Apolipoprotein A-II regulates HDL stability and affects hepatic lipase association and activity

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Abstract The effect of apolipoprotein A-II (apoA-II) on the structure and stability of HDL has been investigated in reconstituted HDL particles. Purified human apoA-II was incorporated into sonicated, spherical LpA-I particles containing apoA-I, phospholipids, and various amounts of triacylglycerol (TG), diacylglycerol (DG), and/or free cholesterol. Although the addition of PC to apoA-I reduces the thermodynamic stability (free energy of denaturation) of its α-helices, PC has the opposite effect on apoA-II and significantly increases its helical stability. Similarly, substitution of apoA-I with various amounts of apoA-II significantly increases the thermodynamic stability of the particle α-helical structure. ApoA-II also increases the size and net negative charge of the lipoprotein particles. ApoA-II directly affects apoA-I conformation and increases the immunoreactivity of epitopes in the N and C termini of apoA-I but decreases the exposure of central domains in the molecule (residues 98–186). ApoA-II appears to increase HL association with HDL and inhibits lipid hydrolysis. ApoA-II mildly inhibits PC hydrolysis in TG-enriched particles but significantly inhibits DG hydrolysis in DG-rich LpA-I. In addition, apoA-II enhances the ability of reconstituted LpA-I particles to inhibit VLDL-TG hydrolysis by HL. Therefore, apoA-II affects both the structure and the dynamic behavior of HDL particles and selectively modifies lipid metabolism.—Boucher, J., T. A. Ramsamy, S. Braschi, D. Sahoo, T. A-M. Neville, and D. L. Sparks. **Apolipoprotein A-II regulates HDL stability and affects hepatic lipase association and activity. *J. Lipid Res.* 2004. 45: 849–858.**

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HDL particles are believed to play an important role in the intravascular transport of lipids. The predominant protein constituent of HDL is apolipoprotein A-I (apoA-I), and the second most abundant protein of this lipoprotein class is apoA-II. HDLs can be subdivided further into those containing only apoA-I and those containing both apoA-I and apoA-II. Several studies have shown that apoA-II can have significant effects on the structural properties of HDL (1–5) and that alterations in these directly affect the functional characteristics of this lipoprotein (4–9).

Early work has shown that apoA-II has a higher lipid affinity than apoA-I and can readily displace apoA-I from lipoprotein particles (1, 10). The apolipoprotein exhibits a higher mean residue hydrophobicity than apoA-I (11, 12), and as such, apoA-II binds to a lipoprotein surface more tightly and is less able to be exchanged between lipid surfaces (13). In the plasma, apoA-II is associated predominantly with smaller and less lipid-enriched HDL particles. The more dense HDL fractions, HDL_3, have been shown to contain higher relative amounts of apoA-II than the larger HDL_2 particles [with apoA-I/apoA-II ratios of 3.7 and 4.8, respectively (14)]. This increased content of apoA-II in the denser HDL classes is associated with a reduced ability of these lipoproteins to shed or exchange apoA-I (10, 16), and this appears to be related to an inability to form the small lipid-poor preβ-HDL lipoprotein classes (5, 10, 16). The present study now confirms the view that apoA-II generates a more stable HDL complex that is less susceptible to dynamic remodeling events.

Abbreviations: apoA-I, apolipoprotein A-I; CE, cholesteryl ester; $D_{1/2}$, midpoint of the guanidine hydrochloride denaturation curve; DG, diacylglycerol; ΔG_D^o, free energy of denaturation; ED_{50}, concentration required to inhibit 50% of the maximal binding of the antibodies to the apoHDL-coated plate; FC, free cholesterol; GdnHCl, guanidine hydrochloride; HL, hepatic lipase; LpA-I, reconstituted HDL complexes containing apoA-I; LpA-I/A-II, reconstituted HDL complexes containing apoA-I and apoA-II; LpA-II, reconstituted HDL complexes containing apoA-II; mAb, monoclonal antibody; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; TG, triacylglycerol.

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ApoA-II has been shown to directly affect HDL remodeling catalyzed by the interfacially active proteins found in the plasma. ApoA-II is less efficient than apoA-I at activating LCAT (17), and studies have shown that displacement of apoA-I in HDL by apoA-II inhibits cholesterol esterification (4). Similarly, apoA-II has been shown to inhibit the lipid transfer activities of cholesteryl ester transfer protein and phospholipid transfer protein (5, 18, 19), and as such, it is thought to directly affect their ability to control the remodeling and speciation of HDL. The effect of apoA-II on hepatic lipase (HL) is less clear, as there are reports that apoA-II can be both stimulatory (6, 20, 21) and inhibitory (7) to lipid hydrolysis. Although the studies appear to be in agreement that apoA-II increases the affinity with which HL binds HDL, the effects of apoA-II on overall hydrolytic rates and the rate of free fatty acid release appear to differ.

In this study, we have comprehensively evaluated the role of apoA-II in HDL structure and function with the use of homogeneous reconstituted lipoprotein systems that have well-defined structures and compositions. Previous work has shown that with comparable compositions, these model lipoproteins can have functional properties almost identical to those of the native HDL classes (22, 23). We have now characterized the effects of apoA-II on the structural properties of homogeneous reconstituted spherical HDL and on the ability of these model lipoproteins to interact with two different plasma proteins. We show that apoA-II uniquely affects the stability of HDL particles and indirectly affects enzyme function by altering the affinity with which interfacially active proteins interact with HDL.

EXPERIMENTAL PROCEDURES

Materials

Triacylglycerol (TG), diacylglycerol (DG), and free cholesterol (FC) were purchased from Sigma Chemical Co. (St. Louis, MO), and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) was obtained from Avanti Polar Lipids (Birmingham, AL). [^{3}H]cholesterol and [^{3}H]TG were purchased from Dupont Canada, Inc. (Mississauga, Ontario, Canada). Di[1-^{14}C]oleoyl phosphatidylcholine was obtained from Amersham Biosciences (Baie d'Urfé, Quebec, Canada). Antibodies 2G11 and 4A12 were purchased from Sanofi (France). Antibodies A03, A05, A07, A11, A17, A16, A44, and A51 were obtained from the Institute Pasteur (Lille, France). Antibodies 3G10, 3D4, 2F1, 4H1, 4F7, and 5F6 were produced in our laboratory (24). All other reagents were of analytical grade.

Purification of lipoproteins and preparation of spherical reconstituted HDL complexes containing apoA-I

Plasma from fasting, normolipidemic subjects was collected by venipuncture. VLDL and HDL were isolated by sequential ultracentrifugation within the density range $\rho < 1.006$ and 1.063–1.21 g/ml, respectively (25). HDL was delipidated in chloroform-methanol as described (26). ApoA-I was isolated by size exclusion chromatography on a Sephacryl S-200 HR column (27), and apoA-II was purified by anion-exchange chromatography on Q-Sepharose (28). Before use, apoA-I and apoA-II were resolubilized in 6 M guanidine hydrochloride (GdnHCl) and 10 mM Tris, pH 7.2, and dialyzed extensively against PBS (50 mM sodium phosphate buffer and 150 M NaCl, pH 7.2). Reconstituted HDL complexes containing apoA-I (LpA-I) and/or apoA-II (LpA-II) were prepared by cosonication of 3.2 mg of POPC, 0.6 mg of TG, and 2 mg of apoA-I and/or apoA-II (at the molar ratios shown in **Table 1**) as previously described (28). In some cases, various amounts of DG and/or FC were also included in the initial mixture. All complexes were filtered through a 0.22 μm syringe tip filter and then reisolated by size-exclusion chromatography on a Superose-6 column.

Physical characteristics of reconstituted HDL complexes containing apoA-I and apoA-II

The size and homogeneity of reconstituted HDL complexes containing apoA-I and apoA-II (LpA-I/A-II) were estimated by nondenaturing gradient gel electrophoresis on precast 8–25% acrylamide gels (Pharmacia Phastgel). After reisolation of the complexes, the protein concentration was determined by the Lowry method as modified by Markwell et al. (29), and the relative proportion of apoA-I was estimated from an RIA using a mixture of anti-apoA-I monoclonal antibodies (mAbs) (30). Total glycerides, FC, and POPC concentrations were determined enzymatically using kits from Roche Diagnostics. In some experiments, radioactive DG was included in the lipids to allow for the determination of particle DG content (31).

The average secondary structure of apoA-I and apoA-II in the LpA-I/A-II complexes was monitored by circular dichroism spectroscopy on a Jasco J40A spectropolarimeter as previously described (32). The effect of GdnHCl concentration on the average secondary structure of apoA-I and apoA-II on the lipoprotein particles was monitored by the changes in molar ellipticity at 222 nm. An apparent free energy of unfolding of apoA-I and apoA-II on the surface of LpA-I/A-II complexes was calculated as described previously (33). Briefly, the ΔG_D^0 values of apoA-I and apoA-II on the reconstituted complexes were calculated from the y-intercept of linear regressions of the free energy of denaturation against RT ln(1+ka), where R is the gas constant (1.98cal/degree mol), T is the temperature (298K) and ka is the average association constant of GdnHCl and protein ($0.6M^{-1}$). The thermodynamic parameters presented in this study represent the average values of the individual contributions of both apoA-I and apoA-II on the lipoprotein complex.

TABLE 1. Effect of apolipoprotein composition on the size and charge of LpA-I complexes

LpA-I Complex[a]	ApoA-I/A-II[a]	Diameter[b]	Surface Potential[c]
	mol/mol	*nm*	*−mV*
LpA-I	1:0	7.7	8.9
LpA-I/A-II	1:1	7.8	9.0
LpA-I/A-II	1:2	8.3[d]	9.1
LpA-II	0:1	8.7[d]	9.1

ApoA-I, apolipoprotein A-I; LpA-I/A-II, reconstituted HDL complexes containing apoA-I and apoA-II.

[a] Reconstituted HDL complexes containing apolipoprotein A-I (apoA-I; LpA-I) were prepared by cosonication of 3.2 mg of 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), 0.6 mg of TG, and 2 mg of apoA-II and/or apoA-I (molar ratios are indicated). Reisolated complexes all contained between 50 and 60 mol of POPC and ~5 mol of TG per particle. Values are representative of three to five different preparations of LpA-I complexes.

[b] Particle diameters from nondenaturing gradient gel electrophoresis.

[c] Charge potential at the particle surface ± 0.2 SD.

[d] Significantly different from LpA-I ($P < 0.001$).

Immunoreactivity of apoA-I in LpA-I/A-II complexes

Immunoreactivity measurements were determined from a competitive solid-phase radioimmunometric assay similar to that described previously (30). The 13 different antibodies recognize defined epitopes (residues) in apoA-I: 4H1 (2-8), 2F1 (8-82), A16 (14-29, 60-82), A05 (25-82), 2G11 (25-96), 3D4 (98-121), A17 (98-121), A11 (98-132), 5F6 (118-141), A03 (135-148), A07 (148-186), A44 (148-186), and 4A12 (173-205). Removawells (Immulon 2; Dynatech Laboratories) were coated with 100 μl of apoHDL and then incubated with mixtures of anti-apoA-I mAb (at a predetermined dilution) and LpA-I/A-II complexes (with 0.1% gelatin in PBS) for 1 h at room temperature. After three washes with 0.05% Tween 20 in PBS, the Removawells were incubated for 1 h with a ^{125}I-labeled anti-mouse IgG antibody (200,000 cpm/well) and then thoroughly washed and counted. The immunoreactivity of apoA-I in the various LpA-I complexes was estimated from the ED_{50}, which represents the LpA-I concentration required to inhibit 50% of the maximal binding of the antibodies to the apoHDL-coated plate. Results are expressed in micrograms per milliliter and are means ± SD of triplicate determinations.

Purification and assay of lecithin:cholesterol acyltransferase

LCAT was purified from normolipidemic plasma, and LCAT activity was determined as previously described (34). LCAT activity was measured by incubating [^{3}H]FC-labeled LpA-I/A-II with purified LCAT under standard incubation conditions and then by measuring the formation of [^{3}H]cholesteryl ester (CE) after extraction and TLC. LCAT activity is expressed as units of enzyme activity, where 1 U represents 1 nmol of cholesterol esterified per nanogram of purified protein per hour of incubation. The V_{max} and apparent K_m of cholesterol esterification by LCAT were determined from double-reciprocal plots of the CE formed as a function of the substrate concentration.

Purification and assay of hepatic lipase

Hepatic lipase was purified from postheparin human plasma by heparin affinity chromatography as previously described (15). LpA-I particles were characterized as substrates for purified HL using a standard assay system. Incubations were carried out for 3 h at 37°C, and fatty acid released during the incubation was determined using a free fatty acid enzyme kit from Roche Diagnostics (Laval, Quebec, Canada). The phospholipid, TG, and DG hydrolytic rates were then determined subtractively after measuring the quantity of radioactive fatty acids liberated from [^{3}H]TG and [^{14}C]DG during the incubation with HL using a liquid-liquid partitioning system as previously described (31). The hydrolytic rates are expressed as pseudo-first-order rate constants in Table 3.

VLDL hydrolysis by HL was characterized in the presence or absence of HDL or reconstituted HDL complexes as previously described (35). Briefly, each sample contained the lipoprotein substrate (350 μM VLDL), purified HL (26 U), 75 μl of incubation buffer (0.33 M Tris-HCl, pH 8.3, 1% fatty acid free-BSA, and 5 mM $CaCl_2$), PBS (to a final volume of 250 μl), and increasing concentrations of HDL or LpA-I/A-II complexes as indicated in Fig. 6. Incubations were carried out for 30 min, and the reactions were terminated on ice. The total amount of fatty acid released during the incubation was determined using free fatty acid diagnostic kits.

Statistical methods

The significance of differences between group mean values was calculated by one-way ANOVA using Instat® GraphPad Software (version 3.00).

RESULTS

Effect of apoA-II on the size and charge of the LpA-I complexes

Sonicated LpA-I particles were prepared to contain various amounts of apoA-I and/or apoA-II (see Table 1 for initial concentrations). All complexes were homogeneous in composition and exhibited only one band on nondenaturing gradient gels (**Fig. 1**), and all contained between 50 and 60 mol of POPC and 5 mol of TG. Inclusion of large amounts of apoA-II in the LpA-I complex caused an increase in the hydrodynamic diameter of the resulting complex. At a molar ratio of 1:1 (apoA-I/apoA-II), the complex exhibited a similar size to a particle containing only apoA-I. However, when greater amounts of apoA-II were complexed with apoA-I, the resulting complex appeared ~0.6 nm larger (Fig. 1). Similarly, when a particle was prepared from apoA-II alone, the LpA-II complex exhibited a size of 8.7 nm, ~1 nm larger than the LpA-I particle.

Western blots of nondenaturing gels treated with an anti-apoA-II antibody (data not shown) confirm the presence of apoA-II in particles with identical molecular weights as the bands shown in Fig. 1. Because minimal protein was lost during the sonication procedure, the stoichiometries of the reisolated heterologous particles (apoA-I/A-II) were similar to the initial composition shown in Table 1. Cross-linking studies confirm this view and show that dimethyl suberimidate cross-linked proteins on the different particles all exhibit an ~60 kDa band on SDS-PAGE gels. This appears to represent a dimer of apoA-I in the LpA-I and low apoA-II particles and an oligomer of apoA-I and two molecules of apoA-II in the apoA-II-enriched particle. Particle size and/or molecular weight measurements from nondenaturing gels are consistent with these stoichiometric estimates.

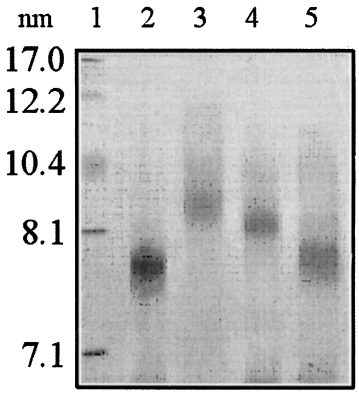

Fig. 1. Nondenaturing gradient gel electrophoresis of reconstituted HDL particles. The size and homogeneity of reconstituted HDL complexes containing apolipoprotein A-I (apoA-I) and apoA-II (LpA-I/A-II) were estimated by nondenaturing gradient gel electrophoresis on precast 8–25% acrylamide gels. Protein bands were visualized by Coomassie Blue R250 staining, and particle size (nanometers) was determined from reference standards. Lane 1, size markers; lane 2, LpA-I; lane 3, LpA-II; lane 4, LpA-I/A-II (1:2); lane 5, LpA-I/A-II (1:1).

The addition of one or two molecules of apoA-II to the sonicated LpA-I complexes also had a small effect on the particle charge and increased the negative surface potential by approximately −0.2 mV. These data are similar to our previously reported observation (36) and show that although differing amounts of apoA-II do not significantly modify HDL charge, the protein is able to contribute to the charge on HDL in a similar manner to apoA-I. It should be noted, however, that although the surface potentials are fairly similar for the different particles, the apoA-II complexes are larger; therefore, it appears that the net valence on these structures is more electronegative but spread out over a larger surface area. This suggests that the net charge on apoA-II is actually slightly higher than on apoA-I. Electrophoresis of the lipid-free proteins in fact shows that the net valence of two apoA-II molecules is ~1.5-fold greater than that for 1 mol of apoA-I (data not shown).

Effect of apoA-II on the secondary structure and stability of LpA-I

The effect of apoA-II on the secondary structure of the LpA-I complexes was evaluated by measuring the average amount of α-helical structures in both apoA-I and apoA-II by circular dichroism. Consistent with the work of others, lipid-free apoA-II was shown to exhibit significantly less α-helical content than lipid-free apoA-I (5, 13, 37, 38). Lipidation of both molecules increases the amount of α-helices in the proteins, but by greatly differing amounts. Complexation of 50 mol of POPC with 2 mol of apoA-I caused an ~20% increase in the amount of α-helix in the apolipoprotein (**Table 2**). In contrast, inclusion of similar amounts of lipid per 4 mol of apoA-II gave rise to a >50% increase in the amount of helical structure in apoA-II. The overall amount of helical structure in LpA-II, however, was still significantly less than that in LpA-I (42% and 58%, respectively). The inclusion of one to two molecules of apoA-II in the lipoprotein complex was associated with total α-helical estimates that were intermediate between those observed for the LpA-I and LpA-II particles.

Varying the apolipoprotein content of the LpA-I complexes had significant effects on the denaturation of the secondary structure of these apolipoproteins by GdnHCl. **Figure 2** shows that the isothermal unfolding of organized secondary structure differs for apoA-I and apoA-II. This difference appears to be primarily attributable to the reduced α-helical structure in apoA-II; midpoints of denaturation ($D_{1/2}$) are actually fairly similar for the two curves (Table 2). Lipidation of these apolipoproteins with POPC and TG has a similar effect on denaturation curves and substantially decreases the ability of GdnHCl to unfold the α-helical structure of apoA-I and apoA-II. These data are similar to what has been observed previously in thermal denaturation studies (2, 38, 35). Table 2 illustrates this point and shows that the $D_{1/2}$ for both apolipoproteins on the lipoprotein particles is significantly increased in the presence of lipids. In addition, similar to observations with α-helix content, LpA-I/A-II containing both apoA-I and apoA-II exhibited intermediate $D_{1/2}$ values relative to those observed for LpA-I and LpA-II particles.

TABLE 2. Effect of apolipoprotein composition on secondary structure and stability

LpA-I Complex[a]	ApoA-I/A-II[a]	α-Helix Content[b]	$D_{1/2}$[c]	Apparent $\Delta G_D{}^{0}$[d]
	mol/mol	*%*	*M*	*kcal/mol apoA-I*
ApoA-I		49[e]	1.0[e]	2.4[e]
ApoA-II		27[e]	0.7[e]	1.5
LpA-I	1:0	58	1.8	1.7
LpA-I/A-II	1:1	50[e]	2.1[f]	1.9
LpA-I/A-II	1:2	53	2.4[e]	2.1[f]
LpA-II	0:1	42[e]	2.8[e]	2.2[e]

$\Delta G_D{}^{o}$, free energy of denaturation; $D_{1/2}$, midpoint of the guanidine hydrochloride denaturation curve.

[a] LpA-I complexes were prepared by cosonication as described. Values are representative of three different preparations of LpA-I/A-II complexes.

[b] Apolipoprotein α-helix content determined from molar ellipticities at 222 nm ± 4% (SD).

[c] Midpoint of the guanidine hydrochloride (GdnHCl) denaturation curve (Fig. 2) ± 0.1 M (SD).

[d] Apparent free energy of denaturation at zero GdnHCl concentration ± 0.2 kcal (SD).

[e] Significantly different from LpA-I ($P < 0.001$).

[f] Significantly different from LpA-I ($P < 0.01$).

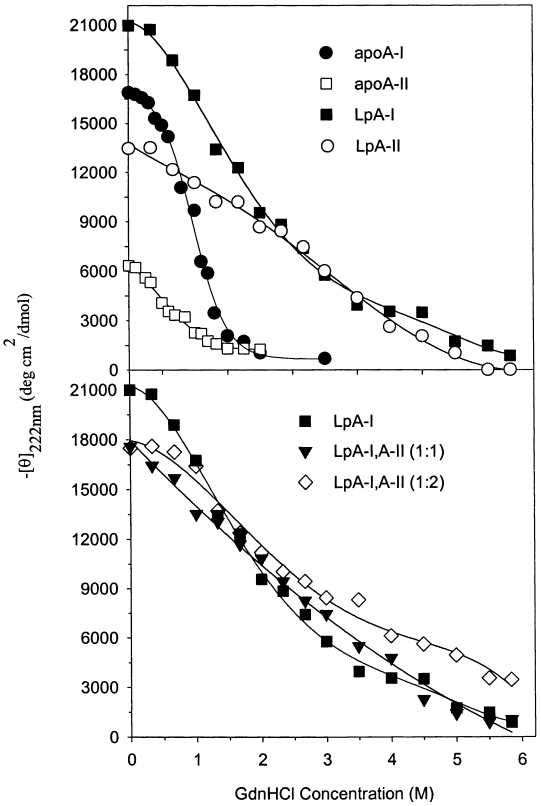

Fig. 2. Effect of guanidine hydrochloride (GdnHCl) concentration on the molar ellipticity of apoA-I on reconstituted HDL particles. Aliquots of LpA-I and LpA-I/A-II particles were incubated with 0–6 M GdnHCl in 0.05 M PBS for 72 h at 4°C. Circular dichroism spectra ($-[\theta]_{220nm}$) were measured at 24°C in a 0.1 cm path length quartz cell with sample protein concentration of 33.3 μg/ml in PBS, and four scans from 230 to 200 nm were collected and averaged at each GdnHCl concentration. Values are representative of three different experiments.

Binding model analysis of the denaturation curves shows that the thermodynamic stabilities of apoA-I and apoA-II are affected very differently by lipidation (**Fig. 3**, Table 2). As previously reported, apoA-I is thermodynamically destabilized in the presence of lipid and exhibits a reduced free energy of denaturation (ΔG_D^o) in a lipoprotein complex (22, 30). In contrast, apoA-II helical stability appears to increase when the apolipoprotein is lipidated and complexed into a lipoprotein particle (Table 2, Figs. 3, 4). **Figure 4** shows that although progressive lipidation reduces the ΔG_D^o for apoA-I, quite the opposite is observed for apoA-II. Reconstituted LpA-I/A-II exhibit ΔG_D^o values intermediate between those observed for particles prepared with the pure proteins, and increased amounts of apoA-II are associated with higher ΔG_D^o values. These data suggest that apoA-II stabilizes the α-helical structure of an HDL particle.

Effect of the apoA-II content of LpA-I/A-II complexes on the conformation of apoA-I

The conformation of the apoA-I molecule on the different LpA-I complexes was evaluated by competitive solid-phase radioimmunometric assay with a panel of 16 antibodies specific for epitopes that range from the N terminus to the C terminus of the apoA-I molecule (24, 39–41). Competitive binding curves were parallel for all mAbs; therefore, ED_{50} values were determined and are presented in **Fig. 5**. ED_{50} values show clear differences between the apoA-I immunoreactivity when apoA-II is present or absent on the lipoprotein complex. As illustrated in Fig. 5, the presence of apoA-II was associated with a decrease in the ED_{50} values for mAbs 2F1, A16, A51, and 2G11, which are located in the N-terminal domain of the apoA-I molecule. The ED_{50} for mAbs A44 and 4A12, located at the end of the C-terminal domain of apoA-I, were also decreased

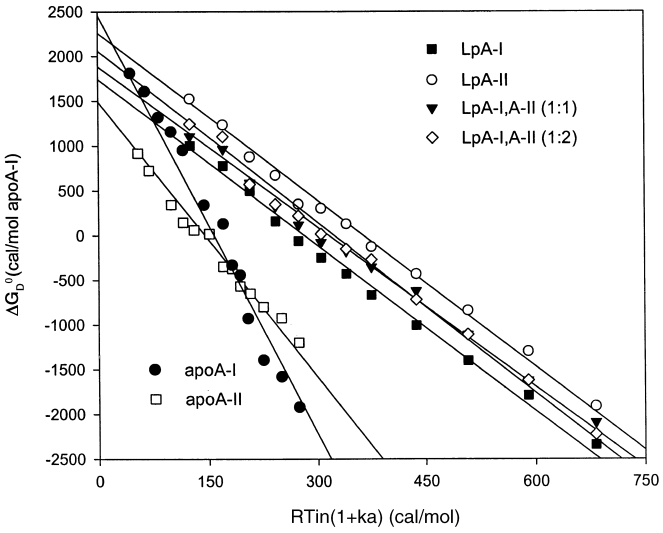

Fig. 3. Effect of apoA-II on the thermodynamic stability of reconstituted HDL. The free energy of denaturation (ΔG_D^o) of apoA-I and apoA-II on the reconstituted complexes was determined as previously described from the *y* intercept of linear regressions of the ΔG_D^o against the RT ln(1 + ka) of the data shown in Fig. 2.

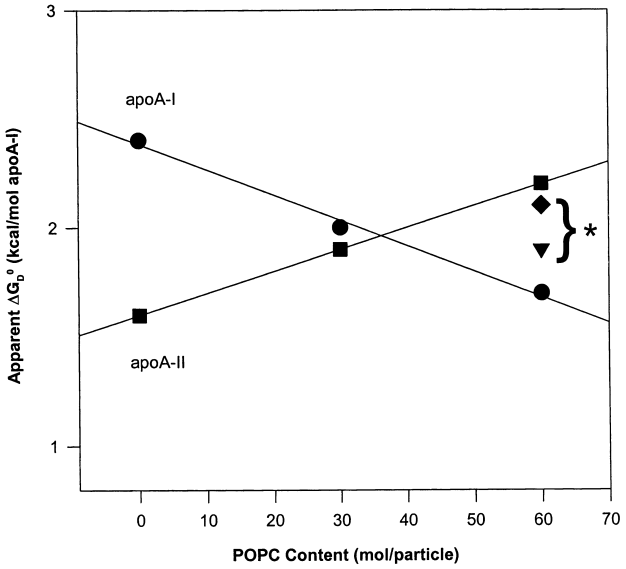

Fig. 4. Effect of phospholipid on the α-helical stability of apoA-I and apoA-II. ΔG_D^o values were determined as previously described and are shown for reconstituted HDL containing increasing amounts of 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) per apoA-I (circles) and apoA-II (squares) and also for reconstituted particles containing both proteins [asterisk (LpA-I/A-II 1:2, diamond; LpA-I/A-II 1:1, inverted triangle)].

in the presence of apoA-II. In contrast, the presence of apoA-II on a LpA-I/A-II complex was associated with an increase in the ED_{50} values for mAbs 3G10, 3D4, A11, 5F6, and A07 located in the middle to the C-terminal region of apoA-I. Overall, the data indicate that the presence of apoA-II molecules on reconstituted HDL profoundly affects apoA-I conformation and promotes the exposure of epitopes located at the N and C termini of the protein, whereas it blocks the access of mAbs specific to domains in the central part of the molecule.

Effect of apoA-II on cholesterol esterification by lecithin:cholesterol acyltransferase

The presence of apoA-II on a reconstituted HDL complex has a significant effect on the lipoprotein structural characteristics and therefore would be expected to also affect LpA-I/A-II functional properties. Previous studies have shown that apoA-II may be inhibitory to LCAT-mediated cholesterol esterification on discoidal reconstituted HDL (4). However, the effect of apoA-II in spherical HDL on LCAT activity has not been studied. Therefore, the effect of HDL apoA-II content on the rate of cholesterol esterification by LCAT was investigated on spherical HDL particles. Reconstituted LpA-I/A-II and LpA-I were prepared to contain a TG core, and the rate of cholesterol esterification was determined in the presence of purified human LCAT. Table 1 illustrates the kinetic rate constants for LCAT and shows that apoA-II had a small stimulatory effect on the rate of cholesterol esterification. However, the small increase in V_{max} was also associated with an almost 2-fold increase in the apparent K_m of apoA-I. This is consistent

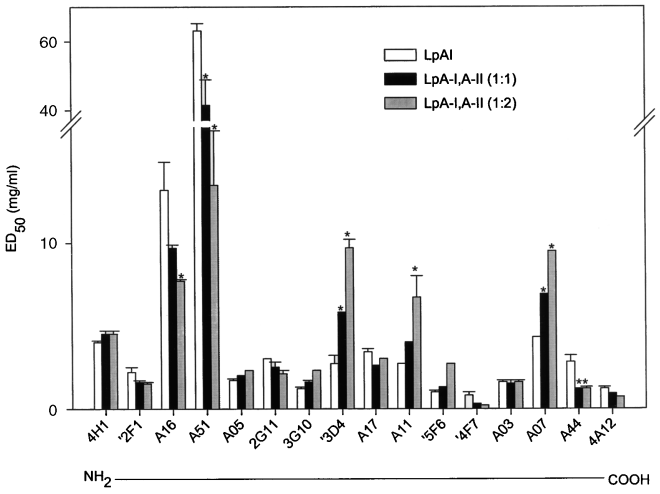

Fig. 5. Effect of apoA-II content on the apoA-I immunoreactivity in LpA-I/A-II complexes. The apoA-I immunoreactivity of the various LpA-I/A-II complexes for each monoclonal antibody (mAb) was evaluated by a radioimmunometric assay. ED_{50} was determined as previously described and represents the LpA-I/A-II concentration required to inhibit half of the maximum binding of the mAb. The mAbs are positioned on the *x* axis as their epitopes appear in the apoA-I sequence and define groups of epitopes localized to the N-terminal (4H1 to 2G11), the central (3G10 to A03), and the C-terminal (A07 to 4A12) regions of the apoA-I molecule. ED_{50} values are expressed in millligrams per milliliter as means ± SD of triplicate determinations and are representative of two different experiments. * Significantly different from LpA-I ($P < 0.01$).

with our previous reports (23, 30, 42) and suggests that a stimulation of LCAT activity is associated with a reduced HDL binding affinity and an increased ability to shuttle between substrates.

Effect of apoA-II on hepatic lipase hydrolytic activity

The effect of apoA-II on the hepatic lipase-mediated hydrolysis of phospholipid and acylglycerides was also evaluated. Previous studies have shown that apoA-II can be both stimulatory (6) and inhibitory (8) to HDL lipid hydrolysis by HL. Reconstituted LpA-I/A-II were prepared to contain various amounts of DG and TG and were incubated with human HL. **Figure 6** illustrates the effect of variations in both apoA-I/apoA-II and DG/TG contents of reconstituted HDL particles. Lipid hydrolytic rates were markedly increased in the DG-enriched particles, consistent with previous reports that DG is a preferred substrate of HL (31). ApoA-II was inhibitory to both sets of particles but was much more inhibitory to the reconstituted particles enriched with DG (Fig. 6A). **Table 3** shows that apoA-II reduced the V_{max} values for both the DG- and TG-enriched particles. In addition, apoA-II also resulted in reduced apparent K_m values, consistent with reports by Hime, Barter, and Rye (8). This suggests that apoA-II affects the affinity with which HL interacts with the lipoprotein particle. Measurement of the specific lipid hydrolytic rates showed that apoA-II selectively inhibited DG hydrolysis and had no effect on phospholipid hydrolysis in the DG-enriched particles, whereas apoA-II preferentially inhibited phospholipid hydrolysis in the DG-poor/TG-rich particles (Fig. 6B).

Previous studies have shown that apoA-I inhibits HL-mediated VLDL hydrolysis (15) and that different HDL fractions can be both inhibitory and stimulatory to VLDL hydrolysis (35). To determine if apoA-II content affects the hydrolytic regulatory ability of HDL, experiments were performed to evaluate the effects of apoA-II in reconstituted HDL particles on VLDL hydrolysis (**Fig. 7**). As in previous reports, reconstituted LpA-I particles inhibited HL-mediated VLDL hydrolysis (35). Inclusion of apoA-II in the reconstituted particle further increased the inhibitory effect of the complex on HL, as did HDL. In contrast, the LpA-II particle had no effect on VLDL hydrolysis by HL.

DISCUSSION

In this study, we have characterized the structure/function effects of apoA-II in highly homogeneous synthetic HDL particles with defined lipid compositions and unique structural properties. We show that homogeneous and well-defined HDL particles can be prepared from various ratios of apoA-I and apoA-II. As the apoA-II content is increased in the reconstituted particles, both the particle size and the negative surface charge are increased in magnitude. Even though lipid-free apoA-II contains fewer α-helices than apoA-I, lipidation of apoA-II was shown to increase its α-helical secondary structure to a greater extent than that observed for apoA-I. Nonetheless, the overall amount of helical secondary structure in HDL prepared with apoA-II was less than that in the apoA-I-only particles. This suggests that phospholipid uniquely affects apoA-II secondary structure in a manner quite different from that observed with apoA-I.

Isothermal denaturation experiments in this study show that apoA-II increases HDL particle stability and significantly blocks the ability of guanidine to denature or unfold HDL apolipoproteins. Binding model analysis of denaturation curves shows that apoA-I is thermodynamically destabilized in the presence of lipid and exhibits a reduced ΔG_D^o in a lipoprotein particle. Conversely, lipidation of apoA-II and formation of a lipoprotein particle increases both its helical content and stability. This lipidation-dependent stability is conferred to reconstituted HDL particles as apoA-II concentration is increased. Reconstituted LpA-I/A-II display ΔG_D^o values intermediate between those observed for LpA-II and LpA-I particles. We have made similar observations with native lipoproteins. HDL_3 has been shown to exhibit a similar ΔG_D^o (2 kcal/mol) as that observed for the particles containing both apoA-I and apoA-II (Table 2). When we removed the apoA-II-containing particles from HDL_3 using an anti-apoA-II column, the amount of guanidine required to reach the $D_{1/2}$ was reduced by >50% in the HDL containing only apoA-I (data not shown). These data confirm that apoA-II stabilizes the overall protein secondary structure

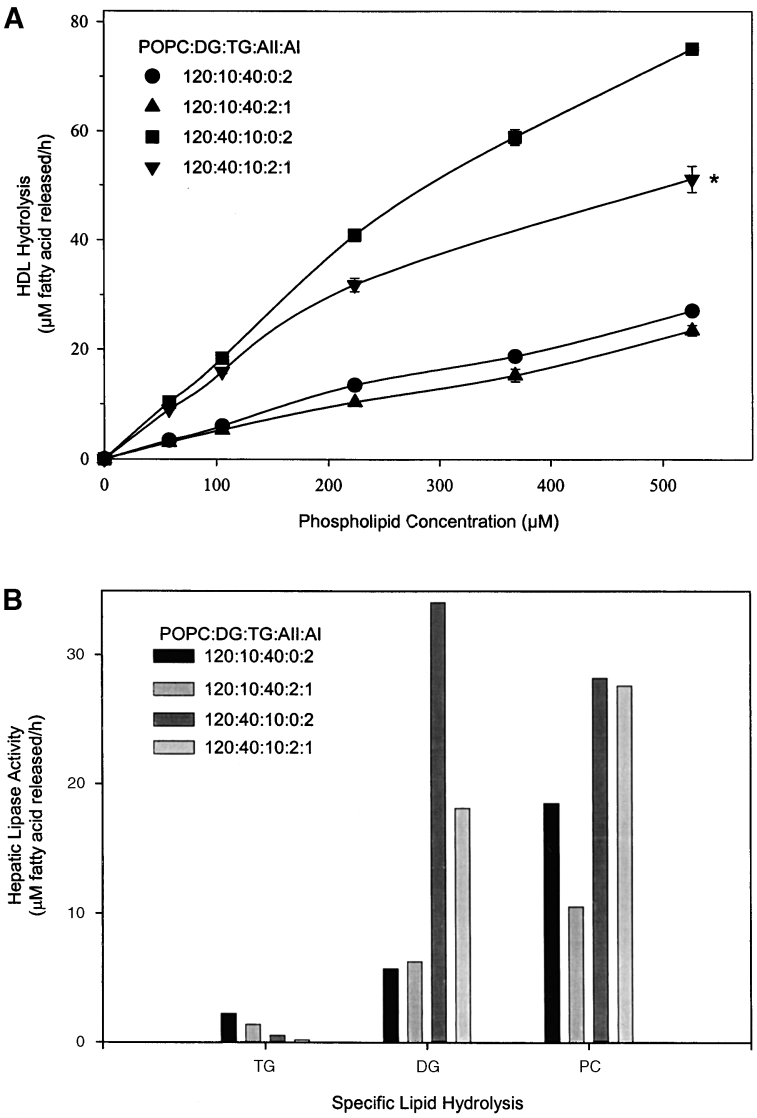

Fig. 6. Effect of apoA-II content of LpA-I/A-II complexes on HL hydrolytic activity. A: LpA-I and LpA-I/A-II particles (POPC/DG/TG/apoA-II/apoA-I molar ratios) were prepared as described and incubated with purified human HL for 3 h at 37°C. Total lipid hydrolysis was determined by measuring the amount of fatty acid released per hour using a commercially available enzyme kit. Values shown are means ± SD of triplicate determinations and are representative of three different preparations of reconstituted complexes. B: The differential hydrolysis of phospholipid, TG, and DG by HL was determined subtractively by measuring the amount of free fatty acid released from [^{3}H]TG and [^{14}C]DG relative to the total fatty acid release for each of the reconstituted particles. Values shown are averages of triplicate determinations and are representative of two different experiments. * Significantly different from DG-enriched LpA-I ($P < 0.001$).

of an HDL particle. This is consistent with another recent report that suggested that apoA-II may promote protein-protein interactions with apoA-I, which inhibit the remodeling of HDL and block the dissociation of lipid-poor apoA-I (5).

It is well established that apoA-II is more lipophilic than apoA-I (1); therefore, the lipidation-dependent stabilization of apoA-II secondary structure may explain why apoA-II is less exchangeable than apoA-I. It has been known for some time that apoA-II is able to displace apoA-I from HDL (1). However, the smaller and denser HDL_3, which contains higher relative amounts of apoA-II, has been shown to be less able to shed or exchange apoA-I (35). This may explain why the denser classes of HDL are less able to form small lipid-poor preβ-HDLs (16). Preβ-HDLs are important in the reverse cholesterol transport pathway as the initial acceptors of lipid from extrahepatic tissues. Therefore, apoA-II stabilization of HDL may explain some of the functional differences between LpA-I and LpA-I/A-II particles and why they exhibit unique metabolic behaviors in vivo.

In this study, we demonstrate that apoA-II content directly affects the conformation of apoA-I in LpA-I/A-II particles. Using a series of 16 different mAbs, we have shown that the addition of apoA-II molecules to reconsti-

TABLE 3. Effect of LpA-I composition on LCAT and HL

LpA-I Complex	FC/DG/TG[a]	V_{max}[b]	V_{max}[b]	K_m[b]
	mol/mol/mol	*nmol FFA/h*	*nmol cholesteryl ester/h*	*μM apoA-I*
LpA-I	30:0:20		6	24
LpA-I/A-II	30:0:20		8	41
LpA-I	0:10:40	94		2,049
LpA-I/A-II	0:10:40	74		990
LpA-I	0:40:10	160		983
LpA-I/A-II	0:40:10	102		789

DG, diacylglycerol; FC, free cholesterol; TG, triacylglycerol.

[a] LpA-I was prepared as described but with the indicated FC, DG, and TG ratios. Reisolated particles exhibited similar stoichiometric ratios. Values are representative of three different preparations of LpA-I/A-II complexes.

[b] V_{max} and apparent K_m values were estimated from double reciprocal plots.

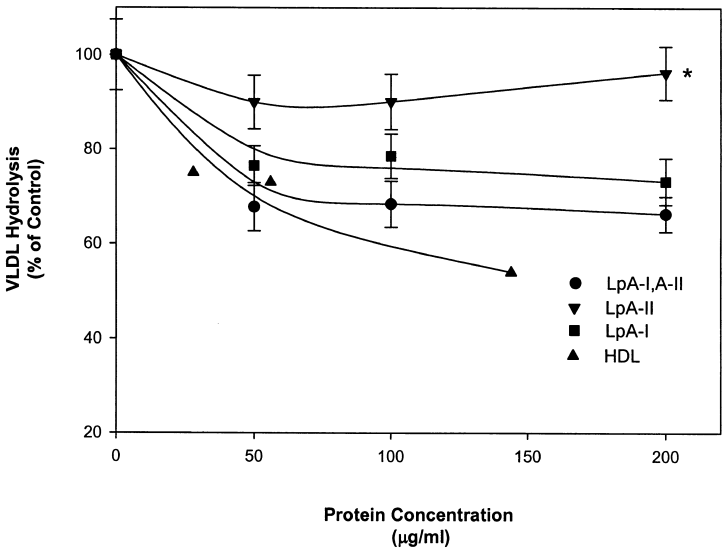

Fig. 7. Effect of HDL apolipoprotein content on VLDL-TG hydrolysis by HL. VLDL (350 μM TG) was incubated with HL and increasing amounts of HDL, LpA-I, LpA-I/A-II, or LpA-II particles for 30 min at 37°C. Hydrolysis was determined by measuring the amount of free fatty acid released using a commercially available enzyme kit. Hydrolytic values shown are means ± SD of triplicate determinations and are representative of two different experiments. * Significantly different from LpA-I ($P < 0.001$).

tuted HDL (LpA-I) exposes epitopes at the N and C termini of apoA-I while blocking the expression of epitopes in the central region of the protein. This suggests that apoA-II is involved in protein-protein interactions with apoA-I. Using a different series of apoA-I-specific mAbs, Rye et al. (5) showed that a region central to the C-terminal domain (residues 143–242) of apoA-I also became less exposed in a reconstituted particle containing apoA-II. They proposed that this may be the result of salt bridges between apoA-II and apoA-I and showed that these structural events can markedly affect the remodeling of HDL particles. Our data support this view but suggest that these protein-protein interactions may be localized closer to the center of the apoA-I molecule (residues 98–186). Our mAbs targeted to the C terminus of apoA-I (residues 173–205) actually showed a small increased binding to the protein. These data show that apoA-II directly affects apoA-I conformation and thereby indirectly affects the functional properties of HDL. ApoA-II appears to generate a more stable HDL that is less susceptible to dynamic remodeling events.

We also investigated the ability of apoA-II in reconstituted spherical HDL to affect LCAT enzymatic activity. ApoA-II caused a small stimulation of cholesterol esterification by LCAT, as evident by a small increase in V_{max}. In contrast, studies by Durbin and Jonas (43) have previously shown that apoA-II has an inhibitory effect on the rate of cholesterol esterification by LCAT with discoidal HDL particles. This suggests that the regulation of LCAT with discoidal versus spherical lipoproteins may differ. However, consistent with this previous report, apoA-II also caused a 2-fold increase in the apparent K_m. This is similar to what was observed in our previous work and suggests that a stimulation of LCAT activity is associated with reduced HDL binding affinity. A reduction in HDL association with LCAT may stimulate activity by promoting an increased ability to shuttle between substrate lipoprotein particles.

To study the role of apoA-II on HL substrate affinity and hydrolytic activity, the effect of apoA-II on the HL-mediated lipid hydrolysis of LpA-I and LpA-I/A-II was evaluated. Lipid hydrolysis was much higher in the DG-enriched particles, confirming our previous observation that DG is the preferred substrate of HL (31). ApoA-II caused a reduction in the lipid hydrolysis of both the DG- and TG-enriched particles; however, the inhibition of HL activity was much greater in the DG-enriched LpA-I/A-II. The addition of apoA-II also resulted in reduced apparent K_m values, suggesting that apoA-II increased the affinity of HL for its lipoprotein substrate. These data suggest that apoA-II is inhibitory to HL-mediated lipid hydrolysis by decreasing the ability of the enzyme to shuttle between substrates. This may partially explain why HDL_2 is a better substrate for HL than is HDL_3 and appears consistent with the work of Mowri et al. (44) showing that HL associates more readily with HDL_3, which contains higher amounts of apoA-II relative to HDL_2. We have made similar observations and have seen a greater association of HL with the reconstituted LpA-I/A-II than with the LpA-I (on Western blots) after reisolation of the complexes by size-exclusion chromatography (data not shown).

Previous studies in our laboratory have shown that apoA-I is inhibitory to VLDL-TG hydrolysis by HL and that different HDL subspecies can be either inhibitory or stimulatory to VLDL-TG hydrolysis (35). In the present study, we show that LpA-I is inhibitory to VLDL-TG hydrolysis, as previously reported (35), and that inclusion of apoA-II in the particle (LpA-I/A-II) further increases this inhibitory effect. In contrast, the particles containing only apoA-II (LpA-II) had no effect on VLDL-TG hydrolysis. This suggests that it is the apoA-I-containing particles that are uniquely inhibitory to HL and that apoA-II can affect this action, potentially by modifying the structural properties of the LpA-I complex. We have reported previously that HDL_3 is inhibitory to VLDL-TG hydrolysis by HL and that HDL_2 is stimulatory. The greater inhibitory effect of HDL_3 may be partly attributable to the relatively higher amount of apoA-II found in this HDL subspecies. Enhanced binding of HL to HDL_3 may directly block the shuttling/asso-

ciation of HL with circulating VLDL and thereby reduce VLDL-TG hydrolysis.

We have now shown that apoA-II can both increase HDL particle stability and cause conformational changes to apoA-I on synthetic HDL particles. These structural alterations have important effects on HDL functional properties. This study suggests that apoA-II may affect the interfacial enzyme association with HDL particles. ApoA-II appears to inversely affect LCAT and HL, decreasing and increasing enzyme association, respectively. Although apoA-II only slightly affects LCAT activation, the apolipoprotein significantly affects HL and inhibits VLDL-TG hydrolysis. By uniquely affecting enzyme and substrate affinity, apoA-II appears to control the dynamic behavior of HDL and directly affects lipid metabolism.

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