Comparative effects of purified apolipoproteins A-I, A-II, and A-IV on cholesteryl ester transfer protein activity

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Abstract The aim of the present study was to determine in vitro the effects of various purified apolipoproteins (apo) on the activity of the cholesteryl ester transfer protein (CETP). It appeared that the ability of apoA-I, A-II, and A-IV to modulate the CETP-mediated transfer of radiolabeled cholesteryl esters between low density lipoproteins (LDL) and high density lipoproteins (HDL) was markedly influenced by the final apolipoprotein:lipoprotein ratio in incubation mixtures. At low apolipoprotein:lipoprotein ratio, the rate of radiolabeled cholesteryl esters transferred from HDL_3 to LDL was significantly increased in the presence of apoA-I and apoA-IV. Under similar conditions, the rate of radiolabeled cholesteryl esters transferred from LDL to HDL_3 was increased in the presence of apoA-I while apoA-IV had no significant effects. At high apolipoprotein:lipoprotein ratio, the ability of apoA-I and apoA-IV to enhance the rate of radiolabeled cholesteryl esters transferred either from HDL_3 to LDL or from LDL to HDL_3 was considerably reduced. At the highest apolipoprotein:lipoprotein ratio studied, apoA-I and A-IV became inhibitors of the CETP-mediated transfer reaction. Interestingly, apoA-II differed markedly from other apolipoproteins as, even at a low apolipoprotein:lipoprotein ratio, it significantly inhibited CETP activity as measured either from HDL_3 to LDL or from LDL to HDL_3. The inhibition by apoA-II was concentration-dependent and, at the highest apolipoprotein:lipoprotein ratio studied, cholesteryl ester transfer activity was totally suppressed. The possibility of a direct interaction between CETP and the two major HDL apolipoproteins, apoA-I and apoA-II, was further investigated by combining crosslinking and immunoblotting techniques. Whereas CETP alone had an apparent molecular mass of 76,000 ± 3,100 Da, crosslinking reactions in incubation mixtures containing CETP and either apoA-I or apoA-II revealed the appearance of additional protein bands with apparent molecular masses of 99,600 ± 6,100 and 86,900 ± 4,500 Da, respectively. These complexes corresponded to the association of one molecule of CETP with one molecule of apoA-I or apoA-II. Interestingly, the mass concentrations of apoA-II needed to produce visible CETP-apolipoprotein complexes appeared to be about ten times higher as compared with apoA-I, suggesting that CETP may have a lower affinity for apoA-II than for apoA-I. In conclusion, data from the present study indicate that apolipoproteins A-I, A-II, and A-IV could be potent modulators of the CETP-mediated transfer of cholesteryl esters between HDL and LDL fractions. As an additional finding of this report was the formation of CETP-apoA-I and CETP-apoA-II complexes, we suggest that the modulating effect of apolipoproteins could be mediated through their direct interaction with CETP.—**Guyard-Dangremont, V., L. Lagrost, and P. Gambert.** Comparative effects of purified apolipoproteins A-I, A-II, and A-IV on cholesteryl ester transfer protein activity. *J. Lipid Res.* 1994. **35:** 982–992.

Supplementary key words high density lipoprotein • low density lipoprotein • radiolabeled cholesteryl esters • polyacrylamide gel electrophoresis • preparative electrophoresis • crosslinking • immunoblotting

In human plasma, cholesteryl ester transfer protein (CETP) catalyzes the exchange of cholesteryl esters and triglycerides between very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) (1). While it is now well accepted that plasma CETP plays a central role in lipoprotein metabolism, it is still uncertain whether it increases the atherogenic potential by transferring cholesteryl esters from HDL to the atherogenic apolipoprotein (apo) B-containing particles (1) or whether it acts as an antiatherogenic factor by favoring the transport of cholesteryl esters from peripheral tissues back to the liver (2).

The precise mechanism of action of CETP is not completely elucidated. However, there is strong evidence that the cholesteryl ester transfer reaction involves a direct interaction of CETP with lipoprotein particles (3, 4). Recent studies demonstrated that many factors may influence CETP activity. For example, in vitro studies have shown that nonesterified fatty acids enhance the

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; CETP, cholesteryl ester transfer protein; SDS, sodium dodecyl sulfate; EDTA, ethylenediamine tetraacetic acid; HMW, high molecular weight; LMW, low molecular weight; TBS, Tris-buffered saline; HSA, human serum albumin; DTT, dithiothreitol; BS, bis(sulfosuccinimidyl)suberate; LCAT, lecithin:cholesterol acyltransferase.

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CETP-mediated transfer of cholesteryl esters from HDL to VLDL or LDL by increasing the interaction of CETP with lipoprotein substrates (4–6). In fact, it appears that the binding of CETP with lipoprotein substrates is dependent on their surface properties as determined by the polar lipid (4, 7) and apolipoprotein (8) composition of the particles. In plasma, apolipoproteins exist in a dynamic state at the surface of lipoproteins and can exchange freely between lipoprotein particles (9, 10). Thus, one can postulate that alterations of the apolipoprotein composition of lipoprotein substrates during their intravascular remodeling could secondarily influence the CETP-mediated lipid transfer reaction by modulating the interaction of CETP with lipoprotein surfaces. Indeed, during the past few years, apolipoprotein-induced alterations of the cholesteryl ester transfer reaction have been reported (11–15). However, comparisons of results from various studies are made difficult as the effect of apolipoproteins on CETP activity has been determined by using experimental systems involving either plasma lipoprotein fractions or artificial lipid emulsions. For example, Son and Zilversmit (11) reported that apoA-I could inhibit cholesteryl ester and triacylglycerol transfers from LDL to HDL whereas, in other studies, plasma apolipoproteins A-I, A-II, C-II, C-III, and E were shown to equally enhance the transfer of cholesteryl esters between LDL and phospholipid/triacylglycerol microemulsions (12, 14). Moreover, in a recent study (16), we observed that cholesteryl ester transfer rates are significantly lower with HDL particles containing both apoA-I and apoA-II as compared with particles containing only apoA-I, whereas observations from another group suggested that purified apoA-II may have a stimulatory effect on cholesteryl ester transfers (17). These controversial data could be explained either by differences in the apolipoprotein concentrations used in the various studies or by the way apolipoproteins were added to the medium, i.e., either directly as free proteins (11, 17) or after their incorporation into lipoproteins (15, 16), recombinant particles (18), or microemulsions (12–14). The mechanism by which apolipoproteins interact with CETP is still unknown and the modulation of cholesteryl ester transfers by apolipoproteins has been interpreted successively as their ability to stabilize lipid emulsions (12) or to enhance the affinity of CETP for lipoprotein particles (15).

In the present study, the effects of apolipoproteins A-I, A-II, and A-IV on the CETP-mediated transfer of radiolabeled cholesteryl esters between HDL_3 and LDL were compared. It appeared that the ability of apolipoproteins to modulate CETP activity was dependent on their structure and concentration. In addition, data brought evidence of formation of CETP-apoA-I and CETP-apoA-II complexes.

MATERIALS AND METHODS

Lipoprotein preparation

Lipoprotein fractions (LDL and HDL_3) were isolated from normolipidemic sera by sequential ultracentrifugation at 100,000 rpm (350,000 *g*) in a 100.2 rotor in a TL-100 ultracentrifuge (Beckman, Palo Alto, CA). Densities were adjusted by the addition of solid KBr and checked with a DMA 35 digital densitymeter (Paar, Graz, Austria).

LDL were isolated as the serum fraction of density 1.019–1.055 g/ml with a 4-h spin at the lowest density and two 5-h spins at the highest density. HDL_3 were isolated as the serum fraction 1.13–1.21 g/ml with a 7-h spin at the lowest density and two 10-h spins at the highest density. Lipoproteins were recovered by tube slicing and were dialyzed overnight against a 10 mmol/l Tris, 150 mmol/l NaCl, pH 7.4, buffer containing 1 mmol/l EDTA-Na_2 and 3 mmol/l NaN_3 (TBS buffer). Ultracentrifugally isolated lipoprotein fractions were deficient in CETP and LCAT activities.

Radiolabeling of LDL and HDL_3

HDL_3 were biosynthetically labeled as previously described (19). Briefly, the d > 1.13 g/ml fraction ultracentrifugally isolated from 20 ml normolipidemic serum and dialyzed against TBS was incubated with 10 nmol of [$1\alpha,2\alpha(n)$-^{3}H]cholesterol (sp act 46 Ci/mmol) (Amersham, Les Ulis, France) for 24 h at 37°C in a shaking water bath to allow cholesterol esterification by the lecithin:cholesterol acyltransferase (LCAT) reaction (19). Subsequently, LDL were labeled by adding to the preceding mixture the d 1.019–1.055 g/ml fraction obtained from 10 ml of normolipidemic serum. The incubation was then prolonged for a 6-h period to allow the exchange of radiolabeled esterified cholesterol between lipoprotein substrates. At the end of the incubation, the labeled LDL and HDL_3 fractions were recovered by sequential ultracentrifugation as described above.

Typical labeled preparations of LDL and HDL_3 obtained with this procedure had specific activities of approximately 4,000 and 10,000 cpm/nmol of cholesterol, respectively. As judged by thin-layer chromatography, more than 95% of total radioactivity of both lipoprotein substrates resided in the cholesteryl ester moiety.

Purification of the cholesteryl ester transfer protein

CETP was purified from 2500 ml of citrated human plasma as previously described (20). Briefly, plasma was subjected to ammonium sulfate precipitation between 35 and 55% saturation and to ultracentrifugation at a density of 1.25 g/ml. The resulting d > 1.25 g/ml fraction was then subjected successively to hydrophobic interaction

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chromatography on a phenyl-Sepharose CL-4B column (Pharmacia, Uppsala, Sweden), to cation exchange chromatography on a carboxymethyl-cellulose column (Whatman, Kent, UK), and to anion exchange chromatography on a MonoQ HR 5/5 column (Pharmacia) (20). The chromatographic procedures were performed using an FPLC system (Pharmacia).

Each fraction recovered from the MonoQ column was assayed for cholesteryl ester transfer activity by measuring its ability to transfer radiolabeled cholesteryl esters from HDL_3 to LDL (21). Active fractions eluted from the MonoQ column were pooled, aliquoted, and stored at −20°C. The preparation of CETP used in the present experiments was deficient in LCAT activity as checked by using the cholesterol esterification assay of Piran and Morin (22). As observed by using gradient gel electrophoresis and immunoblotting, the CETP preparation did not contain detectable amounts of apolipoproteins A-I, A-II, and A-IV (results not shown).

Cholesteryl ester transfer activity assays

Cholesteryl ester transfer activity was determined by measuring the rate of radiolabeled cholesteryl esters transferred either from radiolabeled HDL_3 to unlabeled LDL or from radiolabeled LDL to unlabeled HDL_3. Each incubation mixture contained labeled lipoproteins (2.5 nmol cholesterol), unlabeled lipoproteins (10 nmol cholesterol), and partially purified CETP (0.6 μg protein) in a final volume of 50 μl. Mixtures were incubated for 3 h at 37°C in Eppendorf tubes. At the end of the incubation, tubes were immediately placed on ice for 15 min and centrifuged for 5 min at low speed to remove condensed water. A volume of 45 μl of incubated mixtures was added to 2 ml of a d 1.07 g/ml KBr solution in Beckman centrifugation tubes. The tubes were sealed and ultracentrifuged for 7 h at 50,000 rpm (269,000 *g*) in a 50.4 Ti rotor in a L7 ultracentrifuge (Beckman, Palo Alto, CA). The d $<$ 1.068 g/ml and d $>$ 1.068 g/ml fractions were subsequently recovered in 1-ml volumes after tube slicing and transferred into counting vials. A volume of 2 ml of scintillation fluid (OptiScint Hisafe 3, Pharmacia) was added to each vial and the radioactivity was assayed for 5 min in a Wallac 1410 liquid scintillation counter (Pharmacia). The recovery of total radioactivity in d $<$ 1.068 g/ml and d $>$ 1.068 g/ml fractions was constantly greater than 95%. In nonincubated controls containing radiolabeled HDL_3, the radioactivity in the d $<$ 1.068 g/ml did not exceed 4% of the total. In nonincubated controls containing radiolabeled LDL, less than 10% of the radioactivity was recovered in the d $>$ 1.068 g/ml fraction. Incubation of either radiolabeled HDL_3 with LDL or radiolabeled LDL with HDL_3 in the absence of CETP did not promote significant transfers as compared with nonincubated controls, purified apolipoproteins being added or not.

Cholesteryl ester transfer activity was expressed as the percentage of radiolabeled cholesteryl esters transferred from the radiolabeled donor to the d $<$ 1.068 or d $>$ 1.068 g/ml acceptor fraction after deduction of blank values of control mixtures kept at 4°C. Cholesteryl ester transfer assays were linear as long as the transfer of [^{3}H]cholesteryl esters was less than 40%.

Purification of apolipoproteins

Apolipoproteins A-I, A-II, and A-IV were isolated from human sera by using the preparative electrophoresis procedure previously described (23).

Human apolipoproteins A-I and A-II were purified from high density lipoproteins (HDL) that were ultracentrifugally isolated from fasting human serum as the d 1.070–1.21 g/ml fraction (24). Human apoA-IV was extracted from serum by a lipid emulsion (Intralipid) as described by Weinberg and Scanu (25). Prior to gradient gel electrophoresis, the HDL preparation was delipidated with butanol-diisopropylether 2:3 (26) and the Intralipid extract was delipidated with diethylether–ethanol 3:1 (25).

Apolipoprotein-containing samples were supplemented with either 25 g/l sodium dodecylsulfate (SDS) (for apoA-I and A-II) or with both 25 g/l SDS and 16.5 g/l dithiothreitol (DTT) (for apoA-IV) and were applied to a linear gradient of polyacrylamide ranging from 25 to 300 g/l and containing 1 g/l of SDS (23). The migration buffer was a 49 mmol/l Tris, 380 mmol/l glycine, 1 g/l SDS, pH 8.3 solution. Electrophoresis was conducted for 12 h at 100 V. After electrophoresis, gel portions containing purified apolipoproteins were cut off and apolipoproteins were electrophoretically transferred into agarose gels as previously described (27). Finally, purified apolipoproteins were removed from the gel by a rapid centrifugation (27), SDS was totally removed from the purified protein preparation by using an ExtractiGel D column (Pierce, Rockford, IL), and apolipoproteins were dialyzed overnight against TBS buffer.

Non-denaturating polyacrylamide gradient gel electrophoresis

Non-denaturing electrophoresis was performed in 40–300 g/l polyacrylamide gradient gels (PAA 4/30; Pharmacia) according to the general procedure previously described (28, 29). The migration buffer was a 14 mmol/l Tris, 110 mmol/l glycine, pH 8.3 solution. At the end of electrophoresis, the gels were stained with a solution of 0.4 g/l Coomassie Brilliant Blue G-250 in a solution of 0.33 mol perchloric acid/l and then destained in a 5% methanol, 7.5% acetic acid solution. Apparent molecular weights of separated proteins were determined as compared with protein standards (high molecular weight (HMW) and low molecular weight (LMW) calibration kits; Pharmacia) which were submitted to electrophoresis together with samples under study.

In some experiments, gradient gels were analyzed by

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densitometric scanning of the gels at 633 nm with a 2202 Ultroscan laser densitometer (LKB, Bromma, Sweden) attached to a 2220 integrator (LKB). A semi-quantitative analysis of apolipoprotein amounts in the lipoprotein- and the non-lipoprotein-associated fractions was evaluated by determining the relative areas under the corresponding portions of the scan curves and by relating them to the total area.

SDS-polyacrylamide gradient gel electrophoresis

Homogeneity of purified apolipoprotein preparations was checked by SDS electrophoresis in 80–250 g/l polyacrylamide gradient gels (PAA 8/25; Pharmacia) on a Phastsystem apparatus (Pharmacia). Electrophoresis was performed for 20 min at 250 V in a 0.55 g/l SDS, 0.20 mol/l Tricine, 0.20 mol/l Tris, pH 7.5 buffer, and gels were stained with 0.01% Coomassie Blue R as recommended by the manufacturer.

Crosslinking

Proteins were crosslinked with the membrane impermeant, bifunctional cross-linking reagent bis(sulfosuccinimidyl)suberate (BS) (Pierce Chemical Co., Rockford, IL) according to the general procedure described by Staros (30) with minor modifications (31). Briefly, protein samples were incubated for 30 min at 37°C in the presence of BS (final concentration, 8 mmol/l). The crosslinking reaction was stopped by the addition of SDS (final concentration, 10 g/l) and protein samples were subjected to SDS-polyacrylamide gel electrophoresis on PAA 4/30 gradient gels (Pharmacia) as previously described (31). Apparent molecular weights of crosslinked complexes were determined as compared with protein standards (HMW and LMW calibration kits; Pharmacia).

Immunoblotting

PAA 4/30 gradient gels were blotted to nitrocellulose hybridization transfer membranes (0.45 μm, Hybond-C extra, Amersham) by electrophoretic transfer (32) with a 165 mmol/l Tris, 40 mmol/l glycine, pH 9.5 buffer by using a LKB-2005 Transphor Electroblotting Unit (LKB). After electrotransfer at 500 mA for 6 h, the nitrocellulose membranes were saturated with 3% gelatin in 20 mmol/l Tris, 0.5 mol/l NaCl, pH 7.5, for 1 h at 37°C. Membranes were then incubated overnight with either anti-CETP monoclonal antibodies from mouse (TP6) (33), anti-apoA-I γ-globulin fraction from sheep (Boehringer, Mannheim, Germany), or anti-apoA-II γ-globulin fraction from sheep (Boehringer) that were diluted in 20 mmol/l Tris, 0.5 mol/l NaCl, pH 7.5 buffer containing 1% gelatin. After extensive washes with the Tris/NaCl buffer, membranes were then incubated for 2 h with either goat anti-mouse immunoglobulins G conjugated to horseradish peroxidase (Bio-Rad, Richmond, CA) (anti-CETP immunoblottings) or rabbit anti-sheep immunoglobulins G conjugated to horseradish peroxidase (Biosys, Compiègne, France) (anti-apoA-I and anti-apoA-II immunoblottings) diluted in 1% gelatin Tris/NaCl buffer. The membranes were then extensively washed and detection was done by treating the membranes with a freshly prepared 0.5 g/l 4-chloro-1-naphthol (Bio-Rad), 20% methanol, 0.02% H_2O_2 solution. The reaction was stopped after 15 min with distilled water.

Analytical methods

All chemical assays were performed on a Cobas-Bio Centrifugal Analyser (Roche Diagnostics, Zürich, Switzerland). Total cholesterol was determined by an enzymatic method using Boehringer reagents. Protein concentrations were measured using bicinchoninic acid reagent (Pierce) according to Smith et al. (34).

RESULTS

Apolipoprotein purification

Apolipoproteins A-I, A-II, and A-IV were purified to homogeneity as described in Materials and Methods. As shown in **Fig. 1**, after protein staining with Coomassie blue, purified apolipoproteins appeared as single, homogeneous bands in SDS-polyacrylamide gradient gels. Apparent molecular masses of apoA-I, apoA-II, and apoA-IV as determined by comparison with protein standards (LMW calibration kit, Pharmacia), were 29.0, 16.8, and 45.0 kDa, respectively. These values were in good agreement with those reported in previous studies (35).

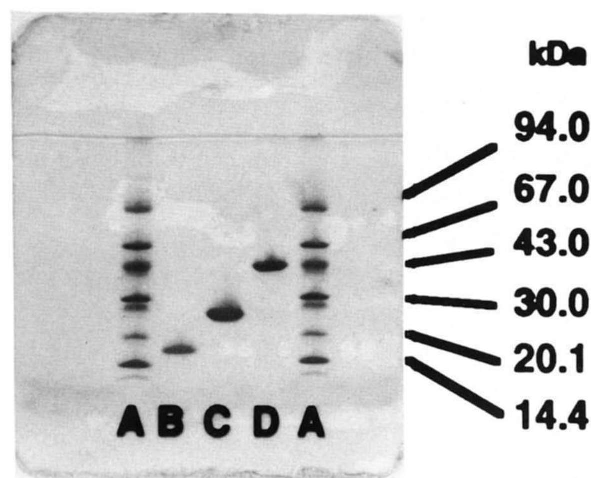

Fig. 1. SDS electrophoresis of purified apolipoproteins on a Phastsystem 8–25% polyacrylamide gradient gel. About 1 μg of each apolipoprotein, apoA-II (B), apoA-I (C), apoA-IV (D) was loaded on the gel and electrophoresis was performed for 80 Vh. Low molecular weight protein standards (Pharmacia) were simultaneously submitted to electrophoresis (A).

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Effects of purified apolipoproteins on the transfer of radiolabeled cholesteryl esters from HDL_3 to LDL

Radiolabeled HDL_3, LDL, and CETP were incubated for 3 h at 37°C in the presence of increasing concentrations of apolipoproteins A-I, A-II, or A-IV. In the presence of CETP but without apolipoprotein supplementation, about 20% of the total radioactivity in the HDL_3 fraction was transferred to LDL. The addition of human serum albumin did not significantly affect the CETP-mediated transfer of radiolabeled cholesteryl esters, whatever the final concentration of albumin was. By contrast, addition of apolipoproteins in incubation mixture had marked effects on cholesteryl ester transfer activity.

At the lowest apolipoprotein concentrations, below 0.2 g/l, apoA-II was the sole apolipoprotein to significantly decrease cholesteryl ester transfers ($P < 0.05$, ANOVA) (**Fig. 2**). By contrast, in the same concentration range, apoA-I and apoA-IV significantly increased the CETP-transfer of radiolabeled cholesteryl esters from HDL_3 to LDL ($P < 0.05$, ANOVA). Transfer rates increased progressively as concentrations of apoA-I and A-IV increased from 0 to 0.2 and 0.1 g/l, respectively. In the presence of 0.5 and 1.0 g/l of apoA-I, the increase in cholesteryl ester transfer activity, as observed with lower concentrations, was significantly reduced (0.5 g/l) and even suppressed (1.0 g/l). Moreover, at concentrations of 0.5 and 1.0 g/l, apoA-IV was no longer an activator but an inhibitor of the cholesteryl ester transfer process. At a concentration of 2 g/l, apoA-I also inhibited the cholesteryl ester transfer (result not shown). At concentrations of 0.5 and 1.0 g/l, apoA-II was shown to completely suppress the ability of CETP to transfer radiolabeled cholesteryl esters from HDL_3 to LDL (Fig. 2).

Therefore, the nature of the effect of purified apolipoproteins on CETP activity was dependent on their concentration in the incubation mixture. At low concentrations, apoA-I and apoA-IV could significantly increase cholesteryl ester transfers while at high concentrations

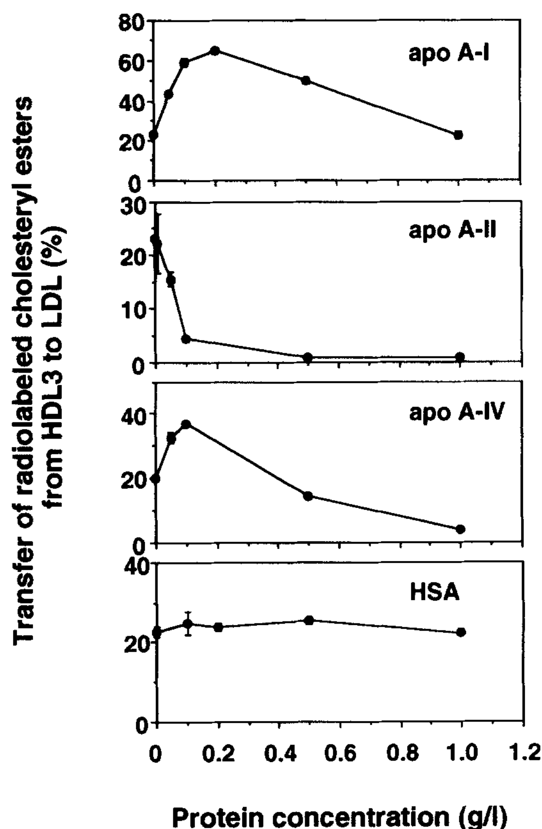

Fig. 2. Comparative effects of albumin (HSA) and apolipoproteins A-I, A-II, and A-IV on the CETP-mediated transfer of radiolabeled cholesteryl esters from HDL_3 to LDL. Each incubation mixture contained radiolabeled HDL_3 (2.5 nmol cholesterol), LDL (10 nmol cholesterol), and CETP (0.6 μg protein) in a total volume of 50 μl. Samples were incubated at 37°C for 180 min with increasing concentrations of albumin or apolipoproteins and then ultracentrifuged at *d* 1.068 g/ml. Values are mean ± SD of triplicate determinations.

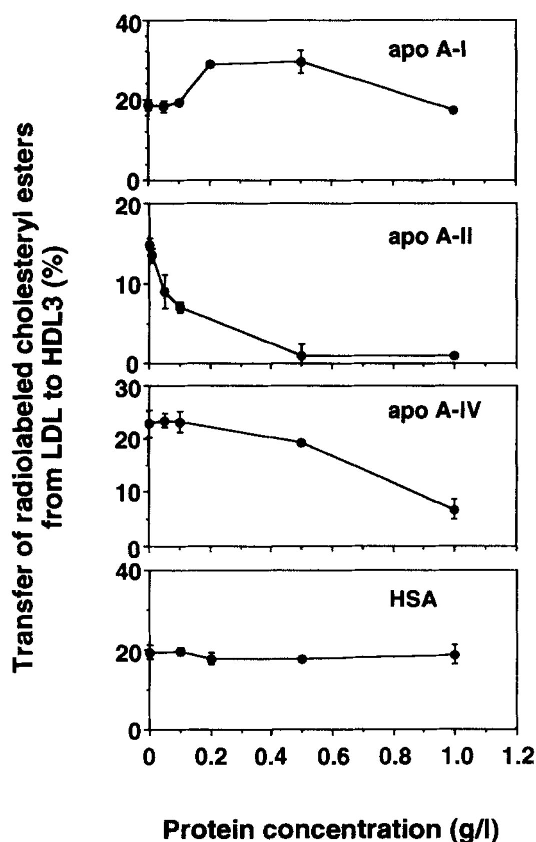

Fig. 3. Comparative effects of albumin (HSA) and apolipoproteins A-I, A-II, and A-IV on the CETP-mediated transfer of radiolabeled cholesteryl esters from LDL to HDL_3. Each incubation mixture contained radiolabeled LDL (2.5 nmol cholesterol), HDL_3 (10 nmol cholesterol), and CETP (0.6 μg protein) in a total volume of 50 μl. Samples were incubated at 37°C for 180 min with increasing concentrations of albumin or apolipoproteins and then ultracentrifuged at d 1.068 g/ml. Values are mean ± SD of triplicate determinations.

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significant inhibiting effects may appear. In the case of apoA-II, no activating effect was observed and a concentration-dependent inhibition of the cholesteryl ester transfer process appeared.

Effect of apolipoproteins on the transfer of radiolabeled cholesteryl esters from LDL to HDL_3

The effect of purified apolipoproteins on CETP activity was investigated by measuring the rate of radiolabeled cholesteryl esters transferred not from HDL_3 to LDL but, in the opposite direction, from LDL to HDL_3.

Globally, results (**Fig. 3**) were similar to those observed while measuring transfer rates from HDL_3 to LDL. However, the effects of various apolipoproteins on transfers from LDL to HDL_3 differed by several points with the transfers from HDL_3 to LDL. At a concentration of 0.2 g/l, apoA-I induced only a 50% increase ($P < 0.05$, ANOVA) in the rate of cholesteryl esters transferred from LDL to HDL_3 (Fig. 3) while, at the same concentration, it induced a 3-fold increase in the transfer rates from HDL_3 to LDL (Fig. 2). In addition, the activating effect of low concentrations of apoA-IV was no longer observed (Fig. 3). However, significant inhibitions of the transfer reaction by apoA-II and A-IV at high concentrations were still apparent. In particular, as observed above, 0.5 and 1.0 g/l of apoA-II totally suppressed the cholesteryl ester transfer process. Albumin did not significantly alter the rate of cholesteryl ester transferred from LDL to HDL_3.

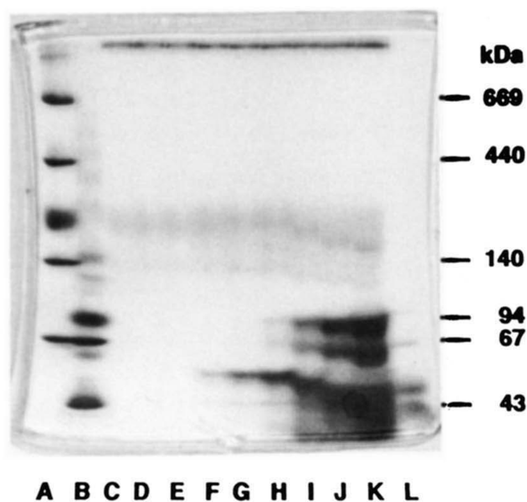

Fig. 4. Polyacrylamide gradient gel electrophoresis of incubation mixtures containing increasing apoA-I concentrations. Each incubation contained radiolabeled HDL_3 (2.5 nmol cholesterol), LDL (10 nmol cholesterol), and CETP (0.6 μg protein) in a final volume of 50 μl. Samples were either kept at 4°C (C) or incubated at 37°C in the absence (D) or in the presence of apoA-I at a final concentration of 0.01 (E), 0.05 (F), 0.1 (G), 0.2 (H), 0.5 (I), 1 (J), or 2 g/l (K). An aliquot of 30 μl of incubation mixtures was then electrophoresed in native 40–300 g/l polyacrylamide gradient gels as described in Materials and Methods. High molecular weight protein standards (A), low molecular weight protein standards (B), and pure apoA-II (L) were also submitted to electrophoresis.

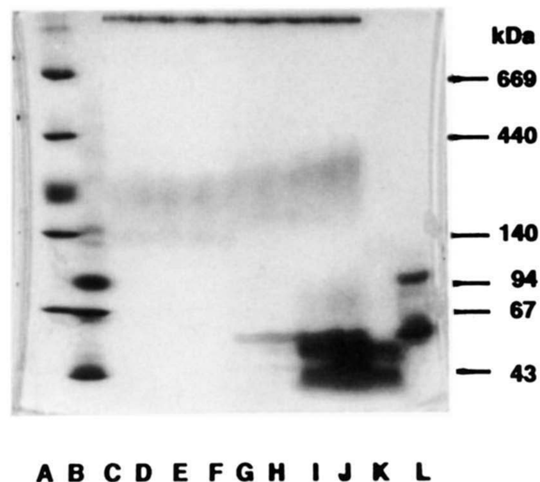

Fig. 5. Polyacrylamide gradient gel electrophoresis of incubation mixtures containing increasing apoA-II concentrations. Each incubation contained radiolabeled HDL_3 (2.5 nmol cholesterol), LDL (10 nmol cholesterol), and CETP (0.6 μg protein) in a final volume of 50 μl. Samples were either kept at 4°C (C) or incubated at 37°C in the absence (D) or in the presence of apoA-II at a final concentration of 0.01 (E), 0.05 (F), 0.1 (G), 0.2 (H), 0.5 (I), or 1 g/l (J). An aliquot of 30 μl of incubation mixtures was then electrophoresed in native 40–300 g/l polyacrylamide gradient gels as described in Materials and Methods. High molecular weight calibration protein standards (A) and low molecular weight protein standards (B), pure apoA-II (K), and pure apoA-I (L) were also electrophoresed.

Therefore, although under our experimental conditions apolipoproteins seemed to be more effective in modulating cholesteryl ester transfers from HDL_3 to LDL, they could also influence cholesteryl ester transfers from LDL to HDL_3 in similar ways. As apoA-I and apoA-II were among the apolipoproteins that were able to markedly influence CETP activity and as they are the two major apolipoproteins of HDL, we chose to focus subsequent experiments on these two apolipoproteins.

Distribution of apoA-I and A-II in incubation mixtures

Distribution of purified apoA-I and A-II in incubation mixtures containing LDL, radiolabeled HDL_3, and CETP was investigated by using native polyacrylamide gradient gel electrophoresis (see Materials and Methods).

As shown in **Fig. 4** and **Fig. 5,** HDL particles were detected in the 150–300 kDa molecular mass interval. When incubation mixtures were supplemented with 0.01 and 0.05 g/l of apolipoproteins A-I (Fig. 4 E, F) or A-II (Fig. 5 E, F), no protein bands in the molecular mass range of purified apoA-I and A-II were observed. This indicated that virtually the totality of apoA-I and A-II was associated with lipoprotein particles. By contrast, when apolipoprotein concentrations increased from 0.05 to 2 g/l, sharp protein bands with molecular masses corresponding to those of non-lipoprotein-associated apolipoproteins appeared (Fig. 4 G to K and Fig. 5 G to J). The molecular

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mass of these protein bands corresponded to polymeric forms of pure apoA-I and apoA-II (Fig. 5 K, L). The appearance of apoA-I and apoA-II in the non-lipoprotein-associated fraction, which was confirmed by immunoblotting (results not shown), is probably a function of the relative physicochemical properties and concentrations of both apolipoproteins.

As evaluated by laser densitometric scanning of gradient gels (**Fig. 6**), the relative proportions of apolipoproteins not bound to lipoprotein particles tended to increase as the total apolipoprotein concentration in incubation mixtures was enhanced. As shown in Fig. 6, constantly higher proportions of unbound apolipoproteins were detected with apoA-I than with apoA-II, probably reflecting the higher hydrophobicity of apoA-II as compared with apoA-I (36).

Effect of apolipoprotein additions on the size distribution of HDL_3

As shown in Figs. 4 and 5, addition of purified apolipoproteins A-I and A-II to incubation mixtures induced some alterations in the size of HDL particles that was either increased in the presence of high concentrations of apoA-II (Fig. 5) or decreased in the presence of high concentrations of apoA-I (Fig. 4). On the one hand, the apoA-II-induced enlargement was in good agreement with previous studies that demonstrated that the substitution of apoA-II for apoA-I in HDL enhanced the mean apparent diameter of the lipoprotein particles (16, 37). On the other hand, the size reduction of HDL particles when adding increasing concentrations of purified apoA-I is consistent with recent characterizations of reconstituted apoA-I-containing HDL particles that revealed that their apparent diameter is lower when the lipid:apoA-I ratio is reduced (38, 39).

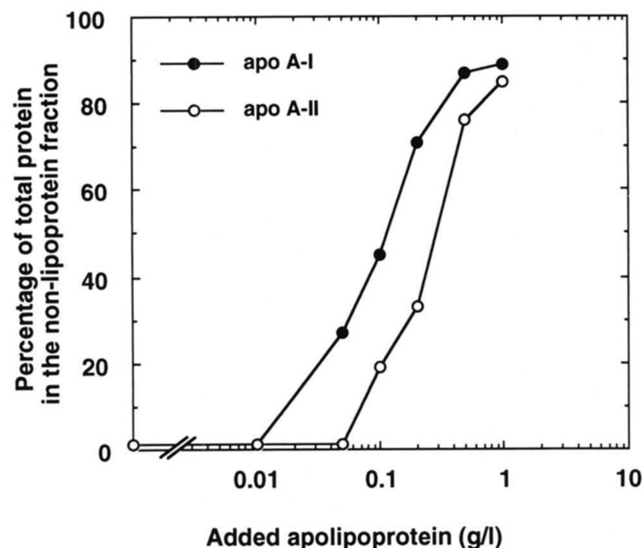

Fig. 6. Effect of apolipoprotein concentrations on the association of apoA-I and apoA-II with lipoprotein particles. The relative proportions of total proteins in the non-lipoprotein fraction were determined by laser densitometric scanning (see Materials and Methods) of gradient gels presented in Figs. 4 and 5.

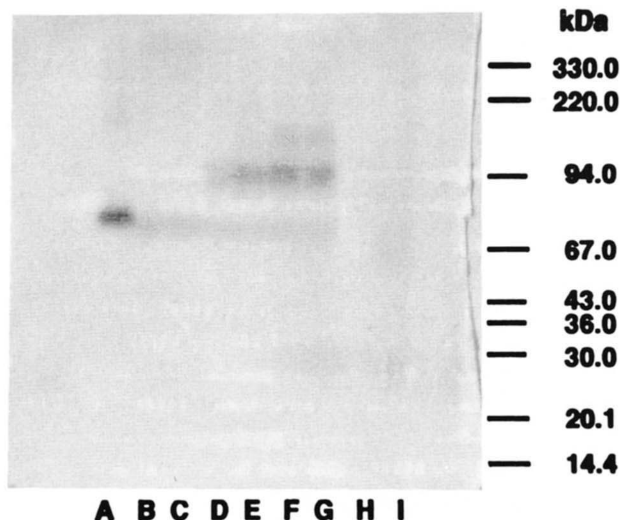

Fig. 7. Immunoblotting with monoclonal anti-CETP (TP6) antibodies showing the binding of CETP with apoA-I. Partially purified fraction of CETP (protein concentration, 0.16 g/l) was incubated at 37°C for 180 min in the absence (A and B) or in the presence of increasing amounts of apoA-I (final concentration of 0.01 (C), 0.05 (D), 0.1 (E), 0.5 (F), and 1 g/l (G)). Pure apoA-I (final concentration, 0.5 g/l) was also incubated alone without CETP (H and I). Protein complexes were crosslinked with BS (B–G, I), subjected to SDS electrophoresis, and electrotransferred to nitrocellulose membranes, and CETP was detected with TP6 antibody as described in Materials and Methods. Non-crosslinked CETP and apoA-I preparations were in A and H, respectively.

Binding of CETP with apoA-I or apoA-II

In experiments described above, the low CETP concentration (12 mg/l) was below levels that could be detected by immunoblotting (results not shown). Consequently, the occurrence of a direct interaction between CETP and apoA-I or apoA-II was investigated by incubating increasing amounts of purified apolipoproteins in the presence of a higher CETP concentration (160 mg/l). After incubation for 3 h at 37°C, protein complexes were crosslinked with BS and samples were subjected to SDS electrophoresis in PAA 4/30 polyacrylamide gradient gels (see Materials and Methods). At the end of electrophoresis, proteins were transferred to nitrocellulose membranes and CETP was specifically detected by using anti-CETP monoclonal antibodies (TP6).

Crosslinking of purified CETP (**Fig. 7B** and **Fig. 8B**) decreased the staining intensity on anti-CETP immunoblots as compared with non-crosslinked controls (Figs. 7A, 8A), probably reflecting a decreased immunoreactivity of CETP induced by covalent binding of the crosslinking reagent. However, BS did not significantly affect the

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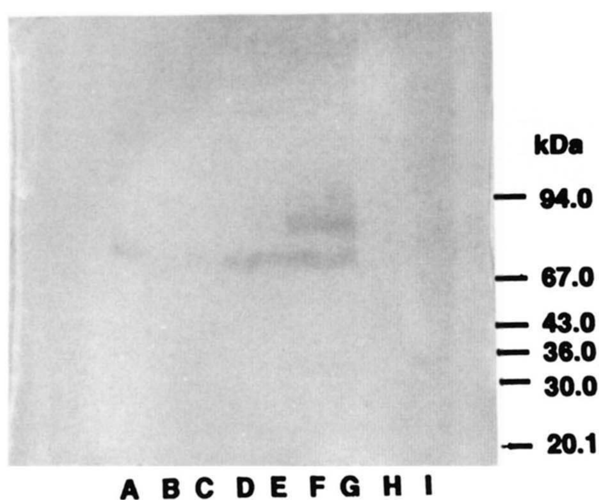

Fig. 8. Immunoblotting with monoclonal anti-CETP (TP6) antibodies showing the binding of CETP with apoA-II. Partially purified fraction of CETP (protein concentration, 0.16 g/l) was incubated at 37°C for 180 min in the absence (A and B) or in the presence of increasing amounts of apoA-II (final concentration of 0.01 (C), 0.05 (D), 0.1 (E), 0.5 (F), and 1 g/l (G)). Pure apoA-II (final concentration, 0.5 g/l) was also incubated alone without CETP (H and I). Protein complexes were crosslinked with BS (B–G, I) and then subjected to SDS electrophoresis and electrotransferred to nitrocellulose membranes, and CETP was detected with TP6 antibody as described in Materials and Methods. Non-crosslinked CETP and apoA-II preparations were in A and H, respectively.

migration of CETP and apparent molecular masses of native and crosslinked CETP molecules were similar (76,000 ± 3,100 and 72,300 ± 3,700 Da, respectively). When CETP was incubated with either apoA-I or apoA-II without crosslinking reaction, an apparent molecular mass of 76,000 Da for CETP was constantly observed (results not shown). By contrast, crosslinking in incubation mixtures containing CETP and increasing amounts of apoA-I (Fig. 7) or apoA-II (Fig. 8) revealed the appearance of new protein bands with apparent molecular masses of 99,600 ± 6,100 Da and 86,900 ± 4,500 Da, respectively. These new protein bands could correspond to associations of one molecule of CETP with either one molecule of apoA-I or one molecule of apoA-II, respectively. The thresholds of apolipoprotein concentration required to observe CETP-apolipoprotein complexes were 0.05 (Fig. 7D) and 0.5 g/l (Fig. 8F) for apoA-I and apoA-II, respectively. With high concentrations of apoA-I (0.5 and 1 g/l), an additional 128,800 ± 5,400 Da band appeared. It could result from the association of one molecule of CETP with two molecules of apoA-I. The presence of apoA-I in 99.6 and 128.8 kDa complexes and of apoA-II in 86.9 kDa complexes was confirmed on anti-apoA-I and anti-apoA-II immunoblottings (results not shown).

DISCUSSION

The results from the present study demonstrated that purified apolipoproteins A-I, A-II, and A-IV can modulate in vitro the CETP-mediated transfer of radiolabeled cholesteryl esters between HDL_3 and LDL particles. The activating or inhibiting potential of purified apolipoproteins was shown to be strongly dependent on the final experimental conditions. An additional finding of the present report was the capacity of apolipoproteins A-I and A-II to bind to CETP. This latter observation suggests that the modulation of CETP activity by apolipoproteins could be mediated by a direct interaction of CETP with apolipoprotein molecules.

At a low apolipoprotein:lipoprotein ratio, apolipoproteins A-I and A-IV significantly increased the rates of radiolabeled cholesteryl esters transferred between HDL_3 and LDL. In the case of apoA-I, consistent results were obtained by measuring cholesteryl ester transfers either from HDL_3 to LDL or, in the opposite direction, from LDL to HDL_3. These results were in good agreement with data from recent studies. Indeed, apoA-I has been shown to enhance the ability of CETP to exchange cholesteryl ester molecules between different lipoprotein fractions (12–14) and we previously demonstrated that apoA-IV can increase the CETP-mediated size redistribution, or conversion, of HDL_3 particles (40). By contrast, at a high apolipoprotein:lipoprotein ratio, the ability of apoA-I to enhance CETP-activity was considerably reduced and apoA-IV no longer appeared as an activator but as an inhibitor of the cholesteryl ester transfer reaction. At the highest apolipoprotein:lipoprotein ratio studied, apoA-I could also significantly inhibit the transfer reaction.

Unlike apoA-I and A-IV, purified apoA-II was not able to significantly enhance CETP activity, even at a low apolipoprotein:lipoprotein ratio. On the contrary, apoA-II always appeared as a potent inhibitor of the cholesteryl ester transfer reaction. It inhibited cholesteryl ester transfers in a concentration-dependent manner and, at the highest apolipoprotein:lipoprotein ratio studied, CETP activity was totally suppressed. Previous studies have shown that apoA-II could also inhibit LCAT activity (41).

In previous studies using synthetic lipid emulsions, apolipoproteins A-I, A-II, C-II, C-III, and E were shown to equally stimulate the cholesteryl ester transfer reaction (12–14). Nishikawa et al. (12) interpreted these data in terms of a stabilization of lipid emulsions by apolipoproteins compared with control emulsions that did not contain a protein moiety. In other words, effects of apolipoproteins might relate more likely to their common physicochemical properties rather than to specific characteristics of each molecule. It is known that HDL apolipoproteins possess α helical domains (42) with a large number of free negative charges (43), and the binding of CETP to lipoprotein,

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an integral step of the cholesteryl ester transfer reaction (3), has been shown to increase considerably while introducing negative charges in the particles (7). Thus, it appears that apolipoproteins could modulate the binding of CETP to lipoprotein particles by introducing negative charges at the lipid–water interface. However, this hypothesis cannot account for all the results presented in this report because at low apolipoprotein:lipoprotein ratios apoA-II differed from all the other apolipoproteins studied by significantly inhibiting the transfer of cholesteryl esters between HDL_3 and LDL. Thus, the mechanism by which apolipoproteins can modulate cholesteryl ester transfers is still unclear and an additional hypothesis must be proposed to explain the observed variations. In an attempt to explain further the modulation of CETP activity by HDL apolipoproteins, we investigated the possibility of a direct interaction between CETP and apolipoproteins A-I and A-II. By combining crosslinking and immunoblotting techniques, the association of one molecule of either apoA-I or apoA-II with one molecule of CETP could be observed. Interestingly, the mass concentration of apoA-II needed to produce visible CETP-apolipoprotein complexes appeared to be about ten times higher as compared with apoA-I, suggesting that CETP may have a lower affinity for apoA-II than for apoA-I. In addition, the binding of two molecules of apolipoproteins per molecule of CETP was observed with the highest concentrations of apoA-I studied. This latter observation might relate both to the capacity of apoA-I to form stable dimers in aqueous medium (44) and to its putative higher affinity for CETP as compared with apoA-II. The hypothesis of a lower affinity of CETP for apoA-II than for apoA-I is sustained further by the observations of Cheung and coworkers (8) who demonstrated that, in human plasma, about 80% of total cholesteryl ester transfer activity is localized in HDL particles containing only apoA-I, while less than 10% is detected in HDL particles containing apoA-II in addition to apoA-I. These observations are consistent with recent data from our laboratory indicating that the interaction of CETP is weaker with HDL particles containing both apoA-I and apoA-II as compared with HDL particles containing only apoA-I (16). Taken together, these observations suggest, therefore, that the modulation of CETP activity by apolipoproteins could relate to a direct interaction of CETP with apolipoprotein molecules. This hypothesis of an apolipoprotein-mediated binding of CETP to lipoprotein particles agrees with recent data that have demonstrated that apoE enhances cholesteryl ester transfer activity by increasing the affinity of CETP for lipoproteins (15). In addition, since Swenson et al. (33) have shown that the CETP-lipoprotein binding step could induce conformational changes of the CETP molecule, the conformation of CETP could vary during its interaction with different apolipoprotein molecules. On the other hand, Morton and Steinbrunner (45) have demonstrated that the actual substrates for CETP are the small amounts of neutral lipids that are solubilized at the surface of the lipoproteins. Thus, according to these data, variations in the capacity of different apolipoproteins to modulate CETP activity might also relate to their ability to modify the availability of cholesteryl esters at the lipoprotein surface.

In the present study, the effect of apoA-I on CETP activity and its distribution as a lipoprotein- or non-lipoprotein-associated protein varied accordingly and were dependent on the final apolipoprotein:lipoprotein ratio in incubation mixtures. At a low apolipoprotein:lipoprotein ratio, lipoprotein particles were able to accommodate virtually the totality of purified apoA-I that was added to the medium. Under these conditions, cholesteryl ester transfers were significantly increased. However, the ability of lipoprotein substrates to accommodate purified apoA-I was limited and at a high apolipoprotein:lipoprotein ratio, we observed that significant proportions of self-associated apolipoprotein remained unbound to lipoproteins. Under these conditions, the ability of apoA-I to stimulate the transfer reaction was significantly reduced. With the highest apolipoprotein:lipoprotein ratio studied, apoA-I significantly inhibited cholesteryl ester transfers. We postulate that unassociated apoA-I might inhibit cholesteryl ester transfers by trapping CETP molecules and suppressing their interaction with lipoprotein substrates. A similar mechanism has been previously proposed to explain the inhibition of the lipid transfer protein-mediated size redistribution of HDL particles by the small proportion of apoA-I that is not associated with any of the major lipoprotein fractions in human plasma (46). Unfortunately, we could not verify this hypothesis because anti-CETP immunoblottings were not sensitive enough to detect the small amounts of CETP protein that were added to the incubation mixtures containing LDL and HDL fractions.

An important finding was that apoA-II differed markedly from other apolipoproteins as, even at a low apolipoprotein:lipoprotein ratio, it exhibited a significant inhibiting effect. This particular behavior of apoA-II could relate to its relative high hydrophobicity as compared with apoA-I, A-IV, and E. Indeed, the discriminant value Z, which accounts for the global hydrophobicity of protein molecules, is clearly higher (0.299) for apoA-II than for apoA-I, A-IV, and E (0.057, −0.195 and −0.018, respectively) (36). Differences of hydrophobicity are associated with marked differences in the relative affinity of apolipoproteins for lipoprotein substrates and two molecules of apoA-II have been shown to displace one molecule of apoA-I from HDL particles without inducing marked alteration of the lipid composition of lipoprotein particles (16, 37, 47). Again, unassociated apoA-I molecules, resulting in that case from the interaction of purified apoA-II with lipoprotein substrates, could secon-

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darily interact with CETP molecules and inhibit the cholesteryl transfer reaction. In addition, differences in the relative hydrophobicity of apoA-I and apoA-II might also account for differences in their ability to bind to CETP, a highly hydrophobic molecule (Z = 0.344) (48).

In conclusion, the present results have shown that purified human apolipoproteins can modulate the CETP-mediated cholesteryl ester transfer reaction. Whereas both apoA-I and apoA-II are able to bind to CETP, they may present different affinities for CETP molecules and they can exhibit opposite effects on the CETP-mediated transfer reaction. These data suggest that the apolipoprotein composition of lipoproteins could be an important parameter in determining the global cholesteryl ester transfer activity in human plasma.

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