to spherical α-migrating HDL by the actions of lecithin:cholesterol acyltransferase (LCAT) as newly synthesized cholesteryl esters partition to the HDL core.

Apolipoprotein A-I (apoA-I) is the predominant protein constituent of HDL, whose contribution to HDL metabolism has been subject to intense investigations (reviewed in Refs. 4–6). In addition to its role as a structural element of HDL, apoA-I stimulates cholesterol efflux, is the most potent activator of LCAT (7) and may be important for HDL interactions with scavenger receptor class B type 1 (8, 9) and ABCA1 (10, 11).

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|| To whom correspondence should be addressed: Lipoprotein and Atherosclerosis Research Group, University of Ottawa Heart Institute, Rm. H460, 40 Ruskin Ave., Ottawa, Ontario K1Y 4W7, Canada. Tel.: 613-761-5255; Fax: 613-761-5281; E-mail: ymarcel@ottawaheart.ca.

1 The abbreviations used are: HDL, high density lipoproteins; PLTP, phospholipid transfer protein; ABCA1, ATP binding cassette transporter A1; preβα-HDL, HDL co-migrating with β-globulins by agarose electrophoresis; α-migrating HDL, HDL co-migrating with α-globulins by agarose gel electrophoresis; LCAT, lecithin:cholesterol acyltransferase; apoA-I, apolipoprotein A-I; aa, amino acid(s); His-Wt apoA-I, histidine-tagged wild-type apoA-I; His-Δ1–65 apoA-I, histidine-tagged Δ1–65 apoA-I; His-Δ1–43 apoA-I, histidine-tagged Δ1–43 apoA-I; His-Δ210–243 apoA-I, histidine-tagged Δ210–243 apoA-I; DMEM, Dulbecco’s modified Eagle’s medium; LDL, low density lipoprotein; BSA, bovine serum albumin; L22A1, reconstituted lipoprotein containing two molecules of apoA-I, PL, phospholipid; FC, free cholesterol; PAGGE, polyacrylamide gradient gel electrophoresis; Δ1βγδδ, standard free energy of denaturation; CE, cholesteryl ester; Δ7–43 apoA-I Ad5, Δ7–43 apoA-I adenovirus; Δ7–65 apoA-I Ad5, Δ7–65 apoA-I adenovirus; Wt apoA-I Ad5, wild-type apoA-I adenovirus; FPLC, fractionation liquid chromatography; TC, total cholesterol; biot-A44 mAb, biotinylated A44 monoclonal antibody, apoE, apolipoprotein E, pfu, plaque-forming units; mAb, monoclonal antibody.
Apox-I is synthesized as a 267-amino acid (aa) preproprotein (reviewed in Ref. 12). An 18-aa signal peptide is cleaved following endoplasmic reticulum translation while a 6-aa pro-sequence is removed following secretion by an unidentified protease to generate the mature 243-aa protein. The secondary structure of apoA-I consists of 10 class A/Y amphipathic α-helices encompassing residues 44–243 and a globular domain (residues 1–43) that includes a G* amphipathic α-helix spanning residues 8–33 (reviewed in Refs. 13, 14).

The study of apoA-I structure and function relationships has yielded valuable information regarding individual steps in HDL metabolism. However, the contribution of the N terminus of apoA-I remains poorly understood. An apoA-I mutant lacking residues 1–43 (Δ1–43 apoA-I) has been characterized previously (15, 17), and it was suggested that the deleted amino acids stabilize the lipid-free conformation of apoA-I. The x-ray crystal structure of this mutant suggests that apoA-I, when bound to HDL, wraps around the periphery of the particle perpendicular to the phospholipid fatty-acid chains (18). Previous work also demonstrates that deletion of these residues confers a 2-fold decrease in the ability of apoA-I to activate LCAT suggesting functional properties of this mutant may be moderately affected, as well (17).

The importance of helix 1 (aa 44–65) for association of apoA-I with phospholipids has been shown previously (15, 19). Using synthetic peptides modeled after repeating 22-mer domains of apoA-I, Palgunachari and co-workers (19) demonstrated that only N- and C-terminal peptides corresponding to aa 44–65 and 220–241 of apoA-I were able to clarify dimyristoylphosphatidylcholine vesicles, implicating both the N and C termini in phospholipid binding. In addition, the characterization of an apoA-I deletion mutant lacking residues 1–65 suggests this protein has significantly less phospholipid binding affinity relative to wild-type and Δ1–43 apoA-I (15). However, the contributions of this helix to cholesterol efflux and to LCAT activation and the in vivo maturation of HDL have not been fully characterized.

To investigate structure-function relationships associated with the N terminus of apoA-I, we have compared wild-type apoA-I and two N-terminal deletion mutants both in vitro, using histidine-tagged proteins purified from Escherichia coli, and, in vivo, by adenovirus-mediated gene transfer. The results of the current in vitro and in vivo studies demonstrate a role for the N terminus of apoA-I in LCAT activation and the requirement of the first class A amphipathic α-helix for the maturation of HDL in vivo.

EXPERIMENTAL PROCEDURES

Construction of cDNAs for His-apoA-I Expression—Histidine (His)-tagged wild-type apoA-I (His-Wt apoA-I) (20) and the deletion mutant lacking residues 1–65 (His-Δ1–65 apoA-I) (15) were described as previously. The apoA-I mutant in which residues 1–43 were deleted (His-Δ1–43 apoA-I) was generated by deletion mutagenesis using the Excite mutagenesis kit from Stratagene and the following oligonucleotides: 5′-CTCATGTTGTAAGGTGCC-3′ (upstream) and 5′-CTAAAGCTCCTTGACAACTGGGACAGCG-3′ (downstream). The apoA-I mutant lacking residues 210–243 (His-Δ210–243 apoA-I) was generated by similar methods using the following oligonucleotides: 5′-GGGCTTGGCCTTCTCGCTGAGCG-3′ (upstream) and 5′-PTAGGAGCCCGCGGCAGGCC-3′ (downstream).

Cell Culture—J774 mouse peritoneal macrophages and 293 human embryonic kidney cells were maintained between passages in Dulbecco’s modified Eagle’s medium (DMEM) and Eagle’s minimal essential medium, respectively, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin sulfate, and 2 mM L-glutamine. Cell culture reagents and plastic ware were purchased from Sigma, Life Technologies, Inc., and Falcon.

Purification of Histidine-tagged ApoA-I—All recombinant His-tagged apoA-I proteins for these studies were expressed and purified in E. coli as described previously (20) with some minor changes (21). After lyophilization, the proteins were solubilized in 6 M guanidine hydrochloride and dialyzed against phosphate-buffered saline.

Cholesterol Efflux—Efflux assays were performed with cholesterol loaded J774 macrophages as described by Sakr et al. (22) with several modifications. Cells were seeded in 12-well plates at a concentration of 104 cells/well and grown for 48 h before cholesterol loading. Cells were cholesterol loaded for 48 h in 1 ml of DMEM containing 1% fetal bovine serum and 100 units/ml penicillin, 100 units/ml streptomycin sulfate, and 2 mM L-glutamine as described previously (23) with or without 0.3 mM 8-bromoadenosine 3′-5′-cylic monophosphate (cAMP) (Sigma Chemical Co.) for 12 h. Cells were then washed 3× in DMEM containing 0.2% BSA. For the efflux assay, cells were incubated for 0, 2, 6, and 24 h in 1 ml of DMEM containing 0.2% BSA with or without 0.3 mM cAMP containing 50 μg of His-Wt apoA-I or equivalent moles of each apoA-I deletion mutant. At the appropriate time point, media samples were removed and centrifuged at 1000 × g to remove floating cells. Cells were solubilized in 1 ml of 0.5 N NaOH for 24 h and assayed for cell protein content by the method of Markwell et al. (23).

The radioactivity in all samples was determined by liquid scintillation counting. Cholesterol efflux was calculated as the percentage of initial cell radioactivity associated with the media as a function of time. Values were corrected for each time point for the efflux of cholesterol to medium not containing apoA-I. The percent stimulation of cholesterol efflux in response to cAMP treatment was calculated according to the following equation: (efflux from J774 cells treated with cAMP − efflux from J774 cells in the absence of cAMP)/cAMP 

Preparation of Reconstituted Lp2A-Is—Reconstituted discoidal lipoproteins containing 2 molecules of apoA-I (Lp2A-I) were prepared by cholate dispersion/Bio-Bead removal protocol using initial molar ratios of 80:10:1 of 1-palmitoyl-2-oleylphosphatidylcholine (PL) (Avanti Polar Lipids, Birmingham, AL), free cholesterol (FC) (Sigma) and His-apoA-I, respectively, as described elsewhere (25). Particle size and homogeneity were confirmed by 4%–20% non-denaturing gradient gel electrophoresis (PAGE). Each particle contained 2 molecules of apoA-I as determined by cross-linking with dimethylsuberimidate, according to an established protocol (26). Enzymatic kits were used to determine the final PL (Wako Chemicals, Neuss, Germany) and FC (Roche Molecular Biochemicals) concentrations. The apoA-I content was measured according to the method of Markwell et al. (23) using a BSA standard.

The stability of the Lp2A-I was assessed by circular dichroism spectroscopy on a Jasco-710 (Japan). The percent ellipticity was determined at 222 nm with increasing concentrations of guanidine hydrochloride to calculate the standard free energy of denaturation (∆G°d).

LCAT Assay—Human LCAT was purified as described previously (27). Lp2A-I was prepared containing [14C]cholesterol and His-Wt, His-Δ1–43, or His-Δ1–65 apoA-I. Two distinct types of experiments were performed. The time course assay was performed as described previously (21) by incubating 3.5 units of LCAT with Lp2A-I for 0, 0.5, 1, 2, 3, 4, and 5 h at 37 °C at a final apoA-I concentration of 2.14 μM. In the second experiment, the rate constants Vmax and apparent Km (Km(app)) were determined by incubating Lp2A-I at the concentrations indicated (expressed in micromolar apoA-I) for 10 min at 37 °C. In both cases, reactions were terminated by the addition of 2 ml of ethanol, and minimal substrate conversion was determined previously (28). The values indicated are the means ± S.E. of triplicate results and are representative of two independent experiments. One unit of LCAT is defined as the amount of enzyme required to convert 1 nmol of FC to cholesteryl ester (CE) per hour using a standard His-Wt Lp2A-I at a final concentration of 2.0 μM.

Production of First Generation Recombinant Adenoviruses—The cDNAs for apoA-I deletion mutants lacking residues 7–43 (Δ7–43 apoA-I) and residues 7–65 (Δ7–65 apoA-I) were sub-cloned into the pCA13 vector (Microbiol Biosystems Inc.). Recombinant adenoviruses (Δ7–43 apoA-I Ad5 and Δ7–65 apoA-I Ad5) were prepared as previously described for the wild-type apoA-I (WT apoA-I) adenovirus (WT apoA-I Ad5) (29). To verify efficient secretion of these deletion mutants, pulse-chase studies were performed using primary hepatocytes isolated from apoA-I-deficient mice (21).

Animals—ApoA-I-deficient (apoA-Idef/def) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were maintained on a 12-h light/12-h dark schedule and were fed a normal chow diet (Charles River rodent diet 5095, 18% protein and 4.5% fat). All experiments were performed in accordance with protocols approved by the University of
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Ottawa Animal Care Committee. Mice used for these studies were all 4- to 12-month-old males.

**Quantification of Apo-A-I in Plasma**—Following adenosine injection, plasma apo-A-I levels were determined by quantitative Western blotting. Equivalent volumes of plasma were separated by 12% SDS-PAGE and analyzed by Western blot using a polyclonal anti-human apo-A-I antibody raised in sheep. The concentrations of WT apo-A-I and the two N-terminal deletion mutants in mouse plasma samples were determined by densitometry and comparison to apo-A-I standards of known concentrations using Quantity One software (Bio-Rad). To eliminate the possibility that differential immunoreactivity may under- or overestimate the plasma levels of the mutant apo-A-Ia expressed in the mouse, we compared the immunoreactivity of His-Wt apo-A-I with the two N-terminal deletion mutants, His-Δ1–43 and His-Δ1–65 apo-A-I. Equivalent amounts of the purified histidine-tagged apo-A-Ia (determined by Markwell et al. (23)) were separated by SDS-PAGE and visualized by Western blot. A mass range of 0.2–5 μg was used, covering the range in apo-A-I mass detected in plasma by this method. The relative intensities was then plotted versus the mass of apo-A-I used for His-Wt, His-Δ1–43, and His-Δ1–65 apo-A-I (data not shown). The plots were virtually identical, indicating that differential immunoreactivity is not a concern in this assay.

**Size Exclusion Chromatography**—Separation of mouse plasma by size exclusion chromatography (FPLC) was performed as described previously (29). Briefly, 500 μl of mouse plasma was loaded onto two Superdex 200 (analytical grade, Amersham Pharmacia Biotech) columns connected in series. Separation was achieved at a flow rate of 0.1 ml/min, and 5-ml fractions were collected. Each fraction was assayed for total cholesterol (TC) (Roche Molecular Biochemicals, Indianapolis, IN), LDL, and HDL content using standard enzymatic kits. 100 μl of each fraction was analyzed for apo-A-I content by slot blot (Bio-Rad Bio-Dot SF unit) followed by Western blot analysis using a biotinylated A44 monoclonal antibody (biot-A44 mAb).

**Discontinuous Gradient Ultracentrifugation**—Apo-A-I-deficient mouse plasma samples were separated by discontinuous gradient ultracentrifugation as described previously (29). Samples were centrifuged for 18 h and 210,000 × *g* at 8 °C. 1-ml fractions were collected from top to bottom, and densities were determined by refractometry (Fisher Scientific, Nepean, Ontario, Canada) analysis. Aliquots of each fraction were dialyzed against phosphate-buffered saline (0.025-μm filter disks, Millipore Corp.) and analyzed by SDS-PAGE (below). Western blot analysis was performed using a polyclonal anti-human apo-A-I antibody from sheep.

**Electron Microscopy**—Negative stain electron microscopy was performed using methods described previously (30). Briefly, discontinuous gradient samples were dialyzed against 0.125 M ammonium acetate, 2.6 mM ammonium carbonate, and 0.26 mM EDTA, pH 7.4, and concentrated to 100–200 μg/ml. Samples were mixed 1:1 with 2% sodium phosphotungstate, pH 7.4, before applying to carbon-coated Formvar grids (200 mesh) and visualized using a Hitachi H-7100 electron microscope at × 50,000 and × 70,000 magnifications.

**Electrophoresis**—SDS-PAGE was performed using 12% acrylamide pre-cast Novex gels (Invitrogen) according to the manufacturer’s specifications. Non-denaturing PAGE was done using 4–20% acrylamide pre-cast Novex gels, and samples were separated for 2000 V h. Pre-β and α-migrating HDL were separated by agarose gel electrophoresis (Beckman Lipogel, Beckman Coulter, Fullerton, CA) followed by neutral lipid staining (Beckman) or Western blotting using the biot-A44 mAb. Samples were separated for 30 min at 100 V.

**Western Blotting**—Samples separated by 12% SDS-PAGE were transferred to nitrocellulose at 125 V for 1 h while those separated by 4–20% PAGE were transferred at 125 V for 2 h. Membranes were probed either with the biot-A44 mAb directed against the central domain of apo-A-I (aa 149–186), an anti-human plasma apo-A-I from sheep (Roche Molecular Biochemicals), or an anti-mouse apoE polyclonal from rabbit (Bioscience International, Kennebunk, ME). Proteins were visualized by chemiluminescence following incubation with either streptavidin (Amersham Biosciences, Inc.), donkey anti-sheep IgG (Sigma), or donkey anti-rabbit IgG (Amersham Biosciences, Inc.) coupled to horseradish peroxidase.

**Statistical Analysis**—The average ± standard error (S.E.) of a minimum of triplicate measurements is reported within. Statistical analysis was performed by Student’s *t* test where indicated and values are considered to be statistically significant at *p* < 0.05 (two-tailed *t* test).

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**Table I**

| Efflux | R² | Stimulation with cAMP |
|-------|----|-----------------------|
| His-Wt apo-A-I | | |
| −cAMP | 0.97 ± 0.02 | 0.99 ± 0.01 | 87 ± 5 |
| +cAMP | 1.83 ± 0.06 | 0.99 ± 0.01 |
| His-Δ1–43 apo-A-I | | |
| −cAMP | 0.88 ± 0.02a | 0.99 ± 0.01 | 78 ± 3 |
| +cAMP | 1.66 ± 0.05 | 0.99 ± 0.01 |
| His-Δ1–65 apo-A-I | | |
| −cAMP | 0.86 ± 0.02a | 1.00 ± 0.01 | 91 ± 1 |
| +cAMP | 1.77 ± 0.01 | 1.00 ± 0.01 |
| His-Δ210–243 apo-A-I | | |
| −cAMP | 0.45 ± 0.03b | 0.99 ± 0.01 | 73 ± 1 |
| +cAMP | 1.19 ± 0.02b | 0.99 ± 0.02 |

* Values statistically different (p < 0.01) relative to His-Wt apo-A-I.

**RESULTS**

**Cholesterol Efflux from J774 Macrophages to His-Wt, His-Δ1–43, His-Δ1–65, and His-Δ210–243 apo-A-I**—His-Wt, His-Δ1–43, His-Δ1–65, and His-Δ210–243 apo-A-I were expressed and purified from *E. coli* and used to evaluate the potential contribution of the N terminus of apo-A-I to cellular cholesterol efflux. Cells were first cholesterol-loaded for 48 h with acetylated LDL and subsequently equilibrated for 12 h in either the absence or presence of 0.3 mM cAMP. Medium containing 50 μg of His-Wt apo-A-I or equivalent moles of His-Δ1–43, His-Δ1–65, and His-Δ210–243 apo-A-I was then added, and the efflux to each acceptor was determined over time (Table I). In the absence of cAMP, efflux to His-Δ1–43 and His-Δ1–65 apo-A-I was slightly, but significantly (p < 0.01), reduced relative to His-Wt apo-A-I while efflux to His-Δ210–243 apo-A-I was greatly reduced (2.2-fold) (p < 0.01) by comparison. In the presence of cAMP, which stimulates expression of ABCA1 (31), efflux to His-Δ1–43 and His-Δ1–65 apo-A-I was not statistically different from His-Wt apo-A-I. In contrast, efflux in the presence of cAMP for His-Δ210–243 apo-A-I was significantly reduced (p < 0.01) relative to His-Wt apo-A-I and the two N-terminal deletion mutants (1.5-fold).

**Effect of Apo-A-I N-terminal Deletions on LCAT Activation**—We next compared the ability of LCAT-mediated cholesterol esterification in *vitro*. Reconstituted, discoidal lipoproteins (Lp2A-I) were prepared with initial molar ratios of 80:10:1 of PL, PC, and His-apo-A-I, respectively. All reconstituted lipoproteins were of similar lipid composition and exhibited similar standard free energies of denaturation (ΔG0′) (data not shown). During a 5-h time course experiment (Fig. 1A), the formation of CE was reduced to 53 and 48% for His-Δ1–43 and His-Δ1–65 Lp2A-I, respectively, relative to His-Wt Lp2A-I (p < 0.05). Analysis of the Michaelis-Menten kinetics by the double-reciprocal Lineweaver-Burke plot (Fig. 1B) indicated that the *V*max values for His-Δ1–43 and His-Δ1–65 Lp2A-I were reduced by 50 and 70%, respectively, relative to His-Wt Lp2A-I (p < 0.05) (Table II).
similar concentrations of Wt apoA-I and two N-terminal deletion mutants (Δ7–43 and Δ7–65 apoA-I) in apoA-I-deficient mice. Residues 1–6 were maintained to allow proper cleavage of the hexapeptide pro-sequence in vivo (see “Discussion”). We have previously shown that apoA-I-deficient mice express high levels of human apoA-I following tail vein administration of 2 × 10^9 pfu of the recombinant adenovirus (29). To determine the expression levels of Δ7–43 and Δ7–65 apoA-I relative to Wt apoA-I, sex- and aged-matched mice were injected with 2 × 10^9 pfu of each respective adenovirus. Analysis of fasting plasma samples 4 days post-injection indicated that Δ7–43 (Fig. 2, lane 4) and Δ7–65 (Fig. 2, lane 6) apoA-I were expressed at ~4-fold lower levels than Wt apoA-I (Fig. 2, lane 4). To attain plasma levels of Δ7–43 and Δ7–65 apoA-I comparable to Wt apoA-I, a 4-fold greater dose (8 × 10^9 pfu) of the Δ7–43 and Δ7–65 apoA-I Ad5 was used. We have shown previously that injection of up to 1 × 10^10 pfu of a luciferase Ad5 is not itself associated with short-term changes in plasma lipid levels relative to non-injected mice (29). This is corroborated by others using similar adenovirus control vectors (32, 33). Injecting 8 × 10^9 pfu of the Δ7–43 (Fig. 2, lane 5) and Δ7–65 (Fig. 2, lane 7) apoA-I Ad5 resulted in plasma apoA-I levels comparable to Wt apoA-I, and this dose was used for all subsequent experiments.

Analysis of plasma lipid levels (Table III) indicated that TC, PL, and phospholipid concentrations were increased significantly (p < 0.05) for Wt, Δ7–43, and Δ7–65 apoA-I above those of the non-injected controls. CE levels were increased (p < 0.05) above background for Wt and Δ7–43 apoA-I but not for Δ7–65 apoA-I. The plasma CE/TC ratio in mice expressing Δ7–43 (0.49 ± 0.12) and Δ7–65 (0.25 ± 0.20) apoA-I was significantly reduced (p < 0.05) relative to Wt apoA-I (0.64 ± 0.07). In addition, plasma PL levels in mice expressing Δ7–43 and Δ7–65 apoA-I were slightly but not significantly reduced relative to those expressing Wt apoA-I.

Analysis of Wt, Δ7–43, and Δ7–65 apoA-I-containing HDL by size exclusion chromatography—Fasting plasma samples from apoA-I-deficient mice 4 days post-injection with 2 × 10^9 pfu of Wt apoA-I Ad5 or 8 × 10^9 pfu of Δ7–43 and Δ7–65 apoA-I Ad5 were separated by size exclusion chromatography. Individual fractions were assayed for TC content (Fig. 3A) and relative apoA-I distribution by slot blot analysis (Fig. 3B). This indicates that Wt, Δ7–43, and Δ7–65 apoA-I associate with lipoproteins of HDL size. In each case, the plasma lipoprotein size...
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distribution is shifted to a similar extent for each of these apoA-I proteins relative to that of the non-injected apoA-I-deficient mouse. The shift in HDL size relative to the non-injected mouse is due to the replacement of apoE with apoA-I on these HDL. We have shown previously that de novo expression of Wt apoA-I reduces plasma concentrations of apoE in the apoA-I-deficient mouse following adenovirus administration (29) and occurs to similar extents for Wt, Δ7–43, and Δ7–65 apoA-I (Fig. 3, inset). Despite lower plasma cholesterol levels associated with Δ7–43 and Δ7–65 apoA-I, HDL and apoA-I distributions were not significantly altered as compared with Wt apoA-I. In addition, neither Wt, Δ7–43, or Δ7–65 apoA-I formed a lipid-free/poor pool (fractions 26–28) as all detectable apoA-I co-localized with the HDL cholesterol peak.

Subsequently, the TC, FC, CE, and PL content in FPLC fractions corresponding to HDL size were normalized to the respective apoA-I concentrations. As compared with Wt apoA-I HDL, Δ7–43, and, more markedly, Δ7–65 apoA-I HDL contained less TC, on average, per HDL particle (Fig. 4A). This is predominantly due to a reduced CE content for Δ7–43 and Δ7–65 apoA-I HDL as compared with Wt apoA-I HDL (Fig. 4C). Furthermore, Wt apoA-I HDL contained more PL than Δ7–43 or Δ7–65 apoA-I HDL (Fig. 4D), however, this reduction was proportionately less than that observed for CE.

Characterization of Wt, Δ7–43, and Δ7–65 apoA-I HDL by Agarose and Non-denaturing PAGGE—Mouse plasma samples were isolated 4 days following injection of either the Wt, Δ7–43, or Δ7–65 apoA-I Ad5. The relative amounts of preβ- and α-migrating HDL associated with each of the apoA-I proteins were evaluated by agarose gel electrophoresis. HDL were visualized either by neutral lipid staining (Fig. 5A) or Western blot analysis with an anti-human apoA-I monoclonal antibody (biot-A44) (Fig. 5B). Two independent apoA-I Ad5 injections are shown in each panel and are representative of four separate injections. Expression of Wt apoA-I was associated with high levels of α-migrating neutral lipid staining (Fig. 5A, lanes 3 and 4) and was consistent with the position of Wt apoA-I by Western blot (Fig. 5B, lanes 3 and 4). Δ7–43 apoA-I associated HDL migrated predominantly to the α-position (Fig. 5B, lanes 5 and 6) and accumulated significantly levels of neutral lipid (Fig. 5A, lanes 5 and 6), although less than observed for Wt apoA-I. In addition, Δ7–43 apoA-I formed more preβ-HDL than was observed for Wt apoA-I. Strikingly, Δ7–65 apoA-I formed only preβ-HDL as demonstrated by Western blot analysis (Fig. 5B, lanes 7 and 8) and neutral lipid staining (Fig. 5A, lanes 7 and 8) (no α-migrating HDL detected).

Non-denaturing PAGGE was next used to determine the size of HDL formed with Wt, Δ7–43, and Δ7–65 apoA-I. Wt (Fig. 5C, lanes 3 and 4) and Δ7–43 (Fig. 5C, lanes 5 and 6) apoA-I HDL formed two distinct HDL pools (10.1–10.7 and 8.8–9.9 nm) similar in size to each other and to a human HDL standard (10.3–11.0 and 8.8–9.9 nm) (Fig. 5C, lane 1). In contrast, Δ7–65 (Fig. 5C, lanes 7 and 8) apoA-I formed a more heterogeneous
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FIG. 4. Relative lipid composition of Wt, Δ7–43, and Δ7–65 apoA-I HDL. TC (A), FC (B), CE (C), and PL (D) levels in FPLC fractions corresponding to HDL size were normalized to the respective apoA-I protein mass. The resultant profiles represent the relative lipid content of apoA-I protein mass. The resultant profiles were normalized to the respective FPLC fractions corresponding to HDL (C), CE (D), FC (E), and PL (F) apoA-I. The lipid measurements were done using enzymatic kits (see “Experimental Procedures”).

FIG. 5. Characterization of Wt, Δ7–43, and Δ7–65 apoA-I HDL by agarose and non-denaturing PAGGE. A and B, mouse plasma 4 days post-injection with Wt (lanes 3, 4), Δ7–43 (lanes 5, 6), and Δ7–65 (lanes 7, 8) apoA-I Ad5 were separated by agarose electrophoresis and visualized by neutral lipid staining (A) and anti-apoA-I Western blotting (B) using the biot-A44 mAb. Human HDL (lane 1), non-injected apoA-I-deficient mouse plasma (lane 2), human LDL (lane 9), and wild-type apoA-I (lane 10) are shown for reference. C, Wt (lanes 3, 4), Δ7–43 (lanes 5, 6), and Δ7–65 (lanes 7, 8) apoA-I Ad5-injected mouse plasma samples were separated by 4–20% non-denaturing PAGGE and visualized by anti-apoA-I Western blotting using the biot-A44 mAb. Human HDL (lane 1) and biotinylated high molecular weight markers (lane 2) are presented for comparison. Two independent apoA-I Ad5 injections are shown in each panel and are representative of four separate injections.

HDL population. The predominant species was of similar size to Wt and Δ7–43 apoA-I HDL (10.3 nm) but included additional species of 11.6, 11.1, 9.4, and 8.8 nm in diameter.

Effect of ApoA-I N-terminal Deletions on HDL Morphology—The morphology of HDL isolated from apoA-I-deficient mice 4 days post-injection with 2 × 10^9 pfu of Wt apoA-I Ad5 or 8 × 10^9 pfu of Δ7–43 and Δ7–65 apoA-I Ad5 was determined by negative stain electron microscopy. Wt apoA-I HDL (Fig. 6B) exhibited a typical hexagonal packing array characteristic of spherical HDL. HDL associated with Δ7–43 (Fig. 6, C and D) apoA-I were predominantly spherical and comparable in size to Wt apoA-I HDL, although discoidal HDL were observed. In contrast, expression of Δ7–65 (Fig. 6, E and F) apoA-I was consistently associated with abnormal HDL morphology. These lipoproteins presented as “rouleaux” of stacked structures characteristic of discoidal HDL. In addition, the density distribution of Δ7–65 apoA-I HDL was shifted to a peak density of 1.12 g/ml relative to Δ7–43 and Wt apoA-I HDL (1.06–1.09 g/ml). These discoidal particles were absent in non-injected (non-shown) and luc-Ad5-injected apoA-I-deficient mice (Fig. 6A), which demonstrated that these HDL formed as a consequence of expressing this mutant apoA-I.

DISCUSSION

Analysis of apoA-I structure-function relationships has benefited greatly from the study of both naturally occurring and engineered mutations. Although many mutations in the N terminus have been described (reviewed in Ref. 34), their effects on HDL metabolism have not been well characterized. ApoA-I Iowa (Gly^266 → Arg) is associated with hereditary systemic amyloidosis (35), low plasma levels of HDL, and apoA-I and has been shown to have enhanced catabolism when infused into normal subjects (36). This mutation lies within the G^4 helix in the globular domain, which suggests that disruption of this helix alters structural and/or functional properties of apoA-I. 3 Point mutations in helix 1 have also been identified (37–39). Unfortunately, it appears that the effects of these mutations on apoA-I and HDL levels have not been fully reported. Because amyloidogenic mutations are proposed to perturb structural properties of apoA-I, a detailed analysis of these mutants may
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ApoA-I-deficient mouse plasma HDL formed 4 days post-injection with luc (A, ρ = 1.09 g/ml), Wt (B, ρ = 1.06 g/ml), Δ7–43 (C, ρ = 1.06 g/ml, D, ρ = 1.09 g/ml), and Δ7–65 (E and F, ρ = 1.12 g/ml) apoA-I Ad5 were isolated by discontinuous gradient ultracentrifugation and visualized by negative stain electron microscopy as described under “Experimental Procedures.” The bar in each panel represents 100 nm. Electron micrographs were taken at × 50,000 magnification in A and E, whereas those for B, C, D, and F were taken at × 70,000 magnification.

Fig. 6. Effect of apoA-I N-terminal deletions on HDL morphology. ApoA-I-deficient mouse plasma HDL formed 4 days post-injection with luc (A, ρ = 1.09 g/ml), Wt (B, ρ = 1.06 g/ml), Δ7–43 (C, ρ = 1.06 g/ml, D, ρ = 1.09 g/ml), and Δ7–65 (E and F, ρ = 1.12 g/ml) apoA-I Ad5 were isolated by discontinuous gradient ultracentrifugation and visualized by negative stain electron microscopy as described under “Experimental Procedures.” The bar in each panel represents 100 nm. Electron micrographs were taken at × 50,000 magnification in A and E, whereas those for B, C, D, and F were taken at × 70,000 magnification.

clarify the role(s) of these helical domains in HDL metabolism. Furthermore, Booth and co-workers (40) reported a novel apoA-I deletion/insertion in which residues 60 to 71 are deleted and replaced by single valine and threonine residues, disrupting a portion of helix 1. This mutant was associated with significantly lower apoA-I and HDL levels.

Therefore, limited analysis of naturally occurring mutations in the N terminus of apoA-I suggests more detailed studies are warranted to delineate functional properties associated with this region. Here we have evaluated the contribution of the globular domain (aa 1–43) and the first class A amphipathic α-helix (aa 44–65) in HDL maturation both in vitro and in vivo.

To address the contributions of the globular domain and helix 1 of apoA-I to HDL metabolism in vitro, histidine-tagged recombinant proteins were expressed and purified from E. coli. This has been shown previously not to significantly perturb physical-chemical properties of apoA-I, including cholesterol efflux and LCAT activation (15, 20). Although the deletions of residues 1–43 and 1–65 bring the N-terminal histidine extension closer to the central domain and could conceivably affect apoA-I structure-function relationships, in vitro expression of similar mutants lacking the histidine tag have the same effects on the LCAT reaction and the maturation of HDL. Thus we conclude that the in vitro experiments truly reflect the effect of the deletions on LCAT.

The importance of the C terminus (aa 187–243) of apoA-I in both phospholipid binding and cholesterol efflux has been shown previously (41–43), suggesting these two properties may be related. To determine if either the N-terminal globular domain or helix 1 are involved in cellular cholesterol efflux, we compared the efflux from cholesterol-loaded J774 macrophages to lipid-free His-Wt, His-Δ1–43, His-Δ1–65, and His-Δ210–243 apoA-I (Table I). Deletion of the globular domain and helix 1 did not impair the ability of apoA-I to elicit cholesterol efflux to an appreciable extent. Interestingly, these observations are inconsistent with studies addressing the ability of individual apoA-I synthetic peptides to elicit cholesterol efflux from human skin fibroblasts (44). From a panel of synthetic peptides modeled after apoA-I helices, only those corresponding to aa 44–65, 44–87, and 209–241 were shown to stimulate cholesterol efflux, suggesting aa 44–65 are important for this property. However, it is difficult to compare individual helical peptides to apoA-I mutants containing many more amphipathic α-helices. Furthermore, comparison of efflux to apoA-I mutants from macrophage and fibroblast cell lines has yielded conflicting results previously (41, 44), suggesting that extrapolation from one cell type to another is not necessarily warranted. Nonetheless, the results here show that the C terminus of apoA-I is more important than the N terminus in promoting cholesterol efflux from macrophages.

Subsequently, we addressed the contribution of the N terminus to LCAT activation. Deletion of the globular domain of apoA-I has been shown previously to confer a 2-fold reduction in the capacity of apoA-I to activate LCAT (17), however, the role of helix 1 was previously unclear. The effect conferred by the deletion of residues 1–65 of apoA-I was assayed previously using an egg PC vesicular assay (15), however, this study could not differentiate between decreased phospholipid binding and a direct affect of this mutation on LCAT activation. Here, we prepared reconstituted LpA2-Is with His-Wt, His-Δ1–43, and His-Δ1–65 apoA-I that were of similar composition and stability, properties that have been shown to affect LCAT activation independently (45). We observed that both His-Δ1–43 and His-Δ1–65 apoA-I were associated with decreases in LCAT activation relative to His-Wt apoA-I (Table II). The observation that these deletions affected LCAT activation is intriguing, because this property is generally associated with class A helices of the central domain, in particular helix 6 (29, 46, 47). Recent work suggests that a cluster of 3 arginine residues at the interface of the hydrophilic and hydrophobic faces in helix 6 contributes to a specific locale of positive surface potential that may stimulate LCAT activity (48). Site-specific mutagenesis of these residues conferred 11- to 12-fold reductions (decrease in Vmax) in LCAT activation without affecting other physical properties of apoA-I. The current study supports previous work implicating residues 1–43 in LCAT activation and suggests residues 44–65 are also involved in activating LCAT. The use of animal models to corroborate apoA-I structure-function relationships observed in vitro has been described previously. To further address the contribution of the globular domain and helix 1 of apoA-I in HDL metabolism, we expressed Wt, Δ7–43, and Δ7–65 apoA-I in apoA-I-deficient mice by adenovirus-mediated gene transfer. ApoA-I is secreted as a proprotein containing 6 additional residues at the N terminus that are removed by a putative metalloprotease, which recognizes residues −2 to +4 and cleaves the pro-sequence thereby generating the mature protein (49). Therefore, residues 1–6 were not removed when constructing the cDNAs for in vivo expression to enable proper processing of these mutants. By injection of an equivalent dose of each recombinant adenovirus, the concentrations of both Δ7–43 and Δ7–65 apoA-I were decreased relative to Wt apoA-I (Fig. 2). We have previously shown that deletions of residues 100–143, 122–165, 144–186 (29), and 210–243 also result in significantly lower levels of these apoA-I mutants in plasma following adenovirus-mediated expression. In the current study, this was not due to impaired secretion, because as pulse-chase analysis indicated that both mutants were secreted as efficiently as Wt apoA-I (primary apoA-I-deficient hepatocytes (data not shown). The lower expression of Δ7–43 and Δ7–65 apoA-I may result from decreased stability of these proteins in the lipid-free form as similar deletions have been shown to significantly alter the...
conformation of apoA-I in solution (15). Alternatively, reduced phospholipid binding affinity may account for this observation as this property associates with a greater fractional catabolic rate among some apoA-I mutants (50). Injection of a 4-fold greater dose of the Δ7–43 and Δ7–65 apoA-I Ad5 resulted in plasma levels of these proteins similar to Wt apoA-I (Fig. 2, Table III). Analysis of plasma lipid concentrations indicated that the CE/TC ratio for both the Δ7–43 and, more markedly, Δ7–65 apoA-I Ad5-injected mice was significantly reduced relative to Wt apoA-I Ad5. This observation corroborates our in vitro observations, because deletion of these residues decreases LCAT activation. The size distribution of the lipoproteins formed by these mutants was analyzed by size exclusion chromatography (Fig. 3) and demonstrated that Wt, Δ7–43 and Δ7–65 apoA-I were associated exclusively with lipoproteins of HDL size. This indicates the plasma CE/TC ratios discussed previously are representative of HDL composition.

Analysis of HDL by agarose electrophoresis (Fig. 5, A and B) indicated both Δ7–43 and Δ7–65 apoA-I formed progressively more preβ-HDL as compared with Wt apoA-I. Davidson and co-workers (51) characterized the molecular basis governing the surface charge of preβ- and α-migrating HDL. They found the greatest contributors were phosphatidylinositol content and the presence of a neutral lipid ester core. Although the lipid core does not directly contribute to the lipoprotein charge, conformational changes in the surface apolipoproteins are believed to confer this effect. The observed staining in Fig. 5A substantiates the importance of a neutral lipid core, because Δ7–43 apoA-I HDL are associated with reduced staining at the α position while Δ7–65 apoA-I HDL exhibits no α-migrating band and only diffuse staining in the preβ position. This work suggests that increases in preβ-HDL and reductions in α-migrating HDL are linked to a gradual impairment in LCAT activation and cholesterol ester accumulation with progressive deletion of the N terminus. Indeed, this interpretation is supported by the in vitro assays, which indicate the ability of apoA-I to activate LCAT is reduced by the deletion of residues 1–43 and further by the additional removal of residues 44–65.

Analysis of HDL size by non-denaturing PAGE indicated that Wt, Δ7–43, and Δ7–65 apoA-I formed HDL species of similar size. However, Δ7–65 apoA-I HDL are significantly more heterogeneous in size than either Wt or Δ7–43 apoA-I HDL (Fig. 5C). In human plasma, preβ-HDL constitutes 4–14% of total HDL and consists of preβ1, preβ2, and preβ3 subtypes (reviewed in Ref. 52). Preβ1-HDL are on average 5.6 nm in diameter and are considered the initial acceptors of cellular FC. Preβ2 and preβ3-HDL have greater FC and PL contents and are significantly larger, in the range of 10–14 nm in diameter. Furthermore, preβ-HDL contains little, if any, CE, which associates with α-migrating HDL. Δ7–65 apoA-I HDL corresponded to preβ2/preβ3 in size and suggests that HDL maturation is impaired at the level of conversion of these subtypes to α-migrating HDL.

Electron microscopic analysis of HDL formed by these mutants (Fig. 6) indicated Δ7–65 apoA-I formed almost exclusively stacked rouleaux of discoidal HDL whereas Δ7–43 and Wt apoA-I formed predominantly spherical HDL with typical hexagonal packing arrays. The appearance of discoidal HDL associated with Δ7–65 apoA-I was anticipated based upon a drastically reduced CE/TC ratio in the plasma and predominant preβ-migration on agarose gels. Discoidal HDL have previously been associated with familial LCAT deficiency (53) and fish-eye disease (54). As supported by our in vitro studies, we propose that both the globular domain and helix 1 are significant LCAT-activating domains in apoA-I and that helix 1 may also be critical for the accumulation and stabilization of core cholesterol esters.

In summary, we have shown that the globular domain (aa 1–43) and helix 1 (aa 44–65) of apoA-I are important for HDL maturation in vivo. Removal of the globular domain alone decreases plasma apoA-I levels and significantly reduces the cholesterol content of HDL (and lowers the CE/TC ratio) but does not prevent formation of spherical α-migrating HDL. This supports current and previous in vitro findings that the globular domain is involved in LCAT activation (17) and suggests this domain contributes to steady-state concentrations of HDL. Similarly, the inability of Δ7–65 apoA-I to form spherical α-migrating HDL is supported by in vitro experiments showing that helix 1 also contributes to LCAT activation by apoA-I. Interestingly, the animal studies suggest that the additional deletion of residues 44–65, in particular, significantly impairs the formation of HDL while the additive effect of this deletion on LCAT activation in vivo is moderate. It is possible that other structural and functional properties of apoA-I, apart from LCAT activation, may be affected by this deletion related to the accumulation or stabilization of core neutral lipids and transformation of preβ- into α-migrating HDL. This is the first demonstration that the globular domain and helix 1 of apoA-I are essential for the formation of mature HDL in a physiologically relevant animal model. Future studies with site-directed mutants are planned to target amino acids within these domains important for HDL maturation.

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The N-terminal Globular Domain and the First Class A Amphipathic Helix of Apolipoprotein A-I Are Important for Lecithin:Cholesterol Acyltransferase Activation and the Maturation of High Density Lipoprotein in Vivo

Brian R. Scott, Dan C. McManus, Vivian Franklin, Andrea G. McKenzie, Tracey Neville, Daniel L. Sparks and Yves L. Marcel

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