Apolipoprotein A-IV promotes the biogenesis of apoA-IV containing HDL particles with the participation of ABCA1 and LCAT

Supplemental Methods

Plasma cholesterol and triglyceride levels, fractionation of plasma by FPLC, and two-dimensional gel electrophoresis of plasma. The concentration of total cholesterol, and triglycerides in plasma drawn four days post-infection was determined using the Total Cholesterol E reagent (Wako Chemicals USA, Inc., Richmond, VA) and INFINITY triglycerides reagent (ThermoScientific, Waltham, MA), respectively, according to the manufacturer's instructions. Levels of the plasma transaminases were determined using commercial available kits (Catachem Inc, CT). For FPLC analysis an aliquot of 15 µl plasma was loaded onto a Sepharose 6 PC column (Amersham Biosciences, Piscataway, NJ) in a SMART micro FPLC system (Amersham Biosciences, Piscataway, NJ) and eluted with PBS. A total of 25 fractions of 50 µl volume each were collected for further analysis. The concentration of lipids in the FPLC fractions was determined as described above for plasma samples. The plasma HDL subpopulations were separated by two-dimensional electrophoresis. The proteins were then transferred to a nitrocellulose membrane and apoA-IV was detected by immunoblotting as described (1), using the goat polyclonal anti-human apoA-IV antibody gift of Dr. Karl Weisgraber, Glaston Foundation.

Fractionation of plasma by density gradient ultracentrifugation and electron microscopy (EM) analysis of the apoA-IV containing fractions. For this analysis, 300 µl of plasma obtained from mice infected with the apoA-IV expressing adenovirus, was diluted with saline to a total volume of 0.5 mL and fractionated by density gradient ultracentrifugation. Following ultracentrifugation, 0.5 mL fractions were collected and analyzed by SDS-PAGE as described (1). Fractions 6-7 obtained by the density ultracentrifugation,

that float in the HDL region, were analyzed by electron microscopy using a Philips CM-120 electron microscope (1).

ApoA-IV mRNA quantification. Total hepatic RNA was isolated by the Trizol® method (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA samples were adjusted to 0.1 μg/μl and cDNA was produced using the high capacity reverse transcriptase cDNA kit (Applied Biosystems, Foster City, CA). ApoA-IV mRNA was quantified using the Applied Biosystems 7300 Real-Time PCR System. Gene Array TaqMan® primers for apoA-IV cDNA (Cat#4331182), and 18s rRNA (Cat# 4319413E) along with the TaqMan® Gene expression PCR Master Mix were used (Applied Biosystems, Foster City, CA).

ApoA-IV production, purification for functional and physicochemical studies. Recombinant apoA-IV was isolated from supernatant of HTB-13 cells grown in roller bottles following infection with recombinant adenovirus containing the apoA-IV gene. The culture medium was collected, concentrated 5-fold, dialyzed against 25 mM ammonium bicarbonate and lyophilized. The lyophilized apoA-IV was combined with β-oleoyl-γ-palmitoyl-L-α-phosphatidylcholine (POPC), cholesterol and sodium cholate at the ratio 1 mg/9.5 mg/0.47 mg/4.5 mg. The proteoliposomes formed were fractionated by density gradient ultracentrifugation and the fractions that contained the pure apoA-IV were collected and delipidated three times using 2:1 v/v chloroform:methanol (2).

ApoA-IV samples preparation. Lyophilized apoA-IV was dissolved to a final concentration of 0.4mg/ml in 8M guanidine hydrochloride in DPBS (pH 7.4). The solution was incubated for 1hour at room temperature and was extensively dialyzed against DPBS to remove all traces of guanidine. After dialysis, the sample was centrifuged at 10000 g for 20min to remove any precipitated protein and quantitated by measuring its absorbance at 280nm.

Preparation of apoA-IV- containing rHDL. Complexes comprised of apolipoprotein, egg yolk phosphatidylcholine (egg-PC[ES1]), and cholesterol were prepared by the sodium cholate dialysis

method (3), using egg-PC, cholesterol, apoA-IV, and sodium cholate in a molar ratio of 100:10:1:100, as previously described (4;5). The sample was extensively dialyzed at 4 °C against 10mM Tris-HCl, pH 8.0, 150mM NaCl and 0.01% EDTA buffer, using dialysis tubing with a molecular weight cut-off of 12,000–14,000. The rHDL particles were stored at 4 °C under nitrogen to prevent the oxidation of lipids.

Circular Dichroism Analyses. Far-UV circular dichroism spectra (200-260nm) were recorded at 20°C and 80°C for ApoA-IV samples and at 20°C and 95°C for apoA-IV- containing rHDL samples using a Jasco-715 spectropolarimeter. The cuvette chamber was thermostated using a Jasco PTC-348WI Peltier temperature controller. ApoA-IV protein samples were at 0.25 mg/ml concentration in DPBS (pH 7.4). rHDL samples contained apoA-IV at a concentration of 0.6 mg/ml in 10mM Tris-HCl, pH 8.0, 150mM NaCl and 0.01% EDTA buffer. A quartz cuvette (Hellma, Germany) with an optical path length of 1 mm was used. Spectra were acquired at 1nm bandwidth, 8 seconds response time, 0.2nm step size and 50nm/min scan speed. Each spectrum was calculated as the average of 5 accumulations. The results were corrected by subtracting the buffer baseline. Helical content was calculated using the molecular ellipticity at 222nm as described by Greenfield et al. (6).

For thermal denaturation measurements the change in molar ellipticity at 222nm was monitored while increasing the temperature up to 80 °C for ApoA-IV samples and up to 95 °C for rHDL samples at a rate of 1°C/min. After the denaturation of the samples, we analyzed the ability of the protein to refold upon cooling by monitoring the change in molar ellipticity at 222nm while gradually reducing the temperature from 80 to 20ºC for apoA-IV and from 95 to 20ºC for rHDL. The thermal denaturation curve was fit to a Boltzman sigmoidal model curve using Graphpad Prism™.

For the chemical denaturation measurements the change in molar ellipticity of the rHDL ApoA-IV particles was monitored at 222nm at 25 ºC upon titration of small amounts of an 8.0M guanidine

hydrochloride solution (Applichem). The spectropolarimeter parameters were the same as described for the typical far-UV CD spectra. Each spectrum was calculated as the average of 3 accumulations.

Fluorescence Spectroscopy. To monitor the chemical denaturation profile of apoA-IV 0.14 mg/mL of freshly refolded protein in DPBS were added in a 4 mL quartz fluorimeter cuvette (Hellma, Germany) and the fluorescence signal of its unique tryptophan was monitored at 340nm after excitation at 295nm by a Quantamaster-4 fluorescence spectrometer (Photon Technology International, New Jersey) after successive additions of small amounts of an 8.0 M guanidine hydrochloride solution. The contents were mixed by repeated pipetting for 5sec, incubated in the dark for 2 min and the fluorescence signal was recorded. The experimental data were fit to a Boltzman sigmoidal model curve using the Graphpad Prism™.

Supplemental tables

Supplemental Table 1: α-helical content and Tm of apoA-IV and HDL-A-IV

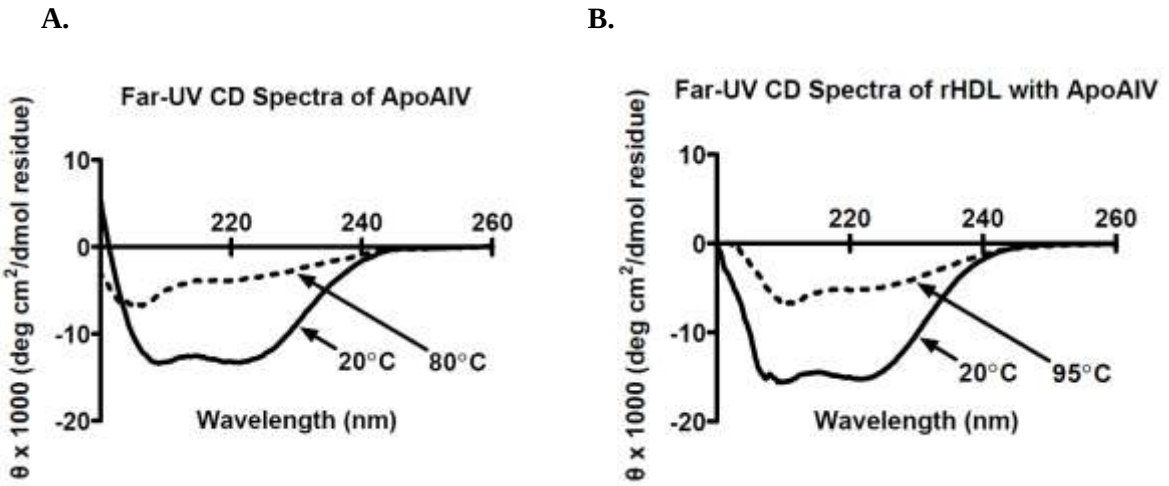
Sample	% α-helix at 222nm at 20˚C	% α-helix at 222nm at 80˚C	% α-helix at 222nm at 95˚C	Tm (˚C)
ApoA-IV	41.4	17.1		45.6
ApoA-IV in rHDL	46.7		20.9	61.4

Supplemental Table 2: Plasma lipids and hepatic apoA-IV mRNA levels

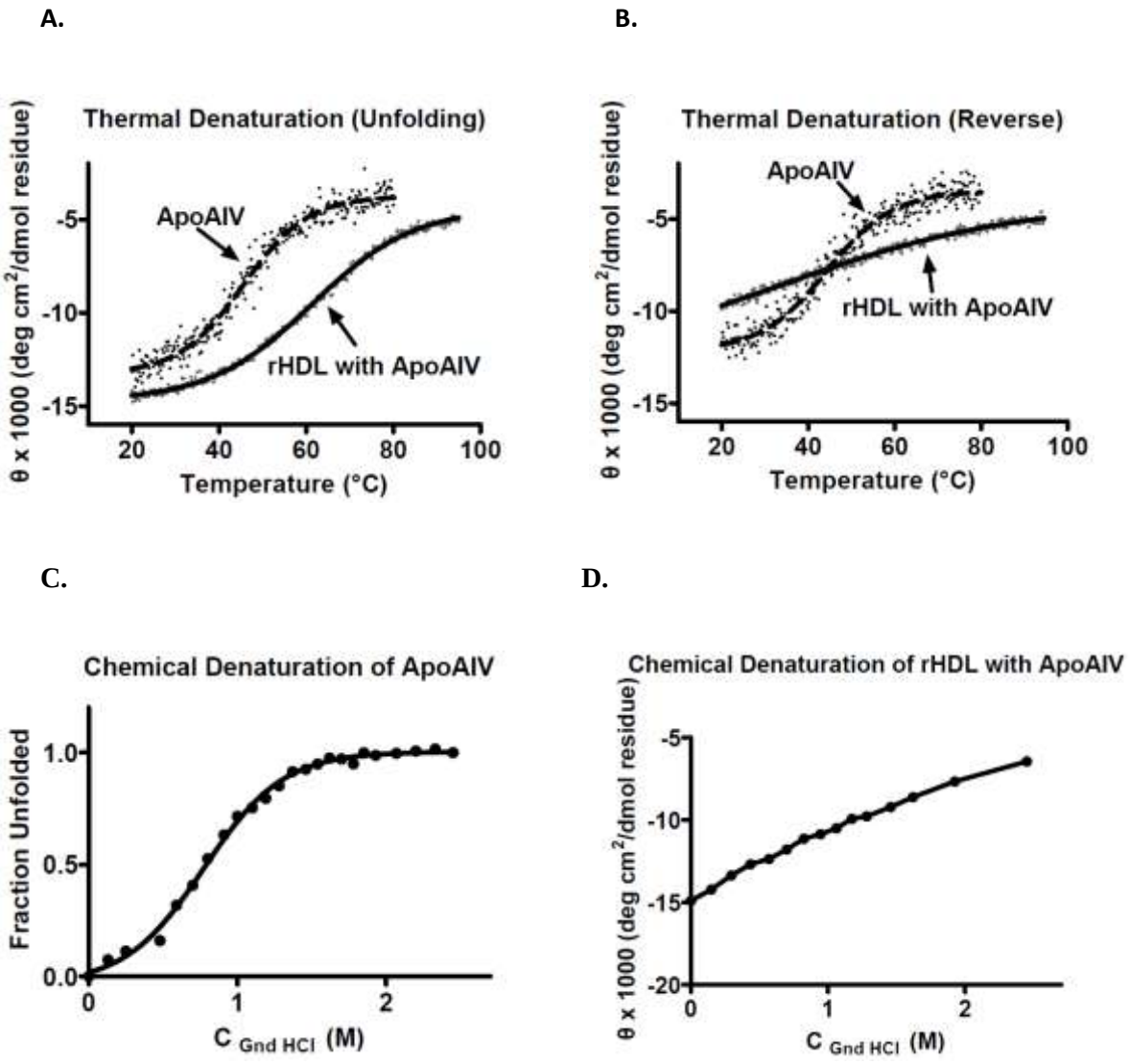
Mouse Strains that received apoAIV	Total Cholesterol (TC) (mg/dL)		Triglycerides (mg/dL)		Relative apoAIV mRNA (%)
	d0	d4	d0	d4	
apoA-I $^{-/-}$	38±4	52±17	13±5	18±12	2±0.3
apoA-I $^{-/-}$ x apoE $^{-/-}$	450±136	746±116	112±80	449±79^{+}	0.7±0.3
ABC A-I $^{-/-}$	22±13	27±25	112±20	46±21^{+}	0.3± 0.1
LCAT $^{-/-}$	36±8	44±10	39±10	68±22	1*

*Expression of apoA-IV is relative to the expression in LCAT$^{-/-}$ mice that was set as 1 (n=3-5 experiments). Statistical significant differences in cholesterol and triglyceride levels ($p<0.05$) between days 0 and 4 are indicated by a ($^{+}$).

Supplemental Figures



Supplemental Figure 1 A,B. Far-UV CD spectra of ApoA-IV (panel A) and rHDL containing apoA-IV (panel B). Solid lines represent spectra at 20˚C, while the dashed lines represent spectra at 80˚C (panel A) and 95˚C (panel B) respectively.



Supplemental Figure 2 A-D. Panels A and B. Thermal denaturation profiles of ApoA-IV and rHDL containing apoA-IV. Samples were denatured by increasing the temperature up to 80˚C for apoA-IV and up to 95˚C for rHDL-A-IV (A). Following denaturation, samples were gradually cooled down at a rate of 1°C/min while following the recovery of the molar ellipticity. Solid or dashed lines represent non-linear regressions to a simple Boltzmann sigmoidal model. Experimental points are indicated as dots. ApoA-IV can be refolded reversibly, while rHDL-A-IV cannot. Panel C, chemical denaturation profile of ApoA-IV. The fluorescence signal of the unique tryptophan of ApoA-IV was monitored while titrating with Gnd HCl. Solid line represents non-linear regression to a simple Boltzmann model. The experimental points

are indicated as dots. Panel D, chemical denaturation of rHDL-A-IV. The molecular ellipticity at 222nm was monitored during titration with Gnd HCl.

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