Structural modification of plasma HDL by phospholipids promotes efficient ABCA1-mediated cholesterol release

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Abstract **It has been suggested that ABCA1 interacts preferentially with lipid-poor apolipoprotein A-I (apoA-I). Here, we show that treatment of plasma with dimyristoyl phosphatidylcholine (DMPC) multilamellar vesicles generates preβ_1-apoA-I-containing lipoproteins (LpA-I)-like particles similar to those of native plasma. Isolated preβ_1-LpA-I-like particles inhibited the binding of ^{125}I-apoA-I to ABCA1 more efficiently than HDL_3 (IC_{50} = 2.20 ± 0.35 vs. 37.60 ± 4.78 μg/ml). We next investigated the ability of DMPC-treated plasma to promote phospholipid and unesterified (free) cholesterol efflux from J774 macrophages stimulated or not with cAMP. At 2 mg DMPC/ml plasma, both phospholipid and free cholesterol efflux were increased (~50% and 40%, respectively) in cAMP-stimulated cells compared with unstimulated cells. Similarly, both phospholipid and free cholesterol efflux to either isolated native preβ_1-LpA-I and preβ_1-LpA-I-like particles were increased significantly in stimulated cells. Furthermore, glyburide significantly inhibited phospholipid and free cholesterol efflux to DMPC-treated plasma. Removal of apoA-I-containing lipoproteins from normolipidemic plasma drastically reduced free cholesterol efflux mediated by DMPC-treated plasma. Finally, treatment of Tangier disease plasma with DMPC affected the amount of neither preβ_1-LpA-I nor free cholesterol efflux. These results indicate that DMPC enrichment of normal plasma resulted in the redistribution of apoA-I from α-HDL to preβ-HDL, allowing for more efficient ABCA1-mediated cellular lipid release. Increasing the plasma preβ_1-LpA-I level by either pharmacological agents or direct infusions might prevent foam cell formation and reduce atherosclerotic vascular disease.**—Hajj Hassan, H., S. Blain, B. Boucher, M. Denis, L. Krimbou, and J. Genest. **Structural modification of plasma HDL by phospholipids promotes efficient ABCA1-mediated cholesterol release.** ***J. Lipid Res.*** **2005.** 46: **1457–1465.**

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HDL is believed to be a potent physiological protective system against atherosclerotic vascular disease. Although it has become generally accepted that this protective effect of HDL is attributable to its pivotal role in the reverse cholesterol transport (RCT) process (1, 2), structural determinants of molecular interactions between circulating HDL particles and key cell proteins governing the RCT process are complex and not well understood.

A growing body of evidence indicates that ABCA1 is a critical cell surface protein required for the transfer of cellular lipid and the maintenance of HDL levels in plasma and is likely important for the first step of RCT from peripheral tissues, including macrophages in the vessel wall (3, 4). Furthermore, Brewer and colleagues (5) have documented that hepatic ABCA1 is a key protein for the formation and maintenance of plasma HDL levels. Moreover, the importance of ABCA1 in the lipidation of apolipoprotein A-I (apoA-I) is highlighted by the finding that >50 mutations in the ABCA1 gene have been associated with a variety of clinically distinct HDL deficiency diseases, including Tangier disease (TD) and familial HDL deficiency (6, 7). These patients are characterized by extremely low HDL-cholesterol levels, caused by defective transport of cellular cholesterol and phospholipids to the extracellular space, leading to hypercatabolism of lipid-poor nascent HDL particles (8).

Earlier studies by Fielding and colleagues (9, 10) have documented that a minor subspecies of human HDL that migrates with preβ mobility on agarose gels can remove free cholesterol from cultured fibroblasts at a faster rate than α-migrating HDL, which constitutes the bulk of plasma HDL. Furthermore, it was documented that preβ-HDL particles were present in the peripheral lymph of dogs (11), suggesting a key role for these particles in the

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Abbreviations: apoA-I, apolipoprotein A-I; BBSM, bovine brain sphingomyelin; DMPC, dimyristoyl phosphatidylcholine; MLV, multilamellar vesicle; PEG, polyethylene glycol; POPC, palmitoyloleoyl phosphatidylcholine; preβ_1-LpA-I, preβ_1 apoA-I-containing lipoproteins; RCT, reverse cholesterol transport; SR-BI, scavenger receptor class B type I; TD, Tangier disease; 2D-PAGGE, two-dimensional polyacrylamide nondenaturing gradient gel electrophoresis.

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initial removal of cholesterol. This is consistent with the concept of Hara and Yokoyama (12) that lipid-free or lipid-poor apoA-I interacts with a site on the cell membrane, removes cellular lipids, and generates nascent preβ-HDL particles. Subsequently, preβ-HDL particles become mature, spherical, and α-migrating HDL by the action of LCAT, which converts free cholesterol to cholesteryl ester. Moreover, this concept is supported by studies demonstrating that preβ-HDL particles act as an initial acceptor of cellular cholesterol and shuttle it into a series of larger preβ particles and ultimately to α-migrating particles (13, 14).

In spite of the importance of preβ$_1$-apoA-I-containing lipoproteins (LpA-I) particles in RCT, very little is known about their contribution to the human plasma ABCA1-dependent cholesterol efflux pathway. This is likely because of the low amount of these particles and the difficulty of isolating them. These problems were circumvented in the present study by increasing the plasma level of preβ$_1$-LpA-I using dimyristoyl phosphatidylcholine (DMPC) multilamellar vesicles (MLVs). Therefore, our experiments were directed at determining the affinity of these newly formed preβ$_1$-LpA-I-like particles for ABCA1 and monitoring their ability to promote cholesterol efflux from a macrophage cell culture model.

MATERIALS AND METHODS

Samples

Blood samples were obtained from normolipidemic male subjects with apoE3/3 phenotype after an overnight fast. Blood was drawn from the antecubital vein into tubes containing EDTA (final concentration, 1.5 mg/ml). Collection tubes were immediately placed on ice before being centrifuged (3,000 rpm, 15 min, 4°C). For experiments in which plasma was incubated with cells, streptokinase was used as the anticoagulant at a final concentration of 150 U/ml blood. Plasma was separated from red blood cells by aspiration and was kept on ice until treatment with phospholipids or electrophoretic separation of apoA-I-containing particles. This study was approved by the ethics committees of the institutions involved. Plasma from TD subjects was provided by Dr. Arnold von Eckardstein from the Institute of Clinical Chemistry, University Hospital Zurich, Switzerland.

Incubation of plasma with phospholipids

Phospholipid MLVs containing DMPC, palmitoyloleoyl phosphatidylcholine (POPC), or bovine brain sphingomyelin (BBSM) were prepared as described previously (15). Plasma from each of either three normolipidemic or three TD subjects (**Table 1**) was incubated with phospholipids at their phase transition temperature for 1 h in the presence of 2 mM DTNB to inhibit LCAT activity. After incubation, the samples were separated by agarose gel electrophoresis or two-dimensional polyacrylamide nondenaturing gradient gel electrophoresis (2D-PAGGE). At the same time, DMPC-treated plasma samples were depleted from apoB-containing lipoprotein by precipitation with polyethylene glycol (PEG) 6000, as described previously (16), before phospholipid and free cholesterol efflux experiments.

Separation of lipoproteins by 2D-PAGGE

ApoA-I-containing particles were separated by 2D-PAGGE as described previously (17). Briefly, samples (30–100 μl) were separated in the first dimension (according to their charge) by 0.75% agarose gel electrophoresis (100 V, 3 h, 4°C) and in the second dimension (according to the size) by 5–23% polyacrylamide concave gradient gel electrophoresis (125 V, 24 h, 4°C). Iodinated high molecular weight protein mixture (7.1–17.0 nm; Pharmacia) was run as a standard on each gel. Electrophoretically separated samples were electrotransferred (30 V, 24 h, 4°C) onto nitrocellulose membranes (Hybond ECL; Amersham). ApoA-I-containing particles were detected by incubating the membranes with immunopurified polyclonal anti-apoA-I antibody (Biodesign).

Isolation of plasma preβ$_1$-LpA-I particles

Either native plasma preβ$_1$-LpA-I or preβ$_1$-LpA-I-like particles were isolated from freshly normolipidemic plasma treated or not with DMPC under nondenaturing conditions, as described previously (18), with the following modifications. Plasma samples were incubated or not with DMPC (2 mg/ml plasma) for 1 h at 24°C in the presence of 2 mM DTNB. DMPC-treated plasma or untreated plasma was subjected to a human immunopurified anti-apoA-I antibody (12171-21A; Genzyme Corp.)-coupled Sepharose column. ApoA-I-containing fractions were then dialyzed and concentrated. Samples were separated by agarose gel electrophoresis, and the preβ-migrating region was excised out. Agarose gel pieces containing the preβ-migrating region were placed at the top of 3–26% nondenaturing gradient gels, as described previously (19). An immunoblot of apoA-I-containing lipoproteins separated by 2D-PAGGE was used as a template to localize preβ$_1$-LpA-I, which is recovered from the gels by electroelution. Preβ$_1$-LpA-I particles were further concentrated by ultrafiltration (spiral ultrafiltration cartridge, molecular weight cut off 50,000; Amicon) to

TABLE 1. Levels of cholesterol, triglyceride, and apolipoprotein in normolipidemic and ABCA1-deficient subjects

	Plasma Concentration					
Subjects	Cholesterol	Triglycerides	HDL-Cholesterol	ApoB	ApoA-I	ApoE
		mmol/l			*mg/dl*	
Controls						
1	3.41	1.12	1.35	90	140	4.14
2	3.97	0.70	1.52	85	138	3.60
3	4.30	0.90	1.65	88	153	3.90
ABCA1-deficient						
TD1	4.14	2.54	0.11	177	6	2.9
TD2	3.50	2.10	0.09	87	3	2.4
TD3	3.20	1.67	0.10	79	5	2.7

apoA-I, apolipoprotein A-I; TD, Tangier disease.

discard any lipid-free apoA-I or proteolytic peptides. The integrity of isolated preβ$_1$-LpA-I and preβ$_1$-LpA-I-like particles was verified by 2D-PAGGE. SDS-PAGE revealed the presence of a single apoA-I band free of proteolytic peptides. Typically, 2 mg/ml native preβ$_1$-LpA-I was obtained from 100 ml of normolipidemic plasma, whereas 5 mg/ml preβ$_1$-LpA-I-like particles was obtained from 100 ml of DMPC-treated plasma, with an overall recovery of ~20% for both native preβ$_1$-LpA-I and preβ$_1$-LpA-I-like particles.

Human plasma apoA-I

Purified plasma apoA-I (Biodesign) was resolubilized in 4M guanidine-HCl and dialyzed extensively against Tris buffer (10 mM Tris and 150 mM NaCl, pH 8.2). Freshly resolubilized apoA-I was used within 48 h.

Competition binding assays

Competition binding assays were performed as described previously (18, 20). Briefly, apoA-I was iodinated with ^{125}I by Iodo-Gen® (Pierce) to a specific activity of 800–1,500 cpm/ng apoA-I. Normal fibroblasts were grown on 24-well plates and were stimulated with 2.5 μg/ml 22-(*R*)-hydroxycholesterol and 10 μM 9-*cis*-retinoic acid for 20 h. Cells were then incubated at 37°C with ^{125}I-apoA-I in DMEM/BSA in the presence of increasing amounts of either native preβ$_1$-LpA-I, preβ$_1$-LpA-I-like particles, HDL$_3$, or unlabeled apoA-I for 2 h. The cells were then washed rapidly two times with ice-cold PBS/BSA and two times with cold PBS and lysed with 0.1 N NaOH. The amount of bound iodinated ligand was determined by γ counting. Control experiments were conducted to examine whether the apparent decrease in cell binding of the labeled apoA-I may be attributable to the ^{125}I-apoA-I binding to different competitor particles instead of the cells. Therefore, an experiment was carried out in which either preβ$_1$-LpA-I-like particles or HDL$_3$ particles were incubated with ^{125}I-apoA-I under similar conditions used for the apoA-I binding assay and then the HDL$_3$ sample was separated by fast-protein liquid chromatography. No significant amount of ^{125}I-apoA-I was found associated with HDL$_3$. On the other hand, because of insufficient separation between lipid-free ^{125}I-apoA-I and preβ$_1$-LpA-I in our fast-protein liquid chromatography system, lipid-free ^{125}I-apoA-I was removed from the incubation medium using a size-exclusion centrifugal filter (MWCO 50,000) combined with a dialysis membrane (MWCO 50,000). This centrifugal filtration system discriminates between lipid-free apoA-I and other lipidated LpA-I particles with molecular mass > 50 kDa. No detectable lipid-free ^{125}I-apoA-I was found associated with preβ$_1$-LpA-I-like particles after filtration followed by dialysis, as assessed by 2D-PAGGE. In separate experiments, we show that both the centrifugal filter and the dialysis membrane retained isolated preβ$_1$-LpA-I with an apparent molecular mass of 67 kDa.

Cellular lipid efflux and lipid labeling

J774 mouse macrophages were cultured in RPMI 1640 with 10% fetal calf serum. At confluence, cells were labeled with 4 μCi/ml [^{3}H]cholesterol or 4 μCi/ml [^{3}H]choline (Perkin-Elmer) for 24 h. After a 24 h labeling period, the cells were washed and then incubated with 0.2% BSA in RPMI with or without 0.3 mM 8-bromo-cAMP for 12 h. Plasma incubated or not with DMPC was depleted of apoB-containing lipoproteins with PEG 6000 and dialyzed. Plasma samples (20 μg of apoA-I) were then incubated with cAMP-stimulated or unstimulated cells for 4 h at 37°C. In some experiments, 300 μM glyburide was added to the medium together with the acceptors. Cellular lipid efflux was determined as follow: ^{3}H cpm in medium/(^{3}H cpm in medium + ^{3}H cpm in cells). The results are expressed as percentages of total radiolabeled phospholipid or cholesterol.

Lipid and lipoprotein assays

Cholesterol and triglyceride concentrations were determined enzymatically on an autoanalyzer (Cobas Mira; Roche Molecular Biochemicals). HDL-cholesterol concentration was determined by measuring cholesterol in the supernatant after precipitation of apoB-containing lipoproteins with heparin-manganese from the d > 1.006 g/ml fraction prepared by ultracentrifugation. Plasma apoA-I and apoB concentrations were determined by nephelometry (Behring Nephelometer 100 Analyzer) or by ELISA. ApoE in total plasma was assayed by ELISA. The number of apoA-I molecules per particle was estimated by cross-linking with dithiobis(succinimidylpropionate) (21). Total phospholipid was determined in native preβ$_1$-LpA-I or preβ$_1$-LpA-I-like particles by the method of Sokoloff and Rothblat (22).

Statistical analysis

Statistical analyses were performed with SigmaPlot statistical software (Jandel Corp., San Rafael, CA). Data are expressed as means ± SD. Student's *t*-test was used for comparisons between groups.

RESULTS

Previous studies by Swaney and colleagues (15) and Tall et al. (23) have documented that enrichment of human serum with phospholipid promotes the formation of new HDL-like complexes. To further investigate the effect of DMPC treatment on the redistribution of apoA-I within HDL subpopulations, the relative concentrations of apoA-I-containing HDL subpopulations were determined by 2D-PAGGE, as described previously by Asztalos and colleagues (24). Plasma samples from three normolipidemic subjects (Table 1) treated or not with DMPC-MLV (2 mg/ml plasma) were separated by 2D-PAGGE, and different HDL subpopulations were quantified by densitometric scanning of radiographic films used to detect the presence of apoA-I associated with HDL subfractions. As shown in **Fig. 1A**, DMPC treatment of plasma significantly increased the concentrations of preβ$_1$-LpA-I (+19%; $P < 0.001$), whereas the concentrations of the small LpA-I α$_3$ particles were decreased significantly (−22%; $P < 0.001$). At the same time, no significant changes were observed in α$_1$ (−4%), α$_2$ (+7%), or preβ$_2$-LpA-I concentrations after DMPC treatment. Interestingly, after treatment of plasma with DMPC, there was a marked shift of α-migrating HDL subpopulations toward preβ mobility. Furthermore, incubation of plasma from a normolipidemic subject (Table 1, control 1) with DMPC generated preβ$_1$-LpA-I particles in a dose-dependent manner, as determined by densitometric scanning of radiographic films used to detect the presence of apoA-I associated with preβ$_1$-LpA-I separated by 2D-PAGGE (Fig. 1B, upper panel). The newly formed preβ-HDL complexes have size and charge similar to those of native plasma preβ$_1$-LpA-I (designated preβ$_1$-LpA-I-like particles) (Fig. 1B, lower panel). In separate experiments, we demonstrate that incubation of plasma with either POPC-MLV or BBSM-MLV did not significantly affect plasma preβ$_1$-LpA-I levels. Analysis of isolated preβ$_1$-LpA-I and preβ$_1$-LpA-I-like particles by silver-stained SDS-PAGE showed a single band in the apoA-I region (28 kDa). Furthermore,

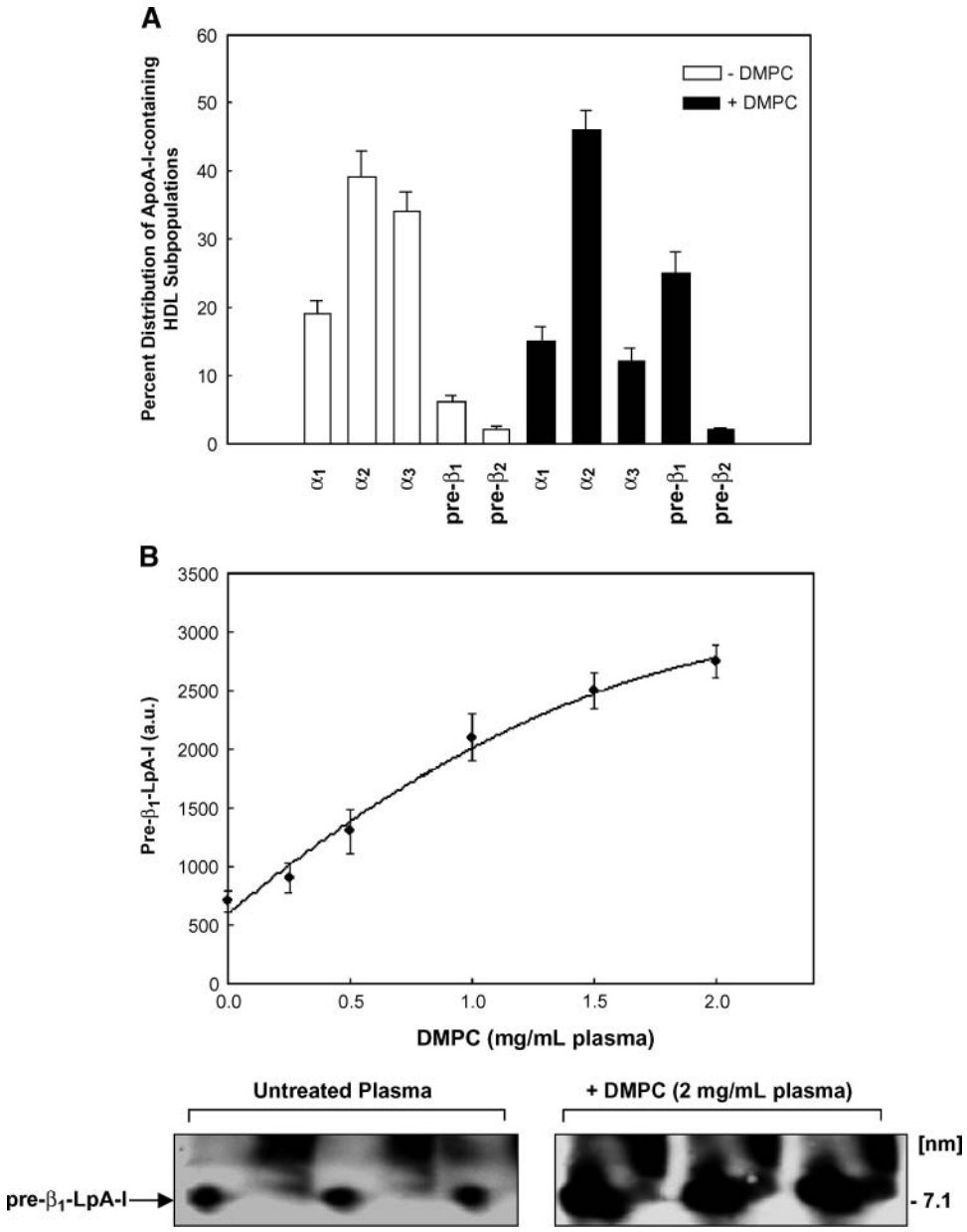
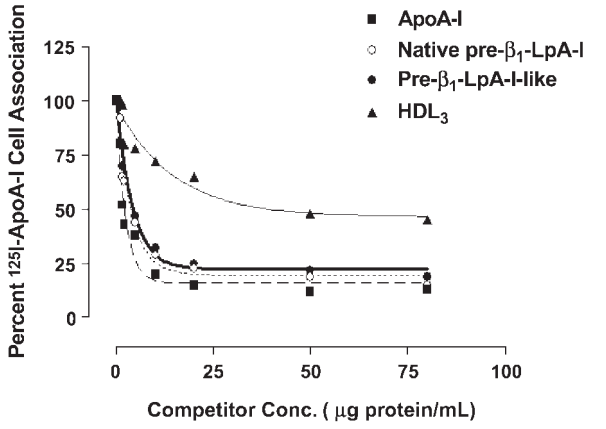


Fig. 1. Effect of dimyristoyl phosphatidylcholine (DMPC) treatment on the distribution of apolipoprotein A-I (apoA-I) in HDL subpopulations. A: One milliliter of plasma from each of three normolipidemic subjects was preincubated or not for 1 h with 2 mg DMPC-multilamellar vesicle (MLV)/ml plasma at 24°C in the presence of 2 mM DTNB to inhibit LCAT, and then plasma samples were separated by two-dimensional polyacrylamide nondenaturing gradient gel electrophoresis (2D-PAGGE). Different HDL subpopulations were quantified by densitometric scanning of radiographic films used to detect the presence of apoA-I associated with HDL subfractions. Means of three different control samples (±SD; n = 3) are shown. B: Upper panel, 1 ml of plasma from a normolipidemic subject (control 1) was preincubated for 1 h with increasing amounts of DMPC-MLV at 24°C in the presence of 2 mM DTNB to inhibit LCAT, and then plasma samples were separated by 2D-PAGGE. After electrophoresis, all samples were electrotransferred together on the same nitrocellulose membrane for appropriate comparison and were examined for apoA-I. ApoA-I associated with preβ_1-preβ-apoA-I-containing lipoproteins was quantitated by densitometric scanning. Results of triplicate measures (means ± SD) are shown. Lower panel, plasma treated or not with 2 mg DMPC-MLV/ml plasma as described above was separated by 2D-PAGGE, and apoA-I associated with preβ_1-LpA-I was detected by human anti-apoA-I antibody. Results from one sample run in triplicate are shown.

cross-linking with dithiobis(succinimidylpropionate)DSP of isolated native preβ_1-LpA-I and preβ_1-LpA-I-like particles showed that both of these particles had one apoA-I molecule per particle (data not shown). On the other hand, the total phospholipid-to-apoA-I molar ratios of isolated native preβ_1-LpA-I and preβ_1-LpA-I-like particles varied with each preparation but clearly demonstrated the presence of several molecules of phospholipid in both of these particles.

Native preβ$_1$-LpA-I and preβ$_1$-LpA-I-like particles contained approximately three and six molecules of phospholipid per molecule of apoA-I, respectively. However, both of these particles contained no detectable or background amounts of cholesterol, as assayed by enzymatic methods.

Having determined that a significant proportion of apoA-I-containing particles were found as preβ$_1$-LpA-I-like particles in DMPC-treated plasma, the question was raised whether these newly formed particles interact with the ABCA1 transporter. Competition assays were performed to determine the ability of isolated preβ$_1$-LpA-I-like particles, as well as native preβ$_1$-LpA-I and spherical HDL particles (HDL$_3$), to compete for the binding of ^{125}I-apoA-I to normal fibroblasts in which ABCA1 was induced with 22(R)-hydroxycholesterol and 9-cis-retinoic acid. As shown in **Fig. 2**, isolated preβ$_1$-LpA-I-like particles inhibited the binding of ^{125}I-apoA-I to ABCA1 more efficiently than HDL$_3$ (IC_{50} = 2.20 ± 0.35 vs. 37.60 ± 4.78 μg/ml, respectively), whereas lipid-free apoA-I was found to have a 1.5-fold greater capacity to bind ABCA1 compared with preβ$_1$-LpA-I- like particles (IC_{50} = 1.37 ± 0.48 vs. 2.20 ± 0.35 μg/ml, respectively). At the same time, no significant differences of binding to ABCA1 were observed between isolated native preβ$_1$-LpA-I and preβ$_1$-LpA-I- like particles (IC_{50} = 1.85 ± 0.50 vs. 2.20 ± 0.35 μg/ml, respectively).



Competitor	IC_{50} (μg protein/mL)
ApoA-I	1.37 ± 0.48
Native pre-β$_1$-LpA-I	1.85 ± 0.50
Pre-β$_1$-LpA-I-like	2.20 ± 0.35
HDL$_3$	37.60 ± 4.78

Fig. 2. Ability of preβ$_1$-apoA-I-containing lipoproteins (LpA-I)-like particles and native HDL$_3$ to interact with ABCA1. Fibroblasts were plated on 24-well plates and stimulated with 22OH/9CRA for 20 h. Cells were then incubated with 1.5 μg/ml ^{125}I-apoA-I for 2 h at 37°C with increasing amounts of either native preβ$_1$-LpA-I, preβ$_1$-LpA-I-like particles, native HDL$_3$, or unlabeled apoA-I (0, 1, 1.5, 2, 5, 10, 20, 50, and 80 μg protein/ml). Cells were then washed rapidly three times with ice-cold PBS/BSA and then with PBS alone. ^{125}I-apoA-I associated with cells was determined as described in Materials and Methods. The values shown represent means of duplicate wells. Similar results were obtained in three independent experiments. The IC_{50} values shown were determined using GraphPad Prism 4.00 software.

Control experiments were conducted to examine whether the apparent decrease in cell binding of the labeled apoA-I may be attributable to the ^{125}I-apoA-I binding to different competitor particles instead of the cells, as described in Materials and Methods. No significant amount of ^{125}I-apoA-I was found associated with either preβ$_1$-LpA-I-like particles or HDL$_3$, supporting the results shown in Fig. 2. Furthermore, we have previously documented that the specific binding of ^{125}I-apoA-I to unstimulated fibroblasts was very low and totally absent in ABCA1 mutant (Q597R) fibroblasts (18).

Because native plasma preβ$_1$-LpA-I has been proposed to be the first acceptor of cellular cholesterol (9), the question was raised whether the newly formed preβ$_1$-LpA-I-like particles affect the cholesterol efflux properties of plasma and to what extent this effect is mediated by the ABCA1 transporter. After treatment of normolipidemic plasma (Table 1, control 2) with increasing amounts of DMPC, as described in Materials and Methods, plasma was depleted of apoB-containing lipoproteins with PEG 6000 precipitation and then dialyzed. ApoB-depleted plasma samples (20 μg of apoA-I) were incubated for 4 h with either [^{3}H]choline- or [^{3}H]cholesterol-labeled J774 macrophages stimulated or not with 0.3 mM cAMP. The current cell culture model has been used by many investigators to examine ABCA1-mediated lipid efflux. Indeed, it was documented that under basal conditions, J774 macrophages express low levels of ABCA1 and scavenger receptor class B type I (SR-BI) and release membrane cholesterol to extracellular acceptors by passive diffusion, whereas stimulation with cAMP upregulates ABCA1-mediated cholesterol efflux (25). As shown in **Fig. 3**, by varying the ratio of DMPC to plasma, we found that DMPC can increase the ability of plasma to promote both phospholipid and free cholesterol efflux in either stimulated or unstimulated cells in a dose-dependent manner. At 2 mg DMPC/ml plasma (saturating DMPC concentration), phospholipid efflux to DMPC-treated plasma from cAMP-stimulated cells was increased by ~50% compared with unstimulated cells (9.60 ± 0.25% vs. 6.30 ± 0.10%, respectively; $P < 0.001$). Similarly, free cholesterol efflux to DMPC-treated plasma was increased significantly compared with unstimulated cells (12.30 ± 0.20% vs. 8.70 ± 0.15%, respectively; $P < 0.001$). Similarly, both phospholipid and free cholesterol efflux from stimulated cells to either isolated native preβ$_1$-LpA-I or preβ$_1$-LpA-I-like particles was increased (+48% and +45%, respectively) compared with unstimulated cells (**Fig. 4**). In separate experiments, we show that free cholesterol efflux to DMPC-MLV alone (2 mg/ml RPMI) represents <1% of free cholesterol efflux to DMPC-treated plasma (2 mg/ml plasma). Furthermore, there were no significant differences in free cholesterol efflux to DMPC-MLV alone between cAMP-stimulated and unstimulated cells (data not shown).

To demonstrate further that preβ$_1$-LpA-I-like particles present in DMPC-treated plasma were responsible for the ABCA1-mediated efflux, we examined the ability of either DMPC-treated or untreated plasma to promote phospholipid and free cholesterol efflux from cAMP-stimulated

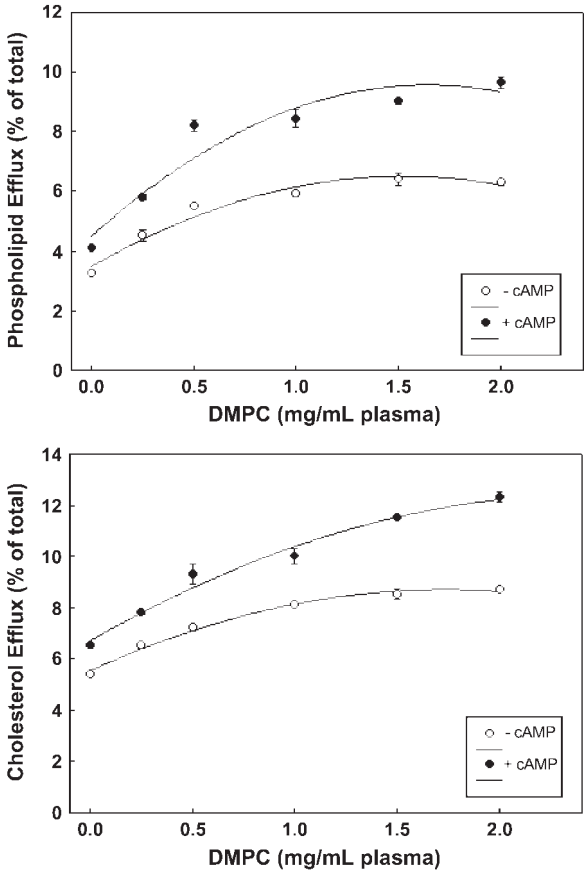


Fig. 3. Concentration dependence of phospholipid and cholesterol efflux from J774 to DMPC-treated plasma. One milliliter of plasma from a normolipidemic subject (control 2) was preincubated for 1 h with increasing amounts of DMPC-MLV at 24°C in the presence of 2 mM DTNB to inhibit LCAT. Plasma was depleted of apoB-containing lipoproteins by PEG 6000 and then dialyzed. ApoB-depleted plasma samples (20 μg of apoA-I) were incubated for 4 h with either [^{3}H]choline- or [^{3}H]cholesterol-labeled J774 cells stimulated or not with 0.3 mM 8-Br-cAMP. Phospholipid and cholesterol efflux were determined as percentages of total (media plus cells) ^{3}H measured in the medium and represent means ± SD from triplicate wells.

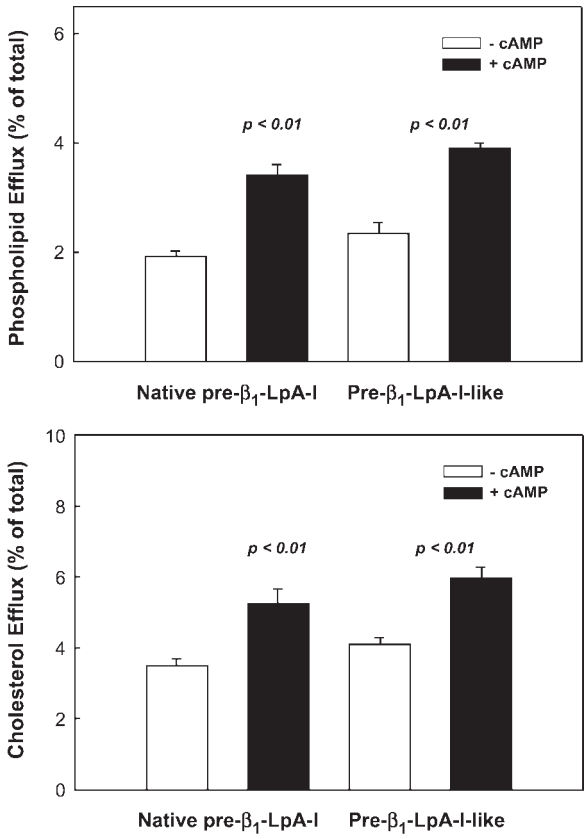


Fig. 4. Phospholipid and cholesterol efflux from J774 macrophages to either isolated native preβ$_1$-apoA-I-containing lipoproteins (LpA-I) or preβ$_1$-LpA-I-like particles. J774 cells were labeled with [^{3}H]choline chloride or [^{3}H]cholesterol and stimulated or not with 0.3 mM 8-Br-cAMP as described in Materials and Methods. Isolated native preβ$_1$-LpA-I or preβ$_1$-LpA-I-like particles (15 μg) were incubated for 8 h with J774 cells. Phospholipid and cholesterol efflux were determined as percentages of total (media plus cells) ^{3}H measured in the medium and represent means ± SD from triplicate wells.

J774 cells in the absence or presence of glyburide (300 μM), an inhibitor of ABCA1 activity. Phospholipid efflux from stimulated cells to DMPC-treated plasma was reduced by ~60% (9.3 ± 0.60% vs. 4.10 ± 0.45%), whereas free cholesterol efflux was reduced by ~45% (12.60 ± 0.60% vs. 7.10 ± 0.30%). In contrast, phospholipid efflux to untreated plasma was reduced by only ~16% (3.90 ± 0.2% vs. 3.25 ± 0.35%), whereas free cholesterol efflux was reduced by ~12% (5.8 ± 0.3% vs. 5.1 ± 0.25%), in the presence of glyburide. Furthermore, treatment of J774 cells with glyburide (300 μM) for 1–12 h did not causes any significant morphological changes or toxicity as measured by trypan blue exclusion.

To verify the specificity of apoA-I-containing particles in the modification of cholesterol efflux properties of DMPC-treated plasma, plasma from a normolipidemic subject (Table 1, control 3) was depleted from apoA-I-containing lipoproteins by immunoaffinity, as described in Materials and Methods, and then treated with DMPC before cholesterol efflux experiments. As shown in **Fig. 5**, removal of apoA-I-containing lipoproteins from plasma drastically reduced free cholesterol efflux mediated by DMPC-treated plasma. To further confirm the role of apoA-I-containing particles in the increased ability of DMPC-treated plasma to promote free cholesterol efflux, plasma from either three patients with TD or three controls (Table 1) was treated or not with 2 mg DMPC/ml plasma, and samples were separated by 2D-PAGGE. Preβ$_1$-LpA-I particles were quantified as described above. As shown in **Fig. 6** (upper panel), despite the presence of a significant amount of preβ$_1$-LpA-I in untreated TD plasma (<50% of that in controls), DMPC treatment did not affect preβ$_1$-LpA-I levels compared with those in controls. Similarly, DMPC treatment of TD plasma did not promote additional cholesterol efflux from cAMP-stimulated cells (Fig. 6, lower panel).

DISCUSSION

A growing body of evidence indicates that lipid-poor apoA-I not only plays a major role in the RCT process but

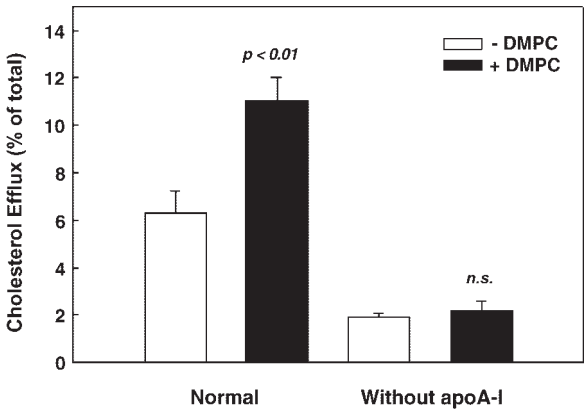


Fig. 5. Effect of the removal of plasma apoA-I-containing lipoproteins on DMPC-mediated [^{3}H]cholesterol efflux from cAMP-stimulated J774 cells. Plasma from a normolipidemic subject (control 2) was depleted or not from apoA-I-containing lipoproteins by immunopurified anti-apoA-I antibody (12171-21A; Genzyme Corp)-coupled Sepharose column as described in Materials and Methods. Either normal plasma or apoA-I-depleted plasma samples were preincubated or not with 2 mg of DMPC-MLV at 24°C for 1 h in the presence of 2 mM DTNB to inhibit LCAT. Different plasma samples were depleted of apoB-containing lipoproteins by PEG 6000 and then dialyzed. ApoB-depleted plasma samples (100 μl) were incubated for 4 h with [^{3}H]cholesterol-labeled J774 cells stimulated with 0.3 mM 8-Br-cAMP. Cholesterol efflux was determined as a percentage of total (media plus cells) ^{3}H cholesterol measured in the medium and represents means ± SD from triplicate wells.

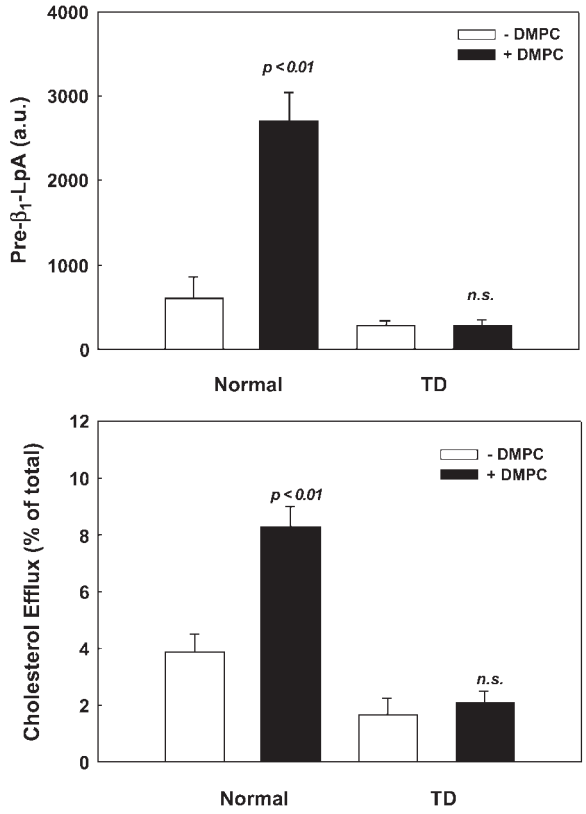


Fig. 6. Effect of preincubation of plasma from TD patients with DMPC-MLV on preβ$_1$-apoA-I-containing lipoproteins (LpA-I) level and [^{3}H]cholesterol efflux from cAMP-stimulated J774 cells. Plasma from each of three normolipidemic subjects and three TD subjects was preincubated or not for 1 h with 2 mg DMPC-MLV/ml plasma at 24°C in the presence of 2 mM DTNB to inhibit LCAT, and then plasma samples were separated by 2D-PAGGE. After electrophoresis, all samples were electrotransferred together on the same nitrocellulose membrane for appropriate comparison and were examined for apoA-I. ApoA-I associated with preβ$_1$-LpA-I was quantitated by densitometric scanning. Results of triplicate measures (means ± SD, n = 3) are shown. ApoB-depleted plasma samples (150 μl) from TD and control subjects were incubated for 4 h with [^{3}H]cholesterol-labeled J774 cells stimulated with 0.3 mM 8-Br-cAMP. Cholesterol efflux was determined as a percentage of total (media plus cells) ^{3}H cholesterol measured in the medium. Results of triplicate measures (means ± SD, n = 3) are shown.

has also emerged as a potential target for therapies designed to inhibit the development of atherosclerotic vascular disease (26). However, whether lipid-poor apoA-I, such as plasma preβ$_1$-LpA-I, has biological properties that make it even more effective than mature α-HDL in preventing or retarding atherosclerosis is currently a topic of many investigations. In the present report, we take advantage of a previous study by Swaney and colleagues (15) demonstrating that enrichment of human serum with phospholipid led to the formation of new HDL complexes, especially preβ-HDL. This allows us to further investigate the affinity of these newly formed particles for ABCA1 and their ability to promote cholesterol efflux.

Although the structural characteristics of DMPC-modified HDL have not been determined, the present study shows that at 2 mg DMPC/ml plasma, a significant proportion of α$_3$ particles were transformed to preβ$_1$-LpA-I-like particles having size and charge similar to those of native plasma preβ$_1$-LpA-I (Fig. 1B). Interestingly, α$_3$ particles contain both apoA-I and apoA-II; although other HDL subpopulations contain apoA-I but not apoA-II (27), it is possible that apoA-II facilitates the dissociation of lipid-poor apoA-I from α$_3$ particles after DMPC treatment. Indeed, previous studies established that the lipid compositions of preβ$_1$-LpA-I species as well as the conformations of apoA-I within these particles differ from those of spherical HDL (28, 29). Furthermore, preβ$_1$-LpA-I is proposed to be an initial acceptor of cell-derived cholesterol, consistent with the idea that lipid-poor apoA-I interacts preferentially with the ABCA1 transporter (18, 21). This concept is supported by our results showing that both isolated native preβ$_1$-LpA-I and preβ$_1$-LpA-I-like particles have an ~17-fold greater capacity to bind ABCA1 compared with HDL$_3$ (Fig. 2). This is consistent with our previous results showing that the association of either lipid-free apoA-I or apoE3 with lipid decreases their affinity for ABCA1 (18, 20).

Previous studies have documented that short-term efflux to plasma (1–5 min) shows that ~50% of the cholesterol released from cells is associated with preβ-HDL, even though it contains only 5–10% of total plasma apoA-I (14). We initially hypothesized that any specific increase in preβ$_1$-LpA-I-like particles after treatment of plasma with DMPC would be associated with a significant increase in the ability of DMPC-treated plasma to promote cholesterol efflux through the ABCA1 transporter pathway. Our hypothesis is supported by the following findings: *i*) DMPC-

treated plasma stimulated greater phospholipid and free cholesterol efflux from cAMP-stimulated cells compared with unstimulated cells (Fig. 3); *ii*) phospholipid and free cholesterol efflux from stimulated cells to isolated native preβ$_1$-LpA-I and preβ$_1$-LpA-I-like particles was increased by ~50% compared with that of unstimulated cells; and *iii*) glyburide strongly inhibited both phospholipid and free cholesterol efflux to DMPC-treated plasma compared with untreated plasma in cAMP-stimulated cells. Based on these results, we have assumed that preβ$_1$-LpA-I-like particles contribute significantly to the release of cholesterol through the ABCA1 pathway in our cAMP-stimulated macrophage cell culture model. However, it should be emphasized that in this system, other cholesterol efflux pathways, such as the passive diffusion pathway or SR-BI, may operate, especially when plasma is used as an acceptor. This is in agreement with a recent study by Favari and colleagues (25) showing that the magnitude of the ABCA1-dependent cholesterol efflux could be modified by increasing the content of preβ-HDL (by phospholipid transfer protein treatment) or by reducing it (by chymase treatment).

Although evidence has been presented demonstrating that preβ$_1$-LpA-I-like particles interact physically with ABCA1 and promote cholesterol efflux through the translocase activity of the same transporter, it remains unclear whether other apolipoproteins known to interact with ABCA1, such as apoE, may be involved in the modification of the cholesterol efflux properties of DMPC-treated plasma. Indeed, it has been proposed that γ-LpE particles play a role in RCT by acting together with preβ$_1$-LpA-I as initial acceptors of cell-derived cholesterol (30). Furthermore, we have documented previously that the ABCA1 transporter mediates the lipidation of lipid-free apoE3 in a cell culture model (20). Interestingly, we observed that DMPC enrichment of plasma increased the level of plasma γ-LpE in a dose-dependent manner (data not shown). It is possible that γ-LpE particles also contribute to the ability of DMPC-treated plasma to promote cholesterol efflux. However, despite an increase in the concentration of γ-LpE after DMPC enrichment of TD plasma, no significant cholesterol release from cAMP-stimulated cells was observed (Fig. 6). It is likely that the HDL-LpE present in plasma of TD subjects, as we have documented previously (16), was transformed to γ-LpE after DMPC enrichment, whereas the absence of a DMPC effect on preβ$_1$-LpA-I is attributable to the total absence of α-migrating apoA-I-containing particles in TD plasma (16, 31). This is consistent with our result showing that removal of apoA-I-containing lipoproteins from normal plasma abolished free cholesterol efflux mediated by DMPC-treated plasma (Fig. 5). These conclusions support the specificity of apoA-I-containing particles in the modification of the cholesterol efflux properties of DMPC-treated plasma.

Earlier studies by Tall and colleagues (23) have documented that incubation of isolated HDL with a mixture of lecithin unilamellar or multilamellar liposomes transforms both HDL_2 and HDL_3 into lipoproteins of decreased density and increased size. Furthermore, the formation of discoidal phospholipid/apoprotein complexes was identified by electron microscopy. It is likely that discoidal lipoproteins were formed as a result of the interactions of liposomes with apoproteins released from HDL. This is consistent with our results showing that both preβ$_1$-LpA-I and γ-LpE particles were increased by DMPC enrichment of plasma.

Although the origin of plasma preβ$_1$-LpA-I has remained enigmatic and its role in preventing atherosclerosis is considered uncertain at this time (28), higher preβ$_1$-LpA-I levels have been observed in coronary heart disease (32). However, a recent study by Asztalos and colleagues (33) documented that preβ$_1$-LpA-I level was not significantly associated with coronary heart disease prevalence, whereas α$_3$ and preα$_1$ particle levels had a positive association with coronary heart disease. Indeed, it was reported by Rothblat and colleagues (34) that preβ$_1$-LpA-I level correlates positively with ABCA1-mediated and inversely with SR-BI-mediated cell cholesterol efflux. Furthermore, it has been shown that intravenous injection of apoA-I/phosphatidylcholine discs into humans increases plasma preβ$_1$-LpA-I concentration, which can stimulate RCT (35). This is supported by a recent study by Nissen and colleagues (26) demonstrating that recombinant apoA-I$_{Milano}$/phospholipid complex produced a regression of coronary atherosclerosis as measured by intravascular ultrasound.

The results of the present report appear reasonably consistent with the concept that preβ$_1$-LpA-I particles or lipid-poor apoA-I are the preferred substrate for the ABCA1 transporter in vitro. Importantly, the structural alterations of plasma HDL by DMPC resulted in the redistribution of plasma apoA-I in favor of the lipid-poor apoA-I pool, allowing for more efficient ABCA1-mediated cholesterol release. Although this observation may have potential therapeutic implications, it remains an unanswered question whether lipid-poor apoA-I such as preβ$_1$-LpA-I has specific properties that result in greater antiatherogenic potential than plasma α-HDL.

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