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

Małgorzata Wróblewska,1,* Barbara Kortas-Stempak,1 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 cholesterol-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 disassociated 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.

Supplementary key words  apolipoprotein A-II • high density lipoproteins • A-II lipid-poor particles

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).

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.

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

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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 into spherical HDL seems to be a stable 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-II 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 HDLs. 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 non-denaturing 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 CaCl₂ 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 MnCl₂ (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, 43 680 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% NaN₃ as a preservative.

In selected experiments, HDL were labeled with [¹⁴C]cholesterol (Polatom, Swierk-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% NaN₃ 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 (Sonophus HD 2070 ultrasonic homogenizer, Bandelin electronic GmbH 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

Incubations 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 lipoproteins-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 CaCl₂ 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 CaCl₂ 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® Millipore, Bedford, MA) by passive transfer and detected with monoclonal antibodies either to human apoA-I, or apoA-II (ICN Pharmaceuticals...
In experiments with HDL \(^{14}\text{C}\)|cholesterol labeled, following electrophoresis, gels were dried and subjected to autoradiography.

The distribution of apoA-I and apoA-II in the precipitate containing non-\(\alpha\)-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\(^{\circledR}\), Millipore Bedford, MA). ApoA-I or apoA-II-containing lipoproteins were detected 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 cholesterol 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 \(\beta\)-mobility fraction containing apoA-I and apoA-II following incubation of HDL with lecithin liposomes**

The formation of new lipoprotein fraction migrating with \(\beta\)-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 \(\beta\)-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 \(^{14}\text{C}\) remained in the \(\alpha\)-mobility position when HDL were incubated alone (Fig. 2, lane 1). In turn, in the mixture of HDL and liposomes \(^{14}\text{C}\) cholesterol appeared to shift to a new \(\beta\)-mobility band and liposomes as well (Fig. 2, lane 2). Immunoblotsing with monoclonal antibodies demonstrated that apoA-I and apoA-II, which in control samples of HDL were detected exclusively in \(\alpha\)-mobility position (Fig. 1B, lane 2 and lane 4, respectively), in incubation mixtures redistributed to \(\beta\)-mobility fraction (Fig. 1B, lane 1 and lane 3, respectively). Moreover, apoA-II was present also in liposomes (Fig. 1B, lane 3).

**Precipitation of non-\(\alpha\)-HDL lipoproteins**

The addition of heparin and CaCl\(_2\) to the mixture of HDL and liposomes produced insoluble complexes of liposomes and \(\beta\)-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...
and incubated for 1 h at 37°C. Heparin-Ca²⁺-lipid suspensions labeled with [³⁵S]methionine and [¹⁴C]cholesterol and EYL liposomes were mixed and incubated at 37°C and 50 μM 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-Ca²⁺ supernatant; Lane 4: post heparin-Ca²⁺ 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-Ca²⁺ 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-Ca²⁺ 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-Ca²⁺ precipitates contained two electrophoretic fractions displaying the mobility of liposomes and pre-β fraction (Fig. 5, lane 4), while post heparin-Ca²⁺ supernatants contained single α-mobility fraction (Fig. 5, lane 3). Immunoblotting with monoclonal antibodies demonstrated the presence of apoA-I and apoA-II in α-mobility bands in post heparin-Ca²⁺ 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 post heparin-Ca²⁺ supernatants, we found that apoA-II in α-mobility bands and apoA-I in post heparin-Ca²⁺ supernatants 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 pre-β mobility fractions from 0.2 to 3% respectively (Fig. 5A).

To avoid high uncertainty in estimating small fluctuations in apo levels in post heparin-Ca²⁺ supernatants, we dissolved post heparin-Ca²⁺ precipitates in small volumes of sodium citrate (see Materials and Methods) and used them for the quantitative assessment of the dissociation of apop 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 pre-β mobility fractions from 0.2 to 3% respectively (Fig. 5A).

### Table 1. Dilution-dependent precipitation of liposomes by heparin and Ca²⁺

| Degree of liposomes dilution | Precipitation of liposomal PL [%] |
|-----------------------------|----------------------------------|
| 2X                          | 99.2 ± 0.9                      |
| 4X                          | 99.7 ± 0.2                      |
| 10X                         | 99.1 ± 0.6                      |
| 40X                         | 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-Ca²⁺ precipitation was performed as described in Materials and Methods. Results (means ± SD from three experiments) are expressed as percent of PL transferred to post heparin-Ca²⁺ precipitates.

### Table 2. Dilution-dependent recovery of HDL-associated lipids and apoA-I in post heparin-Ca²⁺ supernatants

| 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             |
| 1.54X        | 98.7 ± 2.4         | 97.7 ± 1.5         | 100.9 ± 4.4            |
| 2X           | 99.7 ± 0.6         | 98.0 ± 2           | 99.7 ± 1.1             |

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-Ca²⁺ precipitation was performed as described in Materials and Methods. Post heparin-Ca²⁺ supernatants were analyzed for lipids and apoA-I contents. Results (means ± SD from three experiments) are expressed as percent of lipid content in HDL.

### Table 3. Recovery of cholesteryl esters (CE) in post heparin-Ca²⁺ supernatants after precipitation of variable mixtures of liposomes and HDL

| Initial L-PL/HDL-PL ratio | Recovery of CE in post heparin-Ca²⁺ 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-Ca²⁺ precipitation was performed as described in Materials and Methods. Results (mean ± SD from five experiments) are expressed as percent of CE content in HDL.
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 apoA-II released from HDL to post heparin-Ca\(^{2+}\) precipitates correspond to their loss by HDL, we have found a nonlinear inverse relationship between HDL-PL (Fig. 5A, B, inserts, respectively) and the amount of apos accumulating in post heparin-Ca\(^{2+}\) precipitates increased from 0.4 to 1.9% (Fig. 4B). Assuming that apoA-II released from HDL to post heparin-Ca\(^{2+}\) precipitates correspond to their loss by HDL, we have found a nonlinear inverse relationship between HDL-PL and apoA-II released from HDL. The transfer of apoA-I and apoA-II to liposomes became PL enriched in a dose-dependent manner (Fig. 2, lane 3, Fig. 3, lanes 1 and 3). The HDL incubated with liposomes 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. Results for a representative incubation mixture at initial L-PL/HDL-PL ratio 5:1 are shown. Lane 1: post heparin-Ca\(^{2+}\) precipitate and supernatant immunoblotted with anti-human apoA-I antibodies; Lane 2: post heparin-Ca\(^{2+}\) precipitate immunoblotted with anti-human apoA-II antibodies; Lane 3: post heparin-Ca\(^{2+}\) precipitate and supernatant immunoblotted with anti-human apoA-II antibodies; Lane 4: post heparin-Ca\(^{2+}\) precipitate immunoblotted with anti-human apoA-II antibodies.

**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\(^{2+}\) (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-
we demonstrated for the first time that apoA-II may also
added to human plasma and the amount of generated
tween the concentration of dimyristoyl phosphatidylcholine
kelated HDL particles and become a component of new
leave HDL particles and become a component of new
lesterol. We observed also a slight overlapping in the sub-
migrate in the range of Stokes diameters from \(<7.1\) to \(8.4\) nm (Fig. 6B). These sizes overlap
form separate subpopulations of particles (Fig. 6A and
HDL formed separate subpopulations of particles (Fig. 6A and
similar phenomenon was ob-
HDL A-II formed by the conjugation of
As free apoA and sub-
HDL as free apoA and sub-
Cholesterol labeled HDL with liposomes (Fig. 2,
HDL after incubation with liposomes. The initial concentra-
subpopulations of particles with a Stokes diameter of approxi-
PL by lipid-poor particles was accompanied by the accom-
formed separate subpopulations of particles (Fig. 6A and
HDL after incubation with liposomes. The initial concentra-
were precipitated from incubation mixtures by heparin and Ca\(^{2+}\) and dissolved in 2% sodium citrate as described in Materials and Methods. Control incubations contained equivalent amount of HDL but no liposomes. Pre-\(\beta\) mobility fraction and liposomes were precipitated by free apoA-II with lipids derived either from cell mem-
planes or other lipoproteins (11, 36).

At present we cannot determine whether apoA-II dissoci-
med by free apoA-II and PL (35). The majority of apoA-II-
formed separate subpopulations of particles (Fig. 6A and
HDL after incubation with liposomes. The initial concentra-
forming with small amounts of lipids with Stokes diameter of \(<7.1\) to \(7.6\) nm (Fig. 6B). Lipid-poor apoA-II might acquire
migrate in the range of Stokes diam-
and dissolved precipitates (Fig. 2, lane 4). It also re-
and Methods. Concentrations of apoA-I and apoA-II were measured in precipitate samples by nephelometry as described in Materials and Methods. Transfer of apoA 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 postheparin-Ca\(^{2+}\) precipitates. Insert: The relationship between delta percent changes of PL (HDL-PL \(\Delta\)) and apoA-I (HDL-apoA-I \(\Delta\)) content in \(\alpha\)-HDL after incubation with liposomes.
}

**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. Concentrations of apoA-I and apoA-II were measured in precipitate samples by nephelometry as described in Materials and Methods. Transfer of apoA 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 postheparin-Ca\(^{2+}\) precipitates. Insert: The relationship between delta percent changes of PL (HDL-PL \(\Delta\)) and apoA-I (HDL-apoA-I \(\Delta\)) content in \(\alpha\)-HDL after incubation with liposomes.

B: Transfer of HDL apoA-II to postheparin-Ca\(^{2+}\) precipitates. Insert: The relationship between delta percent changes of PL (HDL-PL \(\Delta\)) and apoA-II (HDL-apoA-II \(\Delta\)) content in \(\alpha\)-HDL after incubation with liposomes. The initial concentrations of HDL apoA-I and apoA-II were as follows: apoA-I: \(92.2 \pm 6.6\) mg/dl, apoA-II: \(92.7 \pm 6.6\) mg/dl. Data are means \(\pm\) SEM from five experiments. Value significantly different from control: * \(P < 0.05\).

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-\(\beta\) electrophoretic mobility (Fig. 1B, lane 3). It raises the
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).

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 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 lipid-poor apoA-II in pre-β mobility fraction.
In conclusion, our study demonstrates for the first time that exogenous PL trigger the detaching of apoA-I and apoA-II from HDL. These apop form distinct, specific lipid-apolipoprotein A-II particles and apoA-II 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 apoA-II 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.

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