Phospholipids mediated conversion of HDLs generates specific apoA-II pre-β mobility particles

Małgorzata Wróblewska,[1,*] Barbara Kortas-Stempak,[†] Andrzej Szutowicz,[*] and Tadeusz Badzio[†]

Department of Laboratory Medicine,* Medical University of Gdańsk; and Department of Analytical Chemistry,[†] Medical University of Gdańsk, Gdańsk, Poland

Abstract **Apolipoproteins (apo)A-I and A-II are major proteins of human HDL. The cycling of apoA-I between lipid-poor and lipid-rich forms of HDL plays a key role in the transport of cholesterol by these particles. ApoA-II resides only in part of HDL particles, and little is known about its role in HDL metabolism. Our study investigates the redistribution of apoA-II after HDL remodelling induced by exogenous phospholipids (PL). During incubation with egg yolk lecithin (EYL) liposomes, human HDL became PL-enriched and free cholesterol (FC)-depleted, and lost small amounts of apoA-I and apoA-II. The loss of FC and apolipoproteins correlated with the rise of PL content in HDL. Agarose gel electrophoresis demonstrated the appearance of new pre-β mobility fractions containing apoA-I and apoA-II in liposomes and HDL mixtures. Two-dimensional nondenaturing 2–27% PAGE has shown that the pre-β mobility fraction that appeared at initial liposome-PL/HDL-PL ratio 5:1 consisted of two distinct heterogeneous subpopulations of particles containing either apoA-I or apoA-II. Our study provides evidence that during HDL conversion mediated by PL apoA-II dissociated from HDL particles yielding apoA-II-specific pre-β mobility particles. This observation supports the hypothesis that apoA-II in plasma, like apoA-I, may cycle between lipid-poor and lipid-rich forms of HDL.—**Wróblewska, M., B. Kortas-Stempak, A. Szutowicz, and T. Badzio. **Phospholipids mediated conversion of HDLs generates specific apoA-II pre-β mobility particles. *J. Lipid Res.* 2009.** 50: **667–675.**

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HDLs play a key role in the process of reverse cholesterol transport and thereby fulfill antiatherogenic functions. The bulk of reverse cholesterol transport is linked with the apolipoprotein (apo)A-I, which is a major HDL protein (1). Approximately 2–14% of total plasma apoA-I is located in particles migrating in the agarose gel with pre-β mobility (pre-β HDL) including small, disc-shaped, lipid-poor nascent particles (1, 2). The rest of apoA-I is a component of mature, spherical particles migrating with α-mobility (α-HDL) that can be further divided into two subpopulations: HDL containing apoA-I without apoA-II (HDL A-I) and HDL containing both apoA-I and apoA-II (HDL A-I/A-II) (3). ApoA-I and apoA-II are probably secreted separately into the plasma from hepatocytes in lipid-poor forms (4). Free apoA-I particles acquire phospholipids (PL) and free cholesterol (FC) from cell membranes in the process mediated by the ATP-binding cassette A1 (ABCA-1) transporters. This yields discoid HDL precursors migrating with pre-β mobility in agarose gel electrophoresis (5). Cholesterol esterification catalyzed by LCAT converts pre-β HDL into α-migrating spherical HDL A-I (6, 7). During the remodelling of HDL in plasma, apoA-I continuously cycles between lipid-rich, α migrating spherical HDL and lipid-poor, pre-β migrating discoid particles (7, 8). However, approximately half of the HDL particles in human serum contain also apoA-II, which accounts for approximately 20% of total HDL proteins (3, 7, 8). The formation of HDL A-I/A-II particles is one of the most mysterious steps in HDL metabolism. There are only a few reports concerning the properties of HDL containing apoA-II without apoA-I (HDL A-II) particles purified from normolipidemic human plasma (9, 10). It is generally believed that HDL A-II exist in human plasma in trace amounts. In vitro apoA-II is able to recruit cell membrane PL and cholesterol to form discoid HDL A-II particles with pre-β mobility (11, 12). However, LCAT does not react with these structures and therefore does not transform them into spherical particles (11, 13). The mechanisms mediating the incorporation of apoA-II into HDL A-I/A-II particles in plasma remain unknown. It has been proved recently that free apoA-II and discoid reconstituted HDL containing A-II injected intravenously into rabbits were immediately incorporated into spherical apoA-I-containing HDL, yielding HDL A-I/A-II particles (14). LCAT seems to be a commonly accepted factor promoting the fusion of spherical HDL A-I and discoid HDL A-II (7, 15).

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Abbreviations: apo, apolipoprotein; CE, cholesteryl esters; EYL, egg yolk lecithin; FC, free cholesterol; HDL A-I, HDL containing apoA-I without apoA-II; HDL A-I/A-II, HDL containing apoA-I and apoA-II; PL, phospholipids; TC, total cholesterol.

[1]To whom correspondence should be addressed.
e-mail: wroblew@amg.gda.pl

There is also evidence that free apoA-II can be incorporated into pre-existing HDL by the displacement of A-I from HDL (16). In contrast to apoA-I, apoA-II once built-in into spherical HDL seems to be a stabile component of these particles. Rye, Clay, and Barter (7) suggested that lipid-poor apoA-II can be generated during the remodelling of HDL by plasma factors. However, being extremely hydrophobic (16), it rapidly acquires FC and PL from cell membranes or other lipoproteins. Hence, it could be a pathway for the generation of discoid HDL A-II that would rapidly incorporate into pre-existing HDL. The physiological role of apoA-II remains unclear. Experimental data indicate that apoA-II is likely to maintain the plasma HDL pool and enhance HDL stability by inhibiting hepatic lipase activity and CETP-mediated dissociation of lipid-poor apoA-I (17, 18). It has been also shown that the rate of apoA-II production determines the distribution of apoA-I between HDL A-I and HDL A-I/A-II (19).

A number of experiments have provided evidence that the capability of HDL to promote cholesterol efflux from cells may be enhanced by remodelling with PL (20) and that treatment of plasma with PL generates pre-β mobility apoA-I-containing lipoproteins (21, 22), which may serve as a substrate for ABCA-1 (22). The in vitro interaction between HDL and liposomes causes the dissociation of apoA-I from HDL and spontaneous generation of discoid lipid-protein structures resembling pre-β A-I HDL (23, 24). However, the contribution of a second most abundant HDL apo, apoA-II, in HDL remodelling, in physiological conditions and during the administration of HDL metabolism modulating agents, remains unknown. Tall and Green (24) detected the presence of apoA-I and apoA-II in new lipoproteins formed during incubation of phosphatidylcholine vesicles with isolated HDL_3. However, the process of dissociation of apoA-II from HDL was not defined precisely until today.

The aim of this study was to determine whether apoA-II can participate in HDL remodelling promoted by exogenous PL. This goal has been achieved by the incubation of human HDL with egg yolk lecithin (EYL) liposomes followed by the identification of the reaction products using agarose gel electrophoresis and two-dimensional nondenaturing PAGE. The monitoring of changes in the composition of α-HDL modified by exogenous PL required a simple and rapid procedure for the isolation of these lipoproteins from the incubation mixture. Precipitation with heparin and $CaCl_2$ appeared to be suitable for this purpose (25–27). Our experiments demonstrated that the interaction of HDL with liposomes evokes dissociation of apoA-II from HDL and yields apoA-II-specific lipid-protein particles migrating with pre-β mobility during agarose gel electrophoresis.

MATERIALS AND METHODS

HDL isolation

The sera were obtained from blood drawn from apparently healthy donors after a 12–16 h overnight fast. The HDL fraction was isolated from pooled sera by combined precipitation-ultracentrifugation procedure. At first apoB-containing lipoproteins were precipitated by heparin (200 U/ml) (Polfa, Warszawa, Poland) and $MnCl_2$ (90 mM) (26). HDL fraction was isolated from supernatant by the ultracentrifugation method described by Brousseau et al. (28) with modifications. The density of supernatant was adjusted to 1.21 g/ml by adding solid KBr, and its 2 ml portions were ultracentrifuged in the Beckman TL100 (Beckman Instruments, Fullerton, CA) ultracentrifuge (3 h, 15°C, 435 680 *g*). The top 0.4 ml portions of each tube were collected and dialyzed against 10 mM Tris-HCl buffer pH 7.4 + 0.85% NaCl (6 h, 4°C) and subsequently against 10 mM Tris-HCl buffer pH 7.4 (12 h, 4°C) with 0,1% NaN_3 as a preservative.

In selected experiments, HDL were labeled with [^{14}C]cholesterol (Polatom, Świerk-Otwock, Poland) using filter paper disc (29).

Preparation of liposomes

Liposomes (small unilamellar vesicles) were prepared at a concentration 30 mg/ml. Briefly, portions of EYL purified by aluminum oxide chromatography (Sigma-Aldrich, Poznań, Poland) and dissolved in hexane were dried under a stream of nitrogen. The buffer 10 mM Tris-HCl, pH 7.4 with 0.1% NaN_3 was added to the dried lipids and the suspension was left for 16 h to swell, followed by sonication in the ice bath under nitrogen (Sonoplus HD 2070 ultrasonic homogenizer, Bandelin electronic GmbH and Co. KG, Berlin, Germany). The solution was centrifuged (10,000 rpm, 30 min) to remove titanium shed from the probe. Sepharose CL-4B (Amersham Pharmacia Biotech AB, Sweden) chromatography confirmed that more than 95% of the lipid was present as small unilamellar liposomes.

Incubation of HDL and liposomes and precipitation of non-α-HDL lipoproteins

Incubation mixtures of the same volumes were prepared by mixing a constant volume of HDL with liposomes diluted in 10 mM Tris-HCl buffer pH 7.4 in order to obtain target initial liposomes-PL to HDL-PL (L-PL/HDL-PL) w/w ratios: 1:1, 2:1, 3:1, 4:1, and 5:1. The control incubations (L-PL/HDL-PL ratio 0:1) were prepared by mixing HDL with a respective volume of 10 mM Tris-HCl buffer pH 7.4. Mixtures were incubated at 37°C for 1 h. Incubation was stopped by adding heparin and $CaCl_2$ in final concentrations of 440 U/ml and 35 mM, respectively. Mixtures were allowed to stand for 30 min in room temperature and subsequently centrifuged at 10,000 rpm for 30 min at 4°C. Precipitates containing non-α HDL lipoproteins were washed with 10 mM Tris-HCl buffer pH 7.4 containing 440 U/ml heparin and 35 mM $CaCl_2$ and finally dissolved in 2% sodium citrate stabilized with thimerosal (0.05%) and penicillin-streptomycin (1%). The final volumes of dissolved precipitates were adjusted to 1/10 of the volume of incubation mixtures in order to obtain highly concentrated samples that were analyzed for apoA-I and apoA-II contents. The α-HDL-containing supernatants were analyzed for their total cholesterol (TC), FC, and PL contents.

Electrophoretic analysis

The presence of apoA-I and apoA-II-containing lipoproteins in the mixtures of HDL and liposomes was analyzed by agarose gel electrophoresis (0.75% agarose w/v, 50 mM barbital buffer pH 8.6, 10°C, 10V/cm, 90 min). The positions of lipoprotein fractions were determined on the base of mobility of samples of control HDL, and on liposomes and incubation mixtures that were mixed with Sudan Black B to stain lipid components and electrophoresed in the same conditions. Lipoproteins were transferred from gel to nitrocellulose membrane (Immobilon-P^{SQ}, Millipore, Bedford, MA) by passive transfer and detected with monoclonal antibodies either to human apoA-I, or apoA-II (ICN Pharmaceuticals

Inc., Aurora, OH) and anti-IgG conjugated with alkaline phosphatase using Nitro Blue Tetrazolium and 5-Bromo-4-Chloro-3-Indolyl Phosphate as chromogenic substrates (Sigma-Aldrich, Poland, Poznań).

In experiments with HDL [^{14}C]cholesterol labeled, following electrophoresis, gels were dried and subjected to autoradiography.

The distribution of apoA-I and apoA-II in the precipitate containing non-α-HDL lipoproteins was further analyzed by two-dimensional nondenaturing 2–27% polyacrylamide gradient gel electrophoresis (30). Briefly, in the first dimension, aliquots of precipitate dissolved in 2% sodium citrate were separated by electrophoresis in 0.75% agarose gel as described above. Agarose strips containing appropriate bands were cut and placed on self-made 2–27% polyacrylamide gradient gel. Separation in the second dimension was performed in 25 mM Tris and 190 mM glycine, pH 8.5 for 18 h at 160 V constant current at 10°C. Lipoproteins separated in a gradient gel were then electroblotted to nitrocellulose membrane (Immobilon-P^{SQ}, Millipore Bedford, MA). ApoA-I or apoA-II-containing lipoproteins were detected as described above. The sizes of lipoproteins were assessed by the comparison of their mobility with the mobility of globular protein standards (High Molecular Weight Calibration Kit for Electrophoresis, Amersham Biosciences, UK) electrophoresed on the same gel (31).

Apo analysis

Precipitates dissolved in sodium citrate were mixed with 2% Triton X-100 (1:1 v/v) and incubated for 1 h at 56°C. In such solubilized samples concentrations of apoA-I and apoA-II were assayed by nephelometry (N Antisera to Human apoA-I or apoA-II, N apo Standards Serum, Dade Behring Marburg GmbH, Marburg).

Lipid analysis

Concentrations of TC, FC, and PL were measured using commercially available enzymatic kits. The amount of cholesteryl esters (CE) in supernatants was calculated from the difference between TC and FC.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 4.03 (GraphPad Software Inc., San Diego, CA). Statistical significance (P value) was determined by the Student's t-test for paired samples. Value $P < 0.05$ was considered to be statistically significant.

RESULTS

Formation of pre-β mobility fraction containing apoA-I and apoA-II following incubation of HDL with lecithin liposomes

The formation of new lipoprotein fraction migrating with pre-β mobility in agarose gel electrophoresis following the incubation of ultracentrifuged human HDL with EYL liposomes was demonstrated by Sudan Black B staining (**Fig. 1A**), autoradiography (**Fig. 2**), and immunodetection of apoA-I and apoA-II (Fig. 1B). Lane 1 in Fig. 1A shows that lipids in the incubation mixture of HDL and liposomes redistributed to pre-β mobility fraction. However to the contrary, electrophoregrams of either HDL or liposomes incubated alone revealed the presence of respective single lipid bands (Fig. 1A, lanes 2 and 3, respectively). Also HDL FC labeled with ^{14}C remained in the α-mobility position when HDL were incubated alone (Fig. 2, lane 1). In turn,

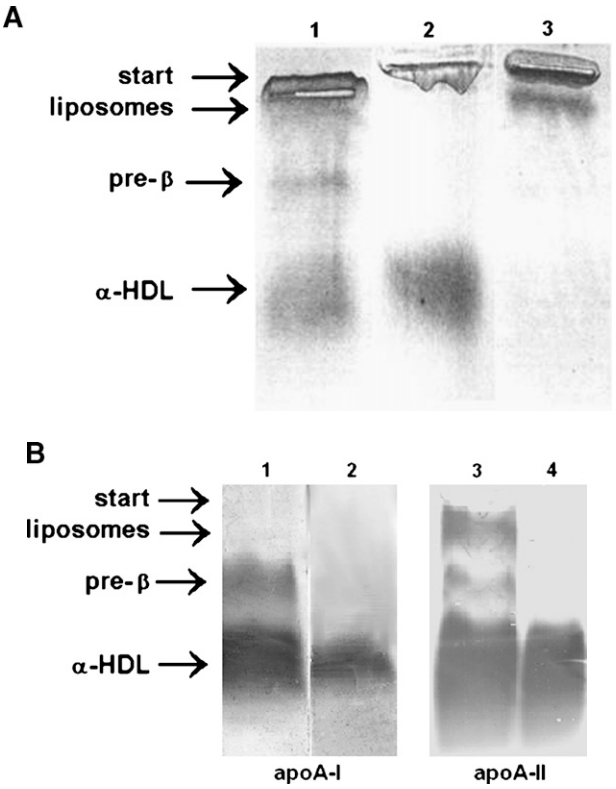

Fig. 1. Lipoprotein fractions in the mixture of HDL and liposomes. The mixtures of ultracentrifuged human HDL and egg yolk lecithin (EYL) liposomes were incubated and subjected to agarose gel electrophoresis as described in Materials and Methods. Control incubations contained equivalent amount of HDL but no liposomes. Immunoblotting to detect either apolipoprotein (apo)A-I or apoA-II was carried out as described in Materials and Methods. Results for a representative incubation mixture at initial L-PL/HDL-PL ratio 5:1 are shown. A: Electrophoretic patterns of samples prestained with Sudan Black B (aliquots of 20 μl were electrophoresed). Lane 1: mixture of HDL and liposomes; Lane 2: control; Lane 3: sole liposomes. B: The results of immunoblotting (aliquots of 5 μl were electrophoresed). Lane 1: mixture of HDL and liposomes immunoblotted with anti-human apoA-I antibodies; Lane 2: control immunoblotted with anti-human apoA-I antibodies; Lane 3: mixture of HDL and liposomes immunoblotted with anti-human apoA-II antibodies; Lane 4: control immunoblotted with anti-human apoA-II antibodies.

in the mixture of HDL and liposomes [^{14}C]cholesterol appeared to shift to a new pre-β mobility band and liposomes as well (Fig. 2, lane 2). Immunoblotting with monoclonal antibodies demonstrated that apoA-I and apoA-II, which in control samples of HDL were detected exclusively in α-mobility position (Fig. 1B, lane 2 and lane 4, respectively), in incubation mixtures redistributed to preβ mobility fraction (Fig. 1 B, lane 1 and 3, respectively). Moreover, apoA-II was present also in liposomes (Fig. 1B, lane 3).

Precipitation of non-α-HDL lipoproteins

The addition of heparin and $CaCl_2$ to the mixture of HDL and liposomes produced insoluble complexes of liposomes and pre-β mobility particles. The effectiveness of the precipitation was assessed on the basis of the precipitation of liposomes and HDL separately, in a wide range of liposomes and HDL concentrations. At 35 mM $CaCl_2$ and

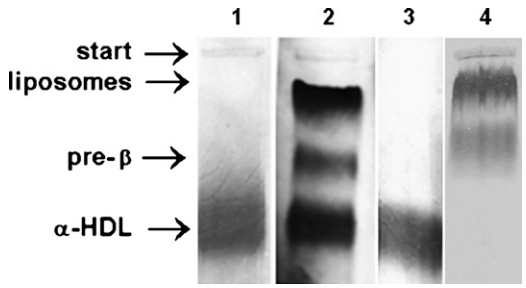

Fig. 2. Distribution of [^{14}C]cholesterol in the mixture of HDL and liposomes, post heparin-Ca^{+2} supernatant and post heparin-Ca^{+2} precipitate. Ultracentrifuged human HDL labeled with [^{14}C]cholesterol and EYL liposomes were mixed and incubated as described in Materials and Methods. Control incubations contained equivalent amount of HDL but no liposomes. Pre-β mobility fraction and liposomes were precipitated from incubation mixtures by heparin and Ca^{+2} and dissolved in 2% sodium citrate as described in Materials and Methods. Aliquots of 10 μl were subjected to agarose gel electrophoresis and autoradiography as described in Materials and Methods. Results for a representative incubation mixture at initial L-PL/HDL-PL ratio 5:1 are shown. Lane 1: control; Lane 2: mixture of HDL and liposomes; Lane 3: post heparin-Ca^{+2} supernatant; Lane 4: post heparin-Ca^{+2} precipitate.

440 U/ml of heparin liposomal PL were precipitated in 99% (**Table 1**), whereas all HDL-associated PL, TC, and apoA-I were recovered in supernatant (**Table 2**). The above conditions were sufficient to reach a plateau of FC, TC, PL, and apoA-I content in post heparin-Ca^{+2} supernatants of liposomes and HDL mixtures. The resistance of HDL to coprecipitation with liposomes and pre-β mobility fraction was confirmed by the 94–97% recovery of CE in post heparin-Ca^{+2} supernatants obtained at different L-PL/HDL-PL ratios (**Table 3**).

This differential precipitation of liposomes and pre-β mobility fractions was verified by agarose gel electrophoresis. Autoradiography demonstrated that post heparin-Ca^{+2} precipitates contained two electrophoretic fractions displaying the mobility of liposomes and pre-β fraction (Fig. 2, lane 4), while post heparin-Ca^{+2} supernatants contained single α-mobility fraction (Fig. 2, lane 3). Immunoblotting with monoclonal antibodies demonstrated the presence of apoA-I and apoA-II in α-mobility bands in post heparin-Ca^{+2} supernatants (**Fig. 3**, lanes 1 and 3, respectively) and also in new pre-β mobility fractions (Fig. 3, lanes 2 and 4, respectively). In accordance with immunoblot analysis of incubation mixture (Fig. 1B, lane 3), only apoA-II was detected in liposomes (Fig. 3, lane 4).

TABLE 1. Dilution-dependent precipitation of liposomes by heparin and Ca^{+2}

Degree of liposomes dilution	Precipitation of liposomal PL [%]
2×	99.2 ± 0.9
4×	99.7 ± 0.2
10×	99.1 ± 0.6
40×	99.3 ± 0.8

PL, phospholipids. Egg yolk lecithin (EYL) small unilamellar vesicles (30 mg/dl of PL) were diluted with 10 mM Tris-HCl buffer pH 7.4 and incubated for 1 h at 37°C. Heparin-Ca^{+2} precipitation was performed as described in Materials and Methods. Post heparin-Ca^{+2} supernatants were analyzed for PL contents. Results (means ± SD from three experiments) are expressed as percent of PL transferred to post heparin-Ca^{+2} precipitates.

TABLE 2. Dilution-dependent recovery of HDL-associated lipids and apoA-I in post heparin-Ca^{+2} supernatants

Degree of HDL dilution	Recovery of PL	Recovery of TC [%]	Recovery of apoA-I
0	100.2 ± 5.9	102.0 ± 5.2	97.0 ± 7.9
1.54×	98.7 ± 2.4	97.7 ± 1.5	100.9 ± 4.4
2×	99.7 ± 0.6	98.0 ± 2	99.7 ± 11.4

PL, phospholipids; TC, total cholesterol. Ultracentrifuged human HDL were diluted with 10 mM Tris-HCl buffer pH 7.4 and incubated for 1 h at 37°C. Heparin-Ca^{+2} precipitation was performed as described in Materials and Methods. Post heparin-Ca^{+2} supernatants were analyzed for lipids and apoA-I contents. Results (means ± SD from three experiments) are expressed as percent of lipids content in HDL.

HDL lipids and apoA-I and A-II distribution after incubation with liposomes

The lipids content in α-migrating HDL remaining in post heparin-Ca^{+2} supernatants markedly changed after incubation with liposomes (**Fig. 4**). As it was expected, HDL incorporated significant amounts of PL from liposomes in a dose-dependent manner. Simultaneously, gradient depletion of TC from HDL was observed. This depletion resulted from the loss of FC, because the CE content in HDL did not change significantly, irrespective of the initial L-PL/HDL-PL ratio in the incubation mixture. We have found significant inverse correlation between the delta percent change of PL content in α HDL (HDL-PL Δ) and delta percent change of FC content in α-HDL (HDL-FC Δ) at $r = -0.8568$ (Fig. 4, insert).

To avoid high uncertainty in estimating small fluctuations in apo levels in post heparin-Ca^{+2} supernatants, we dissolved post heparin-Ca^{+2} precipitates in small volumes of sodium citrate (see Materials and Methods) and used them for the quantitative assessment of the dissociation of apos from HDL. The contents of apoA-I and apoA-II in precipitates increased along with the increase of initial L-PL/HDL-PL ratio in the incubation mixture (**Fig. 5A, B**, respectively). Elevation of L-PL/HDL-PL ratio from 1:1 to 5:1 resulted in the increase of HDL apoA-I transfer to precipitating fractions from 0.2 to 3% respectively (Fig. 5A).

TABLE 3. Recovery of cholesteryl esters (CE) in post heparin-Ca^{+2} supernatants after precipitation of variable mixtures of liposomes and HDL

Initial L-PL/HDL-PL ratio	Recovery of CE in post heparin-Ca^{+2} supernatants [%]
1:1	97.5 ± 4.3
2:1	95.7 ± 3.6
3:1	94.4 ± 5.5
4:1	94.6 ± 6.4
5:1	95.9 ± 9.4

Ultracentrifuged human HDL were mixed with EYL small unilamellar vesicles at different initial L-PL/HDL-PL ratios and incubated for 1 h at 37°C. Heparin-Ca^{+2} precipitation was performed as described in Materials and Methods. Post heparin-Ca^{+2} supernatants were analyzed for CE content as described in Materials and Methods. Results (mean ± SD from five experiments) are expressed as percent of CE content in HDL.

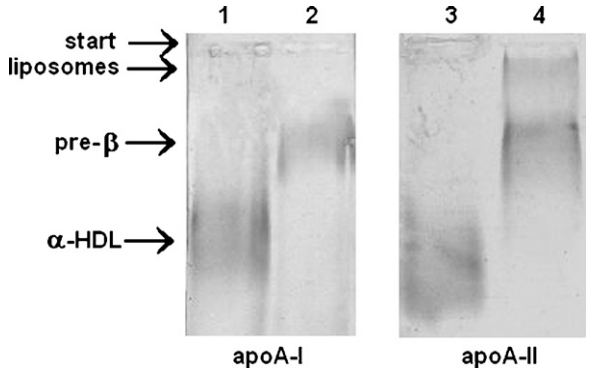

Fig. 3. Distribution of apoA-I and apoA-II in lipoprotein fractions in the post heparin-Ca^{+2} precipitate and supernatant. Ultracentrifuged human HDL and EYL liposomes were mixed and incubated as described in Materials and Methods. Pre-β mobility fraction and liposomes were precipitated from incubation mixtures by heparin and Ca^{+2} and dissolved in 2% sodium citrate as described in Materials and Methods. Aliquots of 5 μl were subjected to agarose gel electrophoresis and immunoblotted for either apoA-I or apoA-II as described in Materials and Methods. Results for a representative incubation mixture at initial L-PL/HDL-PL ratio 5:1 are shown. Lane 1: post heparin-Ca^{+2} supernatant immunoblotted with anti-human apoA-I antibodies; Lane 2: post heparin-Ca^{+2} precipitate immunoblotted with anti-human apoA-I antibodies; Lane 3: post heparin-Ca^{+2} supernatant immunoblotted with anti-human apoA-II antibodies; Lane 4: post heparin-Ca^{+2} precipitate immunoblotted with anti-human apoA-II antibodies.

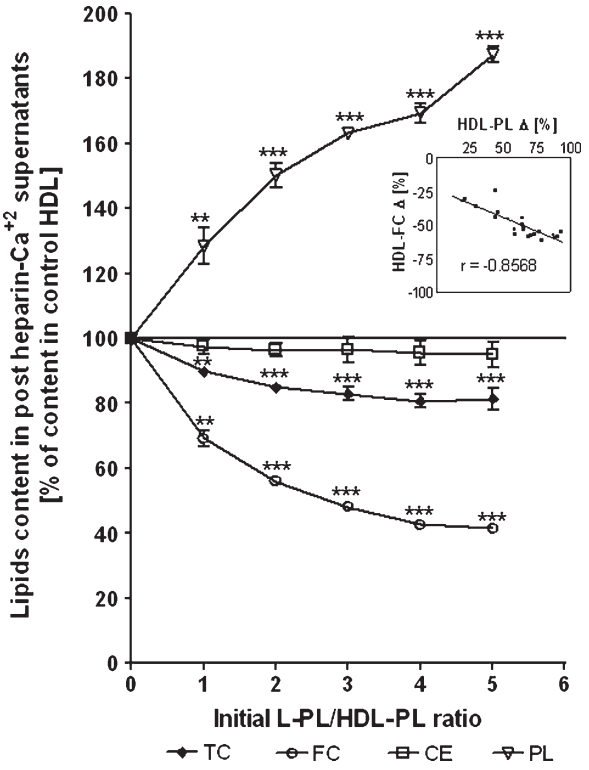

Fig. 4. The changes of lipids content in α-HDL after incubation with liposomes. Ultracentrifuged human HDL and EYL liposomes were mixed and incubated as described in Materials and Methods. Control incubations contained equivalent amount of HDL but no liposomes. Pre-β fraction and liposomes were precipitated from incubation mixtures by heparin and Ca^{+2} and α-HDL-containing postheparin-Ca^{+2} supernatants were analyzed for lipid content as described in Materials and Methods. The changes of lipids content are expressed as a percentage of the lipids content in control incubations. Insert: The relationship between delta percent changes of PL (HDL-PL Δ) and free cholesterol (FC) (HDL-FC Δ) content in α-HDL after incubation with liposomes. The initial lipids concentrations of HDL were as follows: total cholesterol (TC): 169 ± 7.5 mg/dl, FC: 41.9 ± 3 mg/dl, PL: 288.2 ± 12.3 mg/dl. Data are means ± SEM from five experiments. Value significantly different from control: ** $P < 0.05$, *** $P < 0.001$.

On the other hand, in the same conditions, the amount of apoA-II released from HDL to post heparin-Ca^{+2} precipitates increased from 0.4 to 1.9% (Fig. 4B). Assuming that the amount of apos accumulating in post heparin-Ca^{+2}precipitates correspond to their loss by HDL, we have found a nonlinear inverse relationship between HDL-PL Δ and a delta percent change of apoA-I (HDL-apoA-I Δ) and apoA-II (HDL-apoA-II Δ) in α-HDL (Fig. 5A and Fig. 5B, inserts, respectively).

Size heterogeneity of pre-β fraction

The distribution of apoA-I and apoA-II in the new pre-β mobility particles generated at initial L-PL/HDL-PL ratio 5:1 was analyzed by two-dimensional agarose-polyacrylamide gradient gel electrophoresis followed by immunoblotting as described in Materials and Methods. It revealed the existence of two distinct populations of particles containing either apoA-I or apoA-II (**Fig. 6**). ApoA-I-containing population (Fig. 6A) was more homogenous and formed one major band of particles with Stokes diameter of approximately 24.8 nm, and three smaller bands of particles with diameters of approximately 20.6, 16.4, and 8.0 nm. On the other hand, population of apoA-II-containing particles (Fig. 6B) was dominated by heterogeneous cluster of particles with Stokes diameters from less than 7.1 nm to 7.6 nm. In addition, apoA-II was also detected in two bigger and also heterogeneous classes of particles with Stokes diameters from 11.9 to 15.5 nm and from 8.0 to 8.4 nm as well as one more homogenous band with Stokes diameter of 7.7 nm.

DISCUSSION

The reaction between EYL small unilamellar vesicles and human HDL resulted in the redistribution of lipids (Fig. 1A, lane 1, Fig. 2, lane 2) and apoA-I and apoA-II (Fig. 1B, lanes 1 and 3, respectively) to a new lipoprotein fraction migrating with pre-β mobility in agarose gel. We used the known (25–27) differences in the interaction mode of heparin and Ca^{+2} with HDL and PL (Tables 1–3) to separate remodelled α-HDL (Fig. 2, lane 3, Fig. 3, lanes 1 and 3) from the pre-β mobility fraction and liposomes (Fig. 2, lane 4, Fig. 3, lanes 2 and 4). The HDL incubated with liposomes became PL enriched in a dose-dependent manner (Fig. 4). The transfer of apoA-I and apoA-II to fractions precipitating with heparin and Ca^{+} (Fig. 5A, B respectively) was inversely correlated with the accrual of PL in α-HDL (Fig. 5A, B, inserts, respectively). This finding remains in accordance with the recently reported correlation be-

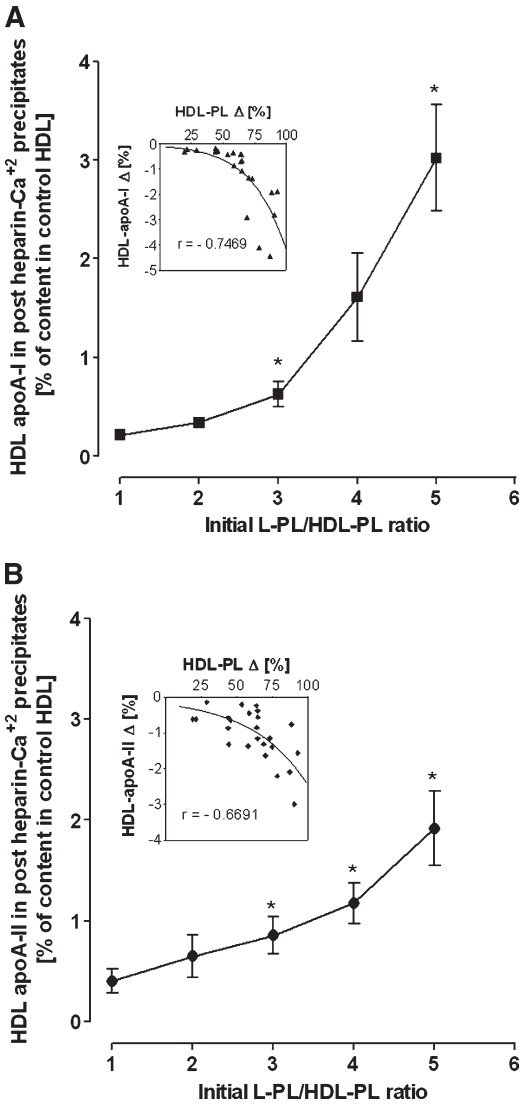

Fig. 5. The transfer of apoA-I and apoA-II to the post heparin-Ca^{+2} precipitates. Ultracentrifuged human HDL and EYL liposomes were mixed and incubated as described in Materials and Methods. Control incubations contained equivalent amount of HDL but no liposomes. Pre-β mobility fraction and liposomes were precipitated from incubation mixtures by heparin and Ca^{+2} and dissolved in 2% sodium citrate as described in Materials and Methods. Concentrations of apoA-I and apoA-II were measured in precipitate samples by nephelometry as described in Materials and Methods. Transfer of apos to postheparin-Ca^{+2} precipitates is expressed as a percentage of either apoA-I or apoA-II content in control incubations. A: Transfer of HDL apoA-I to post heparin-Ca^{+2} precipitates. Insert: The relationship between delta percent changes of PL (HDL-PL Δ) and apoA-I (HDL-apoA-I Δ) content in α-HDL after incubation with liposomes. B: Transfer of HDL apoA-II to post heparin-Ca^{+2} precipitates. Insert: The relationship between delta percent changes of PL (HDL-PL Δ) and apoA-II (HDL-apoA-II Δ) content in α-HDL after incubation with liposomes. The initial concentrations of HDL apos were as follows: apoA-I: 424.2 ± 22.7 mg/dl, apoA-II: 92.7 ± 6.6 mg/dl. Data are means ± SEM from five experiments. Value significantly different from control: * $P < 0.05$.

tween the concentration of dimyristoyl phosphatidylcholine added to human plasma and the amount of generated pre-β HDL-like particles containing apoA-I (22). However, we demonstrated for the first time that apoA-II may also leave HDL particles and become a component of new pre-β mobility lipoproteins (Fig. 1B, lane 3). It is noteworthy that in our experiments apoA-II displayed additional binding with liposomes (Fig. 1B lane 3, Fig. 3, lane 4). The presence of apoA-I exclusively in newly formed pre-β mobility fractions (Fig. 1B, lane 1, Fig. 3, lane 2) can be explained by the fact that the optimal association of apoA-I with PL requires disordered lipid structures, whereas apoA-II more readily associates also with intact lipid bilayers (32, 33).

The majority of apoA-I and apoA-II released from HDL formed separate subpopulations of particles (Fig. 6A and 6B, respectively), presumably due to the resistance of free apoA-I and A-II to from hybrid protein complexes with PL (34). We observed also a slight overlapping in the subpopulations of particles with a Stokes diameter of approximately 8 nm. Therefore, one cannot exclude that a small subpopulation of pre-β mobility particles containing apoA-I and A-II apos was also formed under these conditions. The higher heterogeneity level of apoA-II-containing particles (Fig. 6B) is in accordance with the earlier reported significant diversity of discoid complexes generated by free apoA-II and PL (35). The majority of apoA-II-containing species migrated in the range of Stokes diameters from <7.1 to 8.4 nm (Fig. 6B). These sizes overlap those of pre-β HDL A-II formed by the conjugation of lipid-free apoA-II with lipids derived either from cell membranes or other lipoproteins (11, 36).

At present we cannot determine whether apoA-II dissociated from PL-enriched HDL as free apoA and subsequently associated with lipids to form pre-β migrating particles. Based on the high hydrophobocity of this apo (16) it seems plausible that HDL shed apoA-II connected with small amounts of lipids with Stokes diameter of <7.1 to 7.6 nm (Fig. 6B). Lipid-poor apoA-II might acquire more PL from liposomes and form greater particles represented in Fig. 6B by species with Stokes diameters of 7.7, 8.0–8.4, and 11.9–15.5 nm. Similar phenomenon was observed during the incubation of free apoA-II with cell membranes (11). One cannot rule out that the accrual of PL by lipid-poor particles was accompanied by the accommodation of FC lost by PL-enriched α-HDL (Fig. 4, insert). Such a claim is supported by autoradiograms of mixtures of [^{14}C]cholesterol labeled HDL with liposomes (Fig. 2, lane 2) and dissolved precipitates (Fig. 2, lane 4). It also remains in accordance with the observation, that the inclusion of unesterified cholesterol into apo-lipid complexes increased their size and heterogeneity (31). CE apparently did not participate in this process, as their content in HDL did not change significantly (Fig. 4). It has been shown that the lack of neutral lipid core resulted in the pre-β mobility migration of HDL ssubspecies during electrophoresis in agarose gel (37). Hence, we conclude that the major constituents of particles in the pre-β mobility fraction were apos, either A-I or A-II; PL; and FC. Lipid-apo complexes devoid of apolar lipids are discoid in shape (37).

The major finding of the presented study is the observation that in vitro enrichment of HDL in PL (Fig. 4) generates lipid-poor apoA-II particles (Fig. 6B) with pre-β electrophoretic mobility (Fig. 1B, lane 3). It raises the

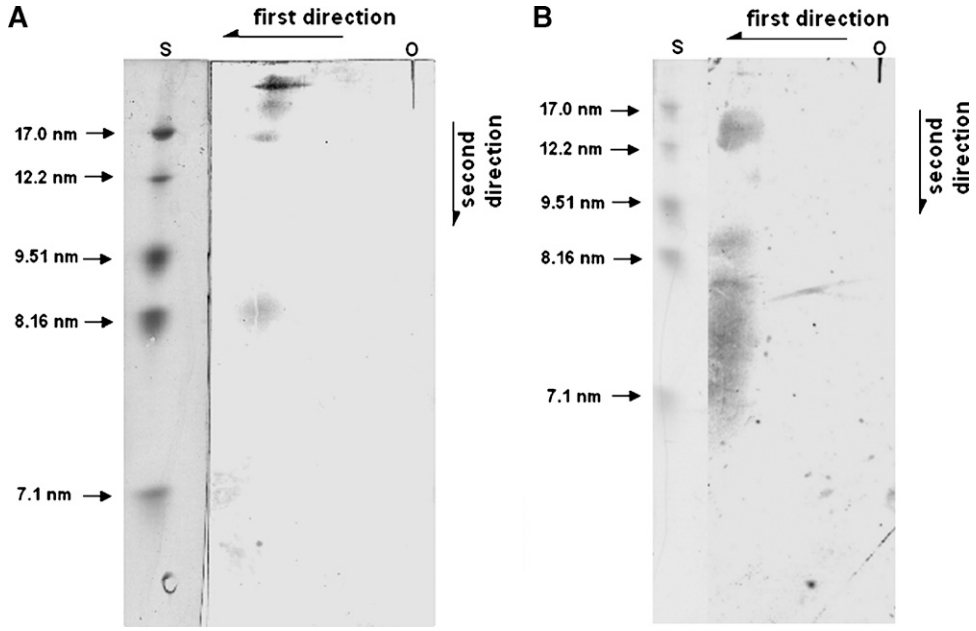

Fig. 6. Two-dimensional separation of pre-β mobility fraction. Ultracentrifuged human HDL and EYL liposomes were incubated at L-PL/HDL-PL ratio 5:1, for 1 h at 37°C. Pre-β particles and liposomes were precipitated from incubation mixtures by heparin and Ca^{+2} and dissolved in 2% sodium citrate as described in Materials and Methods. Aliquots of 15 μl were subjected to two-dimensional nondenaturing PAGE 2–27% and immunoblotted with either anti-human apoA-I or anti-human apoA-II as described in Materials and Methods. Numbers refer to the Stokes diameter of high molecular weight protein standards. O, The origin of agarose gel, S, standard proteins. A: Distribution of apoA-I in pre-β mobility fraction. B: Distribution of apoA-II in pre-β mobility fraction.

question of whether PL-induced HDL remodeling in vivo might induce the formation of such structures; and, if so, what is their physiological importance? In plasma, during lipolysis, phospholipid transfer protein permanently facilitates the transport of PL from triglyceride-rich lipoproteins to HDL particles (38). A previous study indicated that the in vitro lipolysis promoted the transfer of a small amount of apoA-II from HDL to VLDL (39). Castellani et al. (40) recently postulated that apoA-II regulates the metabolism of triglyceride-rich lipoproteins and may be spontaneously transferred from HDL to triglyceride-rich lipoproteins. We believe that the movement of extremely hydrophobic apoA-II across a water medium could be achieved only via the organized structures formed by apoA-II and PL, similar to the small, lipid-poor apoA-II-containing particles generated in our experiments (Fig. 6B). Their sizes were close to those of pre-β mobility HDL particles containing apoA-II as a sole apo, which were formed following over-expression of apoA-II in transgenic mice (41). Such HDL A-II were able to promote ABCA-1-dependent cholesterol efflux from macrophages (42). This is in accordance with the similar binding affinity of apoA-II and apoA-I to ABCA-1 (43); however it is not compliant with previous studies that suggested a proatherogenic effect of apoA-II (44). Strong arguments for the antiatherogenic role of apoA-II delivered in recently published results of a prospective European Prospective Investigation into Cancer and Nutrition -Norfolk study that showed inverse correlation between serum apoA-II levels and risk of future coronary artery disease in apparently healthy subjects (45). Nonetheless, the contribution of lipid-poor apoA-II in reverse cholesterol transport remains to be established.

Our finding, that structural modification of HDL particles by phosphatidylcholine resulted in the generation of lipid-poor apoA-II particles (Fig. 6B), indicates that apoA-II may actively contribute in HDL metabolism and supports the hypothesis expressed by Rye, Clay, and Barter (7), that apoA-II may cycle between lipid-poor and lipid rich forms of HDL. It leads us to speculate on several potential fates of lipid-poor apoA-II particles: *i*) They could be incorporated into small spherical HDL A-I in the process mediated by LCAT (15) and thereby participate in the generation of new spherical HDL A-I/A-II particles. This process might regulate the distribution of apoA-I between HDL A-I and HDL A-I/A-II (19). *ii*) Lipid-poor apoA-II could also be incorporated into pre-existing HDL particles (14). *iii*) It cannot be excluded that lipid-poor apoA-II in plasma could be able to create a discrete subpopulation of lipoprotein particles. This assumption is based on the isolation of apoA-II HDL, most probably spherical due to the presence of neutral lipids, from the plasma of normolipidemic subjects (9). *iv*) ApoA-II could modulate triglyceride hydrolysis following incorporation into triglyceride-rich lipoproteins (39, 40). *v*) Lipid-poor apoA-II could also compete with apoA-I for interaction with ABCA-1 (43). *vi*) Finally, lipid-poor apoA-II could be catabolised in the kidney and be lost from the plasma (46).

In conclusion, our study demonstrates for the first time that exogenous PL trigger the detaching of apoA-I and apoA-II from HDL. These apos form distinct, specific lipid-apo complexes migrating with pre-β mobility in agarose gel. Results presented in this paper support the hypothesis that apoA-II, like apoA-I, may cycle between mature, α-migrating HDL A-I/A-II particles and pre-β migrating, apoA-II-lipid complexes. The generation of lipid-poor apoA-II particles from mature HDL during remodelling mediated by PL may have significant impact on metabolism of HDL particles.

REFERENCES

1. Assmann, G., and J. R. Nofer. 2003. Atheroprotective effects of high-density lipoproteins. *Annu. Rev. Med.* **54:** 321–341.
2. Barrans, A., B. Jaspard, R. Barbaras, H. Chap, B. Perret, and X. Collet. 1996. Pre-β HDL: structure and metabolism. *Biochim. Biophys. Acta.* **1300:** 73–85.
3. Cheung, M. C., and J. J. Albers. 1984. Characterization of lipoprotein particles isolated by immunoaffinity chromatography. Particles containing A-I and A-II and particles containing A-I but no A-II. *J. Biol. Chem.* **259:** 12201–12209.
4. Forte, T. M., M. R. McCall, B. B. Knowles, and V. G. Shore. 1989. Isolation and characterization of lipoproteins produced by human hepatoma-derived cell lines other than HepG2. *J. Lipid Res.* **30:** 817–829.
5. Vedhachalam, C., P. T. Duong, M. Nickel, D. Nguyen, P. Dhanasekaran, H. Saito, G. H. Rothblat, S. Lund-Katz, and M. C. Philips. 2007. Mechanism of ATP-binding cassette transporter A1 mediated cellular lipid efflux to apolipoprotein A-I and formation of high density lipoprotein particles. *J. Biol. Chem.* **282:** 25123–25130.
6. Matz, C. E., and A. Jonas. 1982. Reaction of human lecithin cholesterol acyltransferase with synthetic micellar complexes of apolipoprotein A-I, phosphatidylcholine, and cholesterol. *J. Biol. Chem.* **257:** 4541–4546.
7. Rye, K. A., M. A. Clay, and P. J. Barter. 1999. Remodelling of high density lipoproteins by plasma factors. *Atherosclerosis.* **145:** 227–238.
8. Liang, H. Q., K. A. Rye, and P. J. Barter. 1995. Cycling of apolipoprotein A-I between lipid-associated and lipid-free pools. *Biochim. Biophys. Acta.* **1257:** 31–37.
9. Bekaert, E. D., P. Alaupovic, C. Knight-Gibson, R. A. Norum, M. J. Laux, and M. Ayrault-Jarrier. 1992. Isolation and partial characterization of lipoprotein A-II (LP-A-II) particles of human plasma. *Biochim. Biophys. Acta.* **1126:** 105–113.
10. März, W., and W. Groß. 1988. Immunochemical evidence for the presence in human plasma of lipoproteins with apolipoprotein A-II as the major protein constituent. *Biochim. Biophys. Acta.* **962:** 155–158.
11. Forte, T. M., J. K. Bielicki, R. Goth-Goldstein, J. Selmek, and M. R. McCall. 1995. Recruitment of cell phospholipids and cholesterol by apolipoproteins A-II and A-I: formation of nascent apolipoprotein-specific HDL that differ in size, phospholipid composition, and reactivity with LCAT. *J. Lipid Res.* **36:** 148–157.
12. Hara, H., and S. Yokoyama. 1991. Interaction of free apolipoproteins with macrophages. Formation of high density lipoprotein-like lipoproteins and reduction of cellular cholesterol. *J. Biol. Chem.* **266:** 3080–3086.
13. Jonas, A., S. A. Sweeny, and P. N. Herbert. 1984. Discoidal complexes of A and C apolipoproteins with lipids and their reactions with lecithin: cholesterol acyltransferase. *J. Biol. Chem.* **259:** 6369–6375.
14. Hime, N. J., K. J. Drew, K. Wee, P. J. Barter, and K. A. Rye. 2006. Formation of high density lipoproteins containing both apolipoprotein A-I and A-II in the rabbit. *J. Lipid Res.* **47:** 115–122.
15. Clay, M. A., D. H. Pyle, K. A. Rye, and P. J. Barter. 2000. Formation of spherical, reconstituted high density lipoproteins containing both apolipoproteins A-I and A-II is mediated by lecithin: cholesterol acyltransferase. *J. Biol. Chem.* **275:** 9019–9025.
16. Edelstein, C., M. Halari, and A. M. Scanu. 1982. On the mechanism of the displacement of apolipoprotein A-I by apolipoprotein A-II from the high density lipoprotein surface. Effect of concentration and molecular forms of apolipoprotein A-II. *J. Biol. Chem.* **257:** 7189–7195.
17. Boucher, J., T. A. Ramsamy, S. Braschi, D. Sahoo, T. A. M. Neville, and D. L. Sparks. 2004. Apolipoprotein A-II regulates HDL stability and affects hepatic lipase association and activity. *J. Lipid Res.* **45:** 849–858.
18. Rye, K. A., K. Wee, L. K. Curtiss, D. J. Bonnet, and P. J. Barter. 2003. Apolipoprotein A-II inhibits high density lipoprotein remodeling and lipid-poor apolipoprotein A-I formation. *J. Biol. Chem.* **278:** 22530–22536.
19. Ikewaki, K., L. A. Zech, M. Kindt, H. B. Brewer, and D. J. Rader. 1995. Apolipoprotein A-II production rate is a major factor regulating the distribution of apolipoprotein A-I among HDL subclasses LpA-I and LpA-I:A-II in normolipidemic humans. *Arterioscler. Thromb. Vasc. Biol.* **15:** 306–312.
20. Rodrigueza, W. V., K. J. Williams, G. H. Rothblat, and M. C. Philips. 1997. Remodeling and shuttling. Mechanisms for the synergistic effects between different acceptor particles in the mobilization of cellular cholesterol. *Arterioscler. Thromb. Vasc. Biol.* **17:** 383–393.
21. Jian, B., M. de la Llera-Moya, L. Royer, G. Rothblat, O. Francone, and J. B. Swaney. 1997. Modification of the cholesterol efflux properties of human serum by enrichment with phospholipid. *J. Lipid Res.* **38:** 734–744.
22. Hassan, H. H, S. Blain, B. Boucher, M. Denis, L. Krimbou, and J. Genest. 2005. Structural modification of plasma HDL by phospholipids promotes efficient ABCA1-mediated cholesterol release. *J. Lipid Res.* **46:** 1457–1465.
23. Guo, L. S. S., R. L. Hamilton, J. Goerke, J. N. Weinstein, and R. J. Havel. 1980. Interaction of unilamellar liposomes with serum lipoproteins and apolipoproteins. *J. Lipid Res.* **21:** 993–1003.
24. Tall, A. R., and P. H. R. Green. 1981. Incorporation of phosphatidylcholine into spherical and discoidal lipoproteins during incubation of egg phosphatidylcholine vesicles with isolated high density lipoproteins or with plasma. *J. Biol. Chem.* **256:** 2035–2044.
25. Burstein, M., H. R. Scholnick, and R. Morfin. 1970. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. *J. Lipid Res.* **11:** 583–595.
26. Kim, Y. C., and T. Nishida. 1977. Nature of interaction of dextran sulfate with lecithin dispersions and lysolecithin micelles. *J. Biol. Chem.* **252:** 1243–1249.
27. Kim, Y. C., and T. Nishida. 1979. Nature of the interaction of dextran sulfate with high and low density lipoproteins in the presence of Ca^{2+}. *J. Biol. Chem.* **254:** 9621–9626.
28. Brousseau, T., V. Clavey, J. M. Bard, and J. C. Fruchart. 1993. Sequential ultracentrifugation micromethod for separation of serum lipoproteins and assays of lipids, apolipoproteins, and lipoprotein particles. *Clin. Chem.* **39:** 960–964.
29. Dobiášová, M., L. Adler, T. Ohta, and J. Frohlich. 2000. Effect of labeling of plasma lipoproteins with [^{3}H]cholesterol on values of esterification rate of cholesterol in apolipoprotein B-depleted plasma. *J. Lipid Res.* **41:** 1356–1357.
30. Asztalos, B. F., C. H. Sloop, L. Wong, and P. S. Roheim. 1993. Two dimensional electrophoresis of plasma lipoproteins: recognition of new apoA-I-containing subpopulations. *Biochim. Biophys. Acta.* **1169:** 291–300.
31. Nichols, A. V., E. L. Gong, P. J. Blanche, and T. M. Forte. 1983. Characterization of discoidal complexes of phosphatidylcholine, apolipoprotein A-I and cholesterol by gradient gel electrophoresis. *Biochim. Biophys. Acta.* **750:** 353–364.
32. Van Tornout, P., R. Vercaemst, M. J. Lievens, H. Caster, M. Rosseneu, and G. Assmann. 1980. Reassembly of human apoproteins A-I and A-II with unilamellar phosphatidylcholine-cholesterol liposomes. Association kinetics and characterization of the complexes. *Biochim. Biophys. Acta.* **601:** 509–523.
33. Pownall, H. J., J. B. Massey, S. K. Kusserow, and A. M. Gotto, Jr. 1978. Kinetics of lipid-protein interactions: effect of cholesterol on the association of human plasma high density apolipoprotein A-I with L-α dimyristoylphosphatidylcholine. *Biochemistry.* **17:** 1183–1188.
34. Swaney, J. B., and E. Palmieri. 1984. Hybrid association between human apolipoproteins A-I and A-II in aqueous solution and in phospholipid recombinants. *Biochim. Biophys. Acta.* **792:** 164–171.
35. Blanche, P. J., A. V. Nichols, T. M. Forte, and E. L. Gong. 1988. Characterization of complexes of egg yolk phosphatidylcholine and apolipoprotein A-II prepared in the absence and presence of sodium cholate. *Biochim. Biophys. Acta.* **958:** 143–152.
36. Clay, M. A., D. A. Cehic, D. H. Pyle, K. A. Rye, and P. J. Barter. 1999.

Formation of apolipoprotein specific high-density lipoprotein particles from lipid-free apolipoproteins A-I and A-II. *Biochem. J.* **337:** 445–451.
37. Davidson, W. S., D. L. Sparks, S. Lund-Katz, and M. C. Phillips. 1994. The molecular basis for the difference in charge between pre-β - and α-migrating high density lipoproteins. *J. Biol. Chem.* **269:** 8959–8965.
38. Huuskonen, J., V. M. Olkkonen, M. Jauhiainen, and C. Ehnholm. 2001. The impact of phospholipid transfer protein (PLTP) on HDL metabolism. *Atherosclerosis.* **155:** 269–281.
39. Musliner, T. A., H. J. Michenfelder, and R. M. Krauss. 1988. Interactions of high density lipoproteins with very low and low density lipoproteins during lipolysis. *J. Lipid Res.* **29:** 349–361.
40. Castellani, L. W., C. N. Nguyen, S. Charugundla, M. W. Weinstein, C. X. Doan, W. S. Blaner, N. Wongsiriroj, and A. L. Lusis. 2008. Apolipoprotein A-II is a regulator of VLDL metabolism and insulin resistance. *J. Biol. Chem.* **283:** 11633–11644.
41. Pastier, D., S. Dugué, E. Boisfer, V. Atger, N. Q. Tran, A. van Tol, M. J. Chapman, J. Chambaz, P. M. Laplaud, and A. D. Kalopissis. 2001. Apolipoprotein A-II/A-I ratio is a key determinant in vivo of HDL concentration and formation of pre-β HDL containing apolipoprotein A-II. *Biochemistry.* **40:** 12243–12253.
42. Fournier, N., A. Cogny, V. Atger, D. Pastier, D. Goudouneche, A. Nicoletti, N. Moatti, J. Chambaz, J. L. Paul, and A. D. Kalopissis. 2002. Opposite effects of plasma from human apolipoprotein A-II transgenic mice on cholesterol efflux from J774 macrophages and Fu5AH hepatoma cells. *Arterioscler. Thromb. Vasc. Biol.* **22:** 638–643.
43. Fitzgerald, M. L., A. L. Morris, A. Chroni, A. J. Mendez, V. I. Zannis, and M. W. Freeman. 2004. ABCA1 and amphipathic apolipoproteins form high-affinity molecular complexes required for cholesterol efflux. *J. Lipid Res.* **45:** 287–294.
44. Warden, C. H., C. C. Hedrick, J. H. Qiao, L. W. Castellani, and A. J. Lusis. 1993. Atherosclerosis in transgenic mice overexpressing apolipoprotein A-II. *Science.* **261:** 469–472.
45. Birjmohun, R. S., G. M. Dallinga-Thie, J. A. Kuivenhoven, E. S. G. Stroes, J. D. Otvos, N. J. Wareham, R. Luben, J. J. P. Kastelein, K. T. Khaw, and S. M. Boekholdt. 2007. Apolipoprotein A-II is inversely associated with risk of future coronary artery disease. *Circulation.* **116:** 2029–2035.
46. Dugué-Pujol, S., X. Rousset, D. Château, D. Pastier, C. Klein, J. Demeurie, C. Cywiner-Golenzer, M. Chabert, P. Verroust, J. Chambaz, et al. 2007. Apolipoprotein A-II is catabolized in the kidney as a function of its plasma concentration. *J. Lipid Res.* **48:** 2151–2161.