BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2009,** *48,* 11067–11074 11067
DOI: 10.1021/bi901564g

Small Discoidal Pre-β1 HDL Particles Are Efficient Acceptors of Cell Cholesterol via ABCA1 and ABCG1[†]

Elda Favari,[‡,⊥] Laura Calabresi,[§,⊥] Maria Pia Adorni,[‡] Wendy Jessup,[∥] Sara Simonelli,[§] Guido Franceschini,[§] and Franco Bernini*[,‡]

[‡]*Department of Pharmacological and Biological Sciences, and Applied Chemistries, University of Parma, Parma, Italy,* [§]*Center E. Grossi Paoletti, Department of Pharmacological Sciences, Università degli Studi di Milano, Milano, Italy, and* [∥]*Centre for Vascular Research, University of New South Wales, Sydney, Australia* [⊥]*These authors equally contributed to this work.*

Received September 8, 2009; Revised Manuscript Received October 14, 2009

ABSTRACT: The aim of this study was to correlate the lipid content and size of discoidal reconstituted HDL particles with their ability to promote cellular cholesterol efflux. Homogeneous discoidal rHDL particles containing apoA-I and POPC, with diameters of 7.8, 9.6, 10.8, 12.5, and 17.0 nm, were prepared by the cholate dialysis technique. Cholesterol efflux to rHDL was evaluated in pathway-specific cell models for ABCA1-, ABCG1-, and SR-BI-mediated efflux. ABCA1-mediated efflux was efficiently promoted by the 7.8 nm rHDL containing 82 POPC molecules per particle. This rHDL also promoted ABCG1, but not SR-BI, cholesterol efflux. All large and lipid-rich rHDLs, with a diameter of ≥9.6 nm and a phospholipid content of ≥202 molecules per particle, promoted both SR-BI- and ABCG1-mediated efflux. Our results indicated that the ABCA1-mediated cell cholesterol efflux can be efficiently driven not only by monomolecular lipid free/poor apoA-I but also by a small discoidal phospholipid-containing particle resembling plasma pre-β1 HDL. This same particle also promotes ABCG1- but not SR-BI-mediated efflux. These results help to clarify the role of plasma pre-β1 HDL in reverse cholesterol transport.

It is widely believed that HDL particles mainly inhibit atherosclerosis by promoting reverse cholesterol transport (RCT),[1] a process by which cholesterol is removed from extrahepatic tissues and delivered to the liver for final excretion into the bile (*1*). The first step in the RCT pathway is the efflux of phospholipids and free cholesterol (FC) from cell membranes to an extracellular acceptor (*2*). The process of efflux of FC from cells occurs by at least three major processes: (1) aqueous diffusion, a process that involves the desorption of FC molecules from the donor lipid−water interface and their diffusion through the aqueous phase until they collide with, and are absorbed by, an acceptor; (2) scavenger receptor class B type I (SR-BI)-mediated FC flux, a bidirectional facilitated transport of FC that, like the aqueous diffusion mechanism, depends on cholesterol concentration gradients (*3*); and (3) ATP binding cassette-mediated cholesterol efflux. ABCA1 and ABCG1 are members of a large family of ATP-dependent transporters that share common structural motifs for the active transport of a variety of substrates (*4*). In contrast to aqueous diffusion and SR-BI-mediated FC flux, the movement of FC by ABCA1 and ABCG1 is unidirectional, and net efflux of cellular FC would always occur via these mechanisms (*5*).

HDL represents a heterogeneous family of particles differing in density, size, composition, and surface charge. It is generally accepted that HDL subpopulations may also differ in their ability to activate a specific cellular lipid efflux process (*6*, *7*). In terms of the ability to promote efflux of cholesterol through the different pathways, it has been proposed that HDL, despite the great heterogeneity, can be subdivided into two major functional classes: the lipid-free/poor apoA-I forms that uniquely interact with ABCA1 and the lipid-rich particles that interact with SR-BI and ABCG1, but not with ABCA1. We recently demonstrated that chymase treatment of HDL impairs the ABCA1-dependent pathway without influencing either aqueous or SR-BI-facilitated diffusion and that this effect is caused by depletion of lipid-poor pre-β-migrating particles in HDL. In agreement with this result, Rothblat and colleagues showed that small lipid-poor pre-β1 HDL had the strongest association with ABCA1-mediated cholesterol efflux, while efflux via the SR-BI pathway was associated with large, phospholipid-rich HDL subpopulations (*8*).

The objective of this study was to correlate the lipid content and size of HDL with their ability to specifically promote cholesterol efflux mediated by ABCA1, SR-BI, and ABCG1 proteins. To accomplish this, we used reconstituted HDL (rHDL), made with apoA-I and synthetic phospholipids. These particles resemble nascent HDL (*9*) and mimic most of the physiological properties of plasma HDL, thus providing a valuable tool for identifying structural requirements for some HDL functions (*10*, *11*).

[†]This work was supported by a grant from the Italian Ministry of University (PRIN 2005) to G.F. and F.B. and by grants from Istituto Nazionale per le Ricerche Cardiovascolari (INRC) to F.B.

*To whom correspondence should be addressed: Department of Pharmacological and Biological Sciences, and Applied Chemistries, Viale Usberti 27/A, 43100 Parma, Italy. Phone: +39-0521905039. Fax: +390521905040. E-mail: fbernini@unipr.it.

[1]Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; FC, free cholesterol; POPC, palmitoyloleoylphosphatidylcholine; rHDL, reconstituted HDL; RCT, reverse cholesterol transport; SD, standard deviation; SR-BI, scavenger receptor BI; TD, Tangier disease.

Published on Web 10/19/2009

EXPERIMENTAL PROCEDURES

Materials. Fetal calf serum (FCS), bovine serum albumin (BSA), and 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (cpt-cAMP) were purchased from Sigma. BLT-1 (small molecules that block lipid transport-1) (*12*) was purchased from ChemBridge (San Diego, CA). Organic solvents were purchased from Merck (Darmstadt, Germany). [1,2-^{3}H]Cholesterol was from Amersham Bioscences (Uppsala, Sweden). Tissue culture flasks and plates were from Corning (Corning, NY) and Falcon (Lincoln, NY). Dulbecco's minimal essential medium (DMEM), RPMI 1640, Ham's F12, and phosphate-buffered saline (PBS) were purchased from BioWhitaker (Walkersville, MD). The acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, Sandoz 58-035, was a gift from Novartis (Basel, Switzerland).

Preparation of rHDL. apoA-I was purified from human plasma (*13*). Homogeneous discoidal rHDLs containing apoA-I and POPC, with a diameter of 7.8, 9.6, 10.8, 12.5, and 17.0 nm, were prepared by the cholate dialysis technique, as previously described (*13*, *14*). Initial POPC:apolipoprotein molar ratios of 80:1 and 120:1 were used (*14*). After removal of the sodium cholate by extensive dialysis, the 9.6, 12.5, and 17.0 nm particles were isolated from the reaction mixture containing a 120:1 POPC:apoA-I ratio by gel filtration on three serial Superose 6B columns. The reaction mixture containing an 80:1 POPC:apoA-I ratio, after removal of sodium cholate, was incubated with LDL for 24 h at 37 °C, treatments that lead to the formation of two limiting particles of 7.8 and 10.8 nm (*13*). These two particles were then separated by gel filtration on the Superose columns. Three different preparations of particles were used for the efflux experiments.

Characterization of rHDL. The size of the homogeneous particles was estimated by nondenaturing gradient gel electrophoresis (GGE) (*13*), using precast 4 to 30% gradient gels. The number of apolipoprotein molecules per rHDL particle was determined by cross-linking with dimethyl suberimidate (DMS) (*13*). The phospholipid content of rHDL was determined by an enzymatic method (*15*). Proteins were measured by the method of Lowry et al. (*16*), using bovine serum albumin as the standard. The α-helical content was calculated as previously described (*13*). rHDLs incubated with cells were separated by GGE followed by electroblotting and detection with a sheep anti-human apoA-I antibody.

Cells. J774 mouse macrophages were cultured in RPMI-1640 supplemented with 10% FCS. Fu5AH rat hepatoma cells were grown in DMEM with 10% FCS. Human fibroblasts were grown in DMEM supplemented with glutamine, nonessential amino acids, sodium pyruvate, and 10% FCS. CHO-K1 cells (ATCC) were used for stable transfection of hABCG1 (*17*). All the culture media were supplemented with 50 μg/mL gentamicin.

cpt-cAMP, 22-Hydroxycholesterol, cis-9-Retinoic acid, Probucol, and BLT-1 Solutions. cpt-cAMP was dissolved in distilled water at 30 mM and stored at −20 °C. 22-OH and 9cRA were dissolved in DMSO at 2 mg/mL and 4 mM, respectively, and stored at −20 °C. BLT-1 was purchased from ChemBridge (San Diego, CA) and probucol from Sigma-Aldrich. Both were dissolved in 100% DMSO to form a 10 mM stock solution. These stock solutions were diluted to 1 μM using 0.2% BSA DMEM for BLT-1 and to 20 μM using 0.2% BSA RPMI1640 for probucol. Control solutions consisted of 0.2% BSA in medium. All solutions contained the same amount of DMSO.

Measurement of ABCA1-Mediated Cell Cholesterol Efflux. ABCA1-mediated cholesterol efflux was assessed using control J774 macrophages and J774 macrophages treated with cpt-cAMP to upregulate ABCA1 (*2*). Human normal and Tangier fibroblasts (*18*) were also studied. Cells were grown in medium with 10% FCS, incubated at 37 °C with 5% CO_2, seeded in 24-well plates, and used when they reached 80−90% confluence. Monolayers were washed with PBS and incubated for 24 h in medium containing [^{3}H]cholesterol (2 μCi/mL). The labeling medium contained 1% FCS and an ACAT inhibitor (Sandoz 58-035) at 2 μg/mL. The ACAT inhibitor was added to ensure that all labeled cholesterol was present as free cholesterol. Following this 24 h labeling period, cells were washed and incubated with 0.2% BSA, with or without 0.3 mM cpt-cAMP (J774) or 5 μg/mL 22-OH and 10 μM 9cRA (human fibroblasts) for 18 h. Following this equilibration time, cells were incubated with medium alone or with probucol for 2 h. After this incubation, some wells were washed with PBS, dried, and extracted with 2-propanol; these cells provide baseline (time zero) values for total [^{3}H]cholesterol content. Following this equilibration time, stimulated and unstimulated cells were incubated for the specified time (0−24 h) in the presence of increasing concentrations of rHDL. Media were removed from the monolayers and filtered through a 0.45 μm filter to remove floating cells, and radioactivity in the supernatant was determined by liquid scintillation counting. Cholesterol efflux was calculated as (counts per minute in medium at 4 h/counts per minute at time zero) × 100. The ABCA1-mediated cholesterol efflux was determinaed to be the difference between the percentage efflux from stimulated cells and the percentage efflux from unstimulated cells.

Measurement of ABCG1-Mediated Cell Cholesterol Efflux. CHO-K1 cells (ATCC) stably expressing hABCG1 were generated as previously described (*17*). Parent and hABCG1-expressing cells were labeled for 24 h with [^{3}H]cholesterol, washed, and equilibrated for 90 min in serum-free medium prior to incubation in efflux medium containing BSA (1 mg/mL) in the presence of increasing concentrations of rHDL for an efflux time (6 h). Cell media were filtered through a 0.45 μm filter to remove floating cells, and monolayers were lysed in 0.1% (v/v) Triton X-100. Cells and media were assayed for radioactivity, and cholesterol efflux in medium samples was calculated as a percentage of total cholesterol in the culture (cells and medium). The ABCG1-mediated cholesterol efflux was then calculated as the difference between the percentage efflux from transfected cells and the percentage efflux from CHO-K1 parent cells.

Measurement of SR-BI-Mediated Cell Cholesterol Efflux. SR-BI-mediated cholesterol efflux was measured using Fu5AH rat hepatoma cells, a cell line that expresses high levels of SR-BI (*2*). Cells were seeded in 24-well plates and grown in DMEM medium with 10% FCS for 1 day and then labeled with 2 μCi/mL [^{3}H]cholesterol for 24 h in medium containing 1% FCS and an ACAT inhibitor (Sandoz 58-035) at 2 μg/mL. The ACAT inhibitor was added to ensure that all labeled cholesterol was present as free cholesterol. The remaining monolayers were then incubated for 18 h with 0.2% BSA. Following this equilibration time, cells were incubated with medium alone or with BLT-1 for 2 h. After this incubation, some wells were washed with PBS, dried, and extracted with 2-propanol; these cells provide baseline (time zero) values for total [^{3}H]cholesterol content. Cells were then washed with PBS and incubated in the presence of increasing concentrations of rHDL for 4 h. Cell media were filtered through a 0.45 μm filter to remove floating cells, and radioactivity in the

Table 1: Characteristics of apoA-I-Containing rHDL

particle	size (nm)	POPC:apoA-I[a] mass ratio (w/w)	POPC:apoA-I[a] mole ratio	no. of molecules/particle apoA-I	no. of molecules/particle POPC	% α-helix
7.8 nm apoA-I rHDL	7.8	1.1 ± 0.04:1	41 ± 4:1	2	82	61
9.6 nm apoA-I rHDL	9.6	2.8 ± 0.03:1	101 ± 1:1	2	202	70
10.8 nm apoA-I rHDL	10.8	2.2 ± 0.04:1	82 ± 2:1	3	243	62
12.5 nm apoA-I rHDL	12.5	3.5 ± 0.05:1	127 ± 2:1	3	381	71
17.0 nm apoA-I rHDL	17.0	4.8 ± 0.03:1	178 ± 1:1	4	712	70

[a]Data are expressed as means ± SD of three different preparations.

supernatant was determined by liquid scintillation counting. Cholesterol efflux was calculated as (counts per minute in medium at 4 h/counts per minute at time zero) × 100. The SR-BI-specific efflux was determined as the difference between the percentage efflux from untreated cells and the percentage efflux from cells incubated with BLT-1 (*19*).

Data Analysis. Statistical and kinetic analyses were performed using Prism (version 5.0) (GraphPad Inc., San Diego, CA). Results are expressed as mean ± the standard deviation of triplicate determinations. Significant differences were established by *t* test and defined as $P < 0.05$. In Figure 4, a one-way ANOVA was calculated with all points.

RESULTS

Characterization of rHDL. As previously shown by us and others (*13*, *14*, *20*), apoA-I can form discrete discoidal, pre-*β*-migrating rHDL particles (*21*), differing in size and in apoA-I and phospholipid content. For this study, we prepared five pure homogeneous apoA-I-containing rHDL particles (Table 1 and Figure 1). Two (7.8 and 9.6 nm in diameter) contain two apoA-I molecules per particle but differ in size and POPC content; two others (10.8 and 12.5 nm) contain three apoA-I molecules per particle, again differing in POPC content, and one (17.0 nm) contains four apoA-I molecules per particle (Figure 1). The α-helix content of apoA-I was higher in the 9.6, 12.5, and 17.0 nm compared to the 7.8 and 10.8 nm particles (Table 1), consistent with the concept that apoA-I adopts distinct conformations in different sized particles to form rHDLs containing the same number of apoA-I molecules but with different sizes (*13*, *14*). No detectable lipid-free apoA-I was present in any of the rHDLs (Figure 1).

Efflux of Cholesterol to rHDL. We first evaluated the ability of the rHDL particles that differed in size and phospholipid content to promote efflux of cholesterol from J774 murine macrophages (Figure 2). Under basal conditions, J774 macrophages express a low level of ABCA1, ABCG1, and SR-BI and release membrane cholesterol to extracellular acceptors by passive diffusion (*19*, *22*), whereas stimulation with cAMP upregulates ABCA1 protein and ABCA1-dependent efflux (*2*). The ABCA1-mediated cholesterol efflux was determined as the percentage efflux from stimulated cells minus the percentage efflux from unstimulated cells. As shown in Figure 2 (top and bottom panels), the smallest 7.8 nm particle was the only rHDL able to promote the ABCA1-mediated efflux in a dose-dependent manner, expressed either as a function of protein or as a function of phospholipid concentration in the medium. In the same experiment, probucol, a specific inhibitor of ABCA1-mediated efflux (*23*), was able to slow the release of cholesterol in cAMP-stimulated cells to either apoA-I (from 2.3 ± 0.15 to 1.2 ± 0.08, similar to that under basal conditions, 0.9 ± 0.06) or the 7.8 nm particle (from 4.1 ± 0.19 to 2.6 ± 0.38, similar to that under basal conditions, 2.0 ± 0.09) but had no effect on the efflux driven by the particles with a diameter of ≥9.6 nm. The same particles were tested for their ability to extract cell cholesterol through the ABCG1 pathway. This aim was achieved via comparison of efflux from stably transfected ABCG1-overexpressing CHO and control cells. Nontransfected cells do not express ABCG1 and release cholesterol essentially by passive diffusion. As shown in Figure 3 (top panel), all the rHDL particles were similarly efficient in promoting efflux of cholesterol from

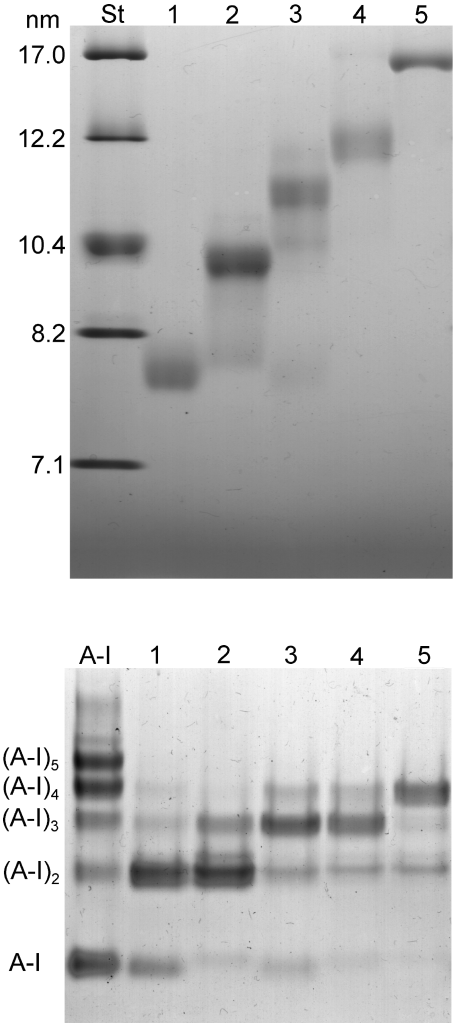

FIGURE 1: Nondenaturing GGE (top) and SDS−PAGE analysis after cross-linking with DMS (bottom) of homogeneous discoidal rHDLs containing apoA-I and POPC with a diameter of 7.8 (lane 1), 9.6 (lane 2), 10.8 (lane 3), 12.5 (lane 4), and 17.0 nm (lane 5). Lipid-free apoA-I (1.0 mg/mL) was cross-linked with DMS and loaded onto the SDS−PAGE gel.

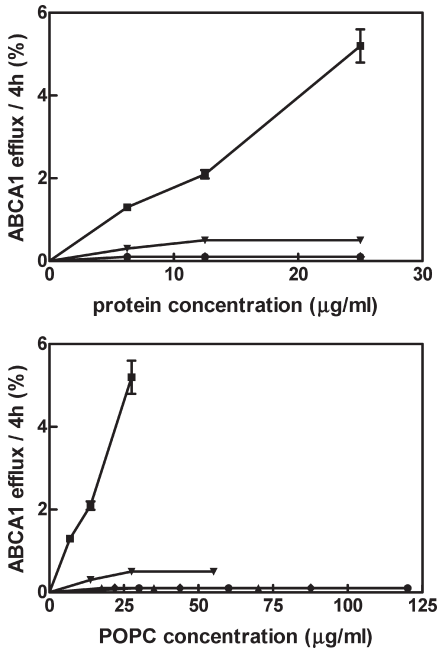

FIGURE 2: Efflux of cholesterol from J774 macrophages to rHDL. Cholesterol efflux is expressed either as a function of protein (top panel) or as a function of phospholipid (bottom panel) concentration in the medium. Macrophages were labeled with 2 μCi/mL [^{3}H]cholesterol for 24 h in RPMI medium with 1% FCS and an ACAT inhibitor (Sandoz 58-035) at 2 μg/mL. Cells were then cultured for 18 h with 0.2% BSA in the absence or presence of 0.3 mM cpt-cAMP, washed, and incubated for 4 h with different concentrations of rHDL: 7.8 (■), 9.6 (▲), 10.8 (▼), 12.5 (◆) and 17.0 nm (●). The ABCA1-mediated cholesterol efflux was determined as the difference between the percentage efflux from stimulated cells and the percentage efflux from unstimulated cells. Each sample was run in triplicate. Values are expressed as means ± SD.

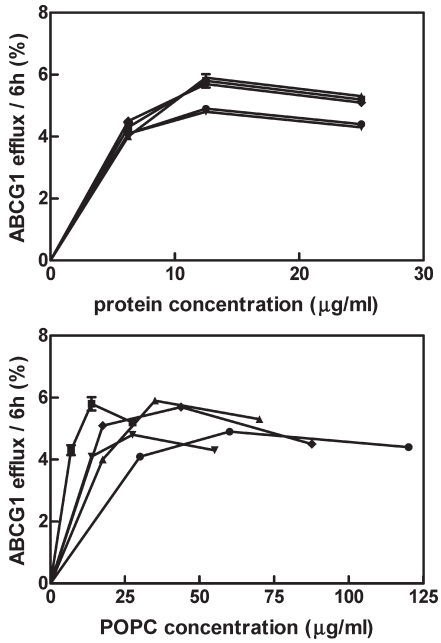

FIGURE 3: Efflux of cholesterol from CHO cells to rHDL. Cholesterol efflux is expressed either as a function of protein (top panel) and as a function of phospholipid (bottom panel) concentration in the medium. Wild-type and human ABCG1-overexpressing CHO cells were labeled with 1 μCi/mL [^{3}H]cholesterol for 24 h, washed, equilibrated for 90 min in serum-free medium, and incubated for 6 h with the indicated concentration of rHDL: 7.8 (■), 9.6 (▲), 10.8 (▼), 12.5 (◆), and 17.0 nm (●). Cells and media were assayed for radioactivity and cholesterol efflux in medium samples calculated as a percentage of total cholesterol in the culture. The ABCG1-mediated cholesterol efflux was then calculated as the difference between the percentage efflux from transfected cells and the percentage efflux from CHO-K1 parent cells. Each sample was run in triplicate. Values are expressed as means ± SD.

ABCG1-overexpressing CHO cells. Since the phospholipid content varies significantly among the different rHDL particles, the ABCG1-mediated cholesterol efflux was also expressed as a function of phospholipid concentration in the medium (Figure 3, bottom panel). The efflux values were comparable for all the tested rHDLs, indicating that all the particles with diameters from 7.8 to 17.0 nm and with 82−712 POPC molecules/particle are efficient acceptors with similar ability to promote ABCG1-dependent cholesterol efflux.

Next, rHDLs were tested for their capacity to promote efflux of cholesterol from Fu5AH hepatoma cells. In this cell model, the SR-BI protein is strongly expressed in the plasma membrane, and thus, the efflux of lipids depends on facilitated diffusion by this receptor. Cells were incubated with rHDLs of different sizes, either in the absence or in the presence of BLT-1, a specific inhibitor of SR-BI cholesterol efflux (*12*). The SR-BI-specific efflux was calculated as the difference between the percentage cholesterol efflux from BLT-1-untreated and treated cells (*24*). As shown in Figure 4 (top and bottom panels), the smallest particle with a diameter of 7.8 nm and 82 POPC molecules/particle was ineffective in promoting SR-BI-mediated cholesterol efflux. In contrast, the larger rHDL particles with a diameter from 9.6 to 17.0 nm and 202−712 POPC molecules/particle were efficient in promoting SR-BI efflux with no differences among the various particles (p = 0.7492 by ANOVA) despite the different phospholipid content.

ABCA1-Mediated Cholesterol Efflux to 7.8 nm rHDL. To confirm the capacity of the 7.8 nm particle to extract cholesterol via ABCA1, we performed efflux experiments on skin fibroblasts from a healthy individual and from a patient with Tangier disease (TD) that do not express ABCA1 (*18*). As shown in Table 2, induction of ABCA1 expression by incubation of cells with 22-OH/9-*cis*-retinoic acid enhanced the efflux of cholesterol to apoA-I and to the 7.8 nm particle in normal cells, but not in TD fibroblasts. We further characterized the ABCA1-mediated cholesterol efflux capacity of the 7.8 nm particle in comparison with lipid-free apoA-I using J774 cells (*2*). First, we compared the efficiency of the 7.8 nm rHDL particle and of lipid-free apoA-I in promoting ABCA1-mediated cell cholesterol efflux by incubating the cells with increasing concentrations of the acceptors (Figure 5, top panel). In control cells, the 7.8 nm particle demonstrated a greater efflux capacity than lipid-free apoA-I, justified by the ability of the particles to extract cholesterol by passive diffusion. In cAMP-stimulated J774 cells, where ABCA1 is upregulated, the 7.8 nm particle exhibited a dose-dependent ability to promote cholesterol efflux (Figure 5, bottom panel). The data in Figure 5 (bottom panel) were analyzed using the Michaelis−Menten equation to provide K_m and V_{max} values and correlated catalytic efficiency (V_{max}/K_m). V_{max} was 10.27 and 6.27% FC efflux/4 h for apoA-I and the 7.8 nm particle, respectively. K_m values were 3.06 μg/mL for apoA-I and 17.31 μg/mL for the 7.8 nm particle. The catalytic efficiency (V_{max}/K_m) of FC efflux was 2.82% FC mL (4 h)$^{-1}$ μg^{-1} for apoA-I and 0.36% FC mL (4 h)$^{-1}$ μg^{-1} for the 7.8 nm particle. These data indicate that the 7.8 nm particle promotes ABCA1-mediated cholesterol efflux via a high-affinity process, even if less efficiently than apoA-I. Next, we compared the 7.8 nm rHDL and lipid-free

apoA-I cholesterol efflux time courses at a protein concentration of 25 μg/mL (Figure 6). The results confirmed that the 7.8 nm rHDL particle can mediate the ABCA1-specific process from 2 to 24 h efflux time. The analysis of media collected at the different incubation time points by nondenaturing GGE showed that no lipid-free apoA-I was formed during the incubation of the 7.8 nm rHDL with cAMP-stimulated J774 cells, and that the particles did not change size over a 24 h incubation (Figure 7, top panel). The same analysis conducted on media collected after incubation of lipid-free apoA-I with cAMP-stimulated J774 cells showed, in agreement with previous data (*25*), that particles with a diameter of 7.5 nm are formed. These products are not detectable after incubation of the 7.8 nm rHDL with cells, confirming that no lipid-free apoA-I is present in the preparation or formed during the incubation.

DISCUSSION

Pre-β-migrating HDLs make up a heterogeneous group of particles represented by either monomolecular lipid-free/lipid-poor apoA-I or discoidal particles consisting of two or three molecules of apoA-I complexed with phospholipids and free cholesterol. It has been suggested that among these apoA-I forms, only the monomolecular, lipid-free/lipid-poor apoA-I interacts with ABCA1, while the lipidated discoidal particles interact with ABCG1 and SR-BI, but not with ABCA1 (*26*). However, the lipid content of the apoA-I discoidal particles, as well as the size, can vary widely; to the best of our knowledge, no direct data demonstrate that all of these lipidated particles have the same efflux activity. The goal of our study was to correlate HDL particle lipid content and size with their ability to specifically promote cholesterol efflux mediated by ABCA1, ABCG1,

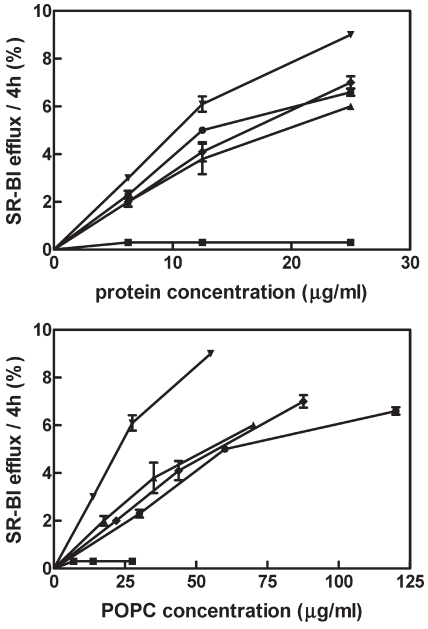

FIGURE 4: Efflux of cholesterol from Fu5AH cells to rHDL. Cholesterol efflux is expressed either as a function of protein (top panel) or as a function of phospholipid (bottom panel) concentration in the medium. Cells were labeled with 2 μCi/mL [^{3}H]cholesterol for 24 h in DMEM medium with 1% FCS and an ACAT inhibitor (Sandoz 58-035) at 2 μg/mL. Cells were then incubated for 18 h with 0.2% BSA, treated with or without BLT-1 for 2 h, washed, and incubated for 4 h with an increasing concentration of rHDL: 7.8 (■), 9.6 (▲), 10.8 (▼), 12.5 (◆), and 17.0 nm (●). The SR-BI-specific efflux was determined as the difference between the percentage efflux from untreated cells and the percentage efflux from BLT-1-incubated cells. Each sample was run in triplicate. Values are expressed as means ± SD.

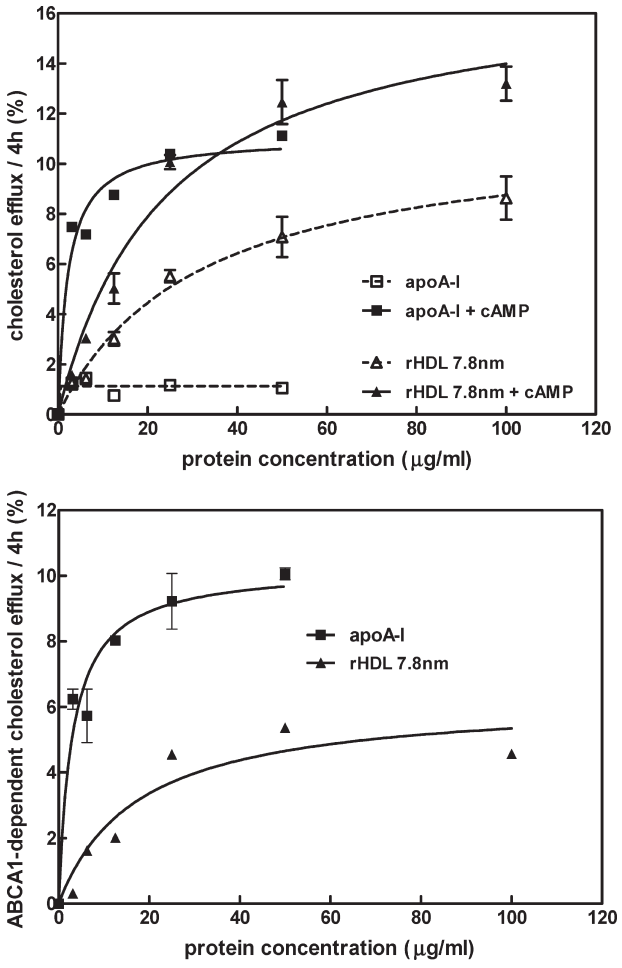

FIGURE 5: apoA-I and 7.8 nm rHDL dose-dependent ABCA1-mediated efflux. The efflux was determined in J774 control cells and cells upregulated for the ABCA1 protein with cpt-cAMP. Efflux was measured as described in the legend of Figure 2, exposing cells to increasing concentrations of either apoA-I or rHDL for 4 h (top panel). The ABCA1-dependent cholesterol efflux (bottom panel) was determined as the difference between the percentage efflux from stimulated cells and the percentage efflux from unstimulated cells. The data are fitted to the Michaelis−Menten equation. Each sample was run in triplicate. Values are expressed as means ± SD.

Table 2: Efflux of Cholesterol[a] from Normal and Tangier Human Fibroblasts

	normal fibroblasts, % cholesterol efflux/4 h			Tangier fibroblasts, % cholesterol efflux/4 h		
	basal	with 22-OH/9cRA	ΔABCA1	basal	with 22-OH/9cRA	ΔABCA1
6.25 μg/mL apoA-I	0.07 ± 0.001	8.73 ± 0.500	8.66 ± 0.500	0.19 ± 0.090	0.23 ± 0.070	0.04 ± 0.001
6.25 μg/mL 7.8 nm rHDL	0.92 ± 0.050	8.65 ± 0.190	7.73 ± 0.179	1.10 ± 0.160	1.13 ± 0.160	0.03 ± 0.020

[a]Each sample was run in triplicate. Values are expressed as means ± SD.

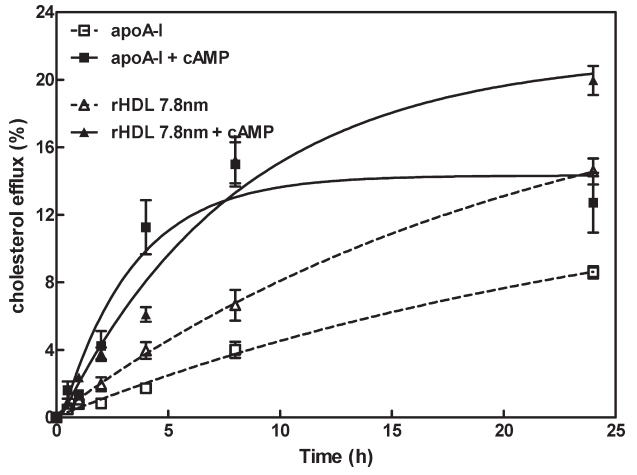

FIGURE 6: Time course of ABCA1-mediated efflux. The efflux was determined in J774 control cells and cells upregulated for the ABCA1 protein with cpt-cAMP. Efflux was measured as described in the legend of Figure 2, exposing cells to 25 μg/mL apoA-I and 25 μg/mL 7.8 nm rHDL. Each sample was run in triplicate. Values are expressed as means ± SD.

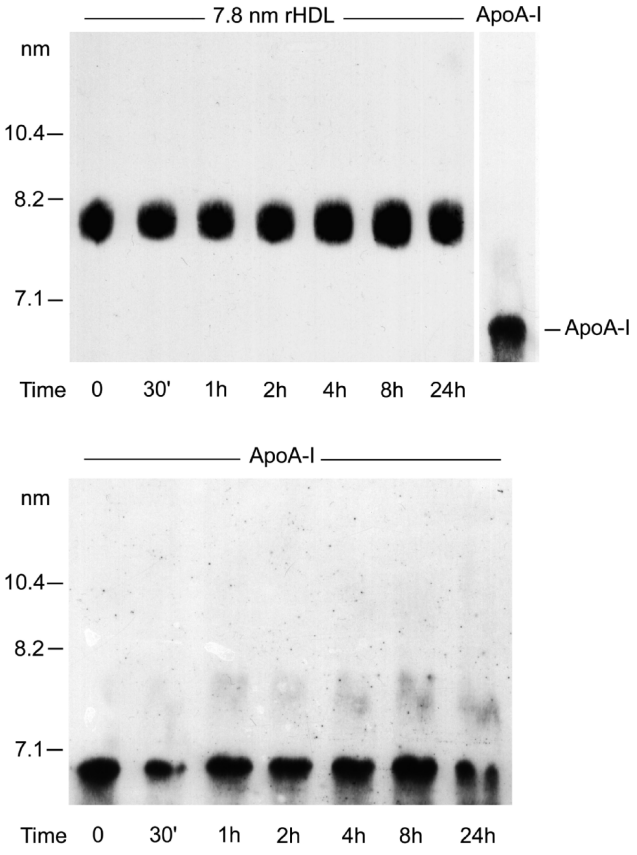

FIGURE 7: Analysis of cell media after incubation with the 7.8 nm rHDL (top panel) or lipid-free apoA-I (bottom panel) during the time course. The 7.8 nm rHDL or lipid-free apoA-I (25 μg/mL) was incubated with cAMP-stimulated J774 macrophages at different time points. Cell media were analyzed by GGE followed by immunoblotting and detection with an anti-apoA-I antibody.

and SR-BI, using five well-characterized pure homogeneous apoA-I-containing rHDLs that varied in the number of POPC molecules per particle and with particle size across the size range of human plasma HDL (7.8−17.0 nm). Consistent with published data (*14*, *20*, *21*), all our particles were discoidal and exhibited pre-β mobility on an agarose gel. The results demonstrate that (i) the ABCA1-mediated cell cholesterol efflux can be efficiently driven not only by monomolecular lipid-free apoA-I but also by a discoidal HDL particle with diameter of 7.8 nm containing two apoA-I and 82 POPC molecules per particle; (ii) the ABCG1-mediated cell cholesterol efflux can be driven, with remarkably similar efficiency, by all the tested HDL particles, with size of ≥7.8 nm and 82 or more POPC molecules; and (iii) the SR-BI-mediated cell cholesterol efflux is also driven by large and lipid-rich HDL particles, but with a minimum diameter of 9.6 nm and a phospholipid content of 202 molecules/particle.

The most striking result of this study is the observation that small discoidal pre-β migrating HDLs are efficient acceptors of cholesterol via ABCA1. The particles used in our experiments are rHDLs but resemble the plasma discoidal pre-β1 HDL in terms of size and apoA-I content (*26*). Thus, our observation indicates that all the plasma apoA-I particles with pre-β1 mobility, which include monomolecular lipid-free/lipid-poor apoA-I and small discoidal particles containing two apoA-I molecules per particle (*26*), can efficiently promote efflux of cell cholesterol through the ABCA1 pathway. Our results clearly indicate that up to 82 phospholipid molecules can bind to apoA-I while maintaining its functionality as a cholesterol acceptor through ABCA1. The reduced efficiency of the 7.8 nm particle in comparison to lipid free apoA-I suggests that the extent of lipidation may gradually weaken the apoA-I ability to promote ABCA1 efflux before achieving its complete inhibition. This observation is apparently in contrast with the conclusion by Mulya et al. (*25*) that a minimal lipidation of apoA-I is sufficient to prevent its interaction with ABCA1 (*25*). However, in that study, the minimal lipidated particles were obtained by interaction of apoA-I with ABCA1. As pointed out by the same authors, under these experimental conditions, apoA-I undergoes a conformational rearrangement that weakens its subsequent binding to ABCA1 (*25*, *27*). Therefore, changes in apoA-I conformation rather than the minimal lipidation could prevent the small pre-β1 migrating apoA-I from interacting with ABCA1. An alternative explanation may be obtained from the observation that apoA-I can adopt different conformations in discoidal rHDLs (*14*). The 7.8 nm rHDL shows a protein structure very similar to that of the 10.8 nm rHDL (*14*); however, the latter is not an acceptor of cholesterol via ABCA1. Thus, the correct apoA-I conformation is a requirement for the interaction of apoA-I with ABCA1, but it is not sufficient. In addition to conformation, the protein or particle has to be able to accommodate further phospholipid molecules. Indeed, the 7.8 nm rHDL used in this study contains 82 POPC molecules but can accommodate at least up to 100 phospholipid molecules (*28*).

The ABCA1 cholesterol efflux to the 7.8 nm rHDL could be also mediated by lipid free apoA-I that dissociates from the particle. This hypothesis is ruled out by different lines of evidence. First, the analysis of the 7.8 nm rHDL by nondenaturing GGE before and after incubation with cells (Figure 7, top panel) showed that no lipid-free apoA-I is present during the incubation. The possibility that lipid-free apoA-I in the incubation media could not be detected because it reassociates with lipids upon interaction with cells is not supported by the presence of a single-particle species. As shown in Figure 7 (bottom panel) and by others (*25*, *29*), interaction of free-apoA-I with cells leads to the formation of particles with different sizes. Second, if the ABCA1 efflux we observed with the 7.8 nm particles were driven by dissociated free apoA-I, we should expect a similar result with

the larger particles. Instead, these particles are totally unable to promote ABCA1-mediated cholesterol efflux (Figure 2).

Our data are only apparently in contradiction with published data. Oram and colleagues (*30*) demonstrated that mild trypsinization of HDL completely abolished efflux of ABCA1 to treated HDL. They concluded that the efflux must have been promoted by dissociated, trypsin-sensitive, free apoA-I. However, since the tested HDL preparations did not contain pre-β1 HDL particles (*30*), which are also trypsin-sensitive (*31*), no conclusion was drawn about the ability of these particles to promote ABCA1 efflux. Indeed, consistent with the results presented here, Denis et al. (*32*) reported that pre-β1 particles isolated from human serum and not containing lipid-free apoA-I could promote ABCA1 efflux.

The small 7.8 nm rHDL is also an efficient acceptor of cholesterol through ABCG1, and this efflux was similar to that obtained with all the other tested rHDLs. However, it was unable to promote efflux by SR-BI. These results are in agreement with data recently published by Lorenzi et al., who showed that small rHDLs, with a size similar to that of our smallest particle, interact with ABCG1 but not with SR-BI (*33*). Our results also indicate that no difference in ABCG1 and SR-BI efflux is present among phospholipid-rich particles, with diameter from 9.6 to 17.0 nm and more than 200 phospholipid molecules/particle.

To the best of our knowledge, this is the first time that a phospholipid/apoA-I-containing particle, resembling plasma pre-β1 HDL, is identified as an acceptor of ABCA1-mediated cholesterol efflux. In addition, our results demonstrate that a single HDL particle may drive both ABCA1- and ABCG1-mediated efflux. Finally, we provide conclusive evidence that a particle may discriminate between ABCG1 and SR-BI processes.

We may speculate that plasma small discoidal pre-β1 particles may represent a major player in the first step of reverse cholesterol transport, being able to remove macrophage cholesterol through the two active pathways.

The reported concentration of pre-β1 particles in plasma varies widely, in part reflecting biological variations but possibly also because of differences in techniques that are used to quantify them (*26*). We have recently shown that pre-β1 particles can accumulate in some HDL deficiencies, such as the apoA-I Milano mutant (*34*) and LCAT deficiency (*35*). Such particles can significantly contribute to efflux of macrophage cholesterol to serum (*34*, *36*). The small discoidal pre-β1 particles also resemble the synthetic HDLs that have been shown to be antiatheroscleotic in preclinical and human studies (*37*). This observation supports the concept that the infusion of small HDLs, or the use of strategies to increase their plasma concentration, represents a tool for enhancing the antiatherogenic potential of HDL.

ACKNOWLEDGMENT

We thank Prof. S. Calandra of University of Modena (Modena, Italy) for providing the human normal and Tangier fibroblasts.

REFERENCES

1. Barter, P. J., and Rye, K. A. (1996) Molecular mechanisms of reverse cholesterol transport. *Curr. Opin. Lipidol. 7*, 82–87.
2. Yancey, P. G., Bortnick, A. E., Kellner-Weibel, G., de la Llera-Moya, M., Phillips, M. C., and Rothblat, G. H. (2003) Importance of different pathways of cellular cholesterol efflux. *Arterioscler., Thromb., Vasc. Biol. 23*, 712–719.
3. de La Llera-Moya, M., Connelly, M. A., Drazul, D., Klein, S. M., Favari, E., Yancey, P. G., Williams, D. L., and Rothblat, G. H. (2001) Scavenger receptor class B type I affects cholesterol homeostasis by magnifying cholesterol flux between cells and HDL. *J. Lipid Res. 42*, 1969–1978.
4. Dean, M., Hamon, Y., and Chimini, G. (2001) The human ATP-binding cassette (ABC) transporter superfamily. *J. Lipid Res. 42*, 1007–1017.
5. Yancey, P. G., de la Llera-Moya, M., Swarnakar, S., Monzo, P., Klein, S. M., Connelly, M. A., Johnson, W. J., Williams, D. L., and Rothblat, G. H. (2000) High density lipoprotein phospholipid composition is a major determinant of the bi-directional flux and net movement of cellular free cholesterol mediated by scavenger receptor BI. *J. Biol. Chem. 275*, 36596–36604.
6. Campos, H., Roederer, G. O., Lussier-Cacan, S., Davignon, J., and Krauss, R. M. (1995) Predominance of large LDL and reduced HDL2 cholesterol in normolipidemic men with coronary artery disease. *Arterioscler., Thromb., Vasc. Biol. 15*, 1043–1048.
7. von Eckardstein, A., Huang, Y., and Assmann, G. (1994) Physiological role and clinical relevance of high-density lipoprotein subclasses. *Curr. Opin. Lipidol. 5*, 404–416.
8. Asztalos, B. F., de la Llera-Moya, M., Dallal, G. E., Horvath, K. V., Schaefer, E. J., and Rothblat, G. H. (2005) Differential effects of HDL subpopulations on cellular ABCA1- and SR-BI-mediated cholesterol efflux. *J. Lipid Res. 46*, 2246–2253.
9. Jonas, A. (1986) Reconstitution of high-density lipoproteins. *Methods Enzymol. 128*, 553–582.
10. Calabresi, L., Franceschini, G., Burkybile, A., and Jonas, A. (1997) Activation of lecithin cholesterol acyltransferase by a disulfide-linked apolipoprotein A-I dimer. *Biochem. Biophys. Res. Commun. 232*, 345–349.
11. Calabresi, L., Canavesi, M., Bernini, F., and Franceschini, G. (1999) Cell cholesterol efflux to reconstituted high-density lipoproteins containing the apolipoprotein A-IMilano dimer. *Biochemistry 38*, 16307–16314.
12. Nieland, T. J., Tan, M. C., Monne-van Muijen, M., Koning, F., Kruisbeek, A. M., and van Bleek, G. M. (1996) Isolation of an immunodominant viral peptide that is endogenously bound to the stress protein GP96/GRP94. *Proc. Natl. Acad. Sci. U.S.A. 93*, 6135–6139.
13. Calabresi, L., Vecchio, G., Frigerio, F., Vavassori, L., Sirtori, C. R., and Franceschini, G. (1997) Reconstituted high-density lipoproteins with a disulfide-linked apolipoprotein A-I dimer: Evidence for restricted particle size heterogeneity. *Biochemistry 36*, 12428–12433.
14. Calabresi, L., Meng, Q. H., Castro, G. R., and Marcel, Y. L. (1993) Apolipoprotein A-I conformation in discoidal particles: Evidence for alternate structures. *Biochemistry 32*, 6477–6484.
15. Takayama, M., Itoh, S., Nagasaki, T., and Tanimizu, I. (1977) A new enzymatic method for determination of serum choline-containing phospholipids. *Clin. Chim. Acta 79*, 93–98.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem. 193*, 265–275.
17. Gelissen, I. C., Harris, M., Rye, K. A., Quinn, C., Brown, A. J., Kockx, M., Cartland, S., Packianathan, M., Kritharides, L., and Jessup, W. (2006) ABCA1 and ABCG1 synergize to mediate cholesterol export to apoA-I. *Arterioscler., Thromb., Vasc. Biol. 26*, 534–540.
18. Brooks-Wilson, A., Marcil, M., Clee, S. M., Zhang, L. H., Roomp, K., van Dam, M., Yu, L., Brewer, C., Collins, J. A., Molhuizen, H. O., Loubser, O., Ouelette, B. F., Fichter, K., Ashbourne-Excoffon, K. J., Sensen, C. W., Scherer, S., Mott, S., Denis, M., Martindale, D., Frohlich, J., Morgan, K., Koop, B., Pimstone, S., Kastelein, J. J., Genest, J.Jr., and Hayden, M. R. (1999) Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat. Genet. 22*, 336–345.
19. Duong, M., Collins, H. L., Jin, W., Zanotti, I., Favari, E., and Rothblat, G. H. (2006) Relative contributions of ABCA1 and SR-BI to cholesterol efflux to serum from fibroblasts and macrophages. *Arterioscler., Thromb., Vasc. Biol. 26*, 541–547.
20. Jonas, A., Wald, J. H., Toohill, K. L., Krul, E. S., and Kezdy, K. E. (1990) Apolipoprotein A-I structure and lipid properties in homogeneous, reconstituted spherical and discoidal high density lipoproteins. *J. Biol. Chem. 265*, 22123–22129.
21. Sparks, D. L., Lund-Katz, S., and Phillips, M. C. (1992) The charge and structural stability of apolipoprotein A-I in discoidal and spherical recombinant high density lipoprotein particles. *J. Biol. Chem. 267*, 25839–25847.

22. Rothblat, G. H., de la Llera-Moya, M., Favari, E., Yancey, P. G., and Kellner-Weibel, G. (2002) Cellular cholesterol flux studies: Methodological considerations. *Atherosclerosis 163*, 1–8.
23. Favari, E., Zanotti, I., Zimetti, F., Ronda, N., Bernini, F., and Rothblat, G. H. (2004) Probucol inhibits ABCA1-mediated cellular lipid efflux. *Arterioscler., Thromb., Vasc. Biol. 24*, 2345–2350.
24. Adorni, M. P., Zimetti, F., Billheimer, J. T., Wang, N., Rader, D. J., Phillips, M. C., and Rothblat, G. H. (2007) The roles of different pathways in the release of cholesterol from macrophages. *J. Lipid Res. 48*, 2453–2462.
25. Mulya, A., Lee, J. Y., Gebre, A. K., Thomas, M. J., Colvin, P. L., and Parks, J. S. (2007) Minimal lipidation of pre-β HDL by ABCA1 results in reduced ability to interact with ABCA1. *Arterioscler., Thromb., Vasc. Biol. 27*, 1828–1836.
26. Rye, K. A., and Barter, P. J. (2004) Formation and metabolism of preβ-migrating, lipid-poor apolipoprotein A-I. *Arterioscler., Thromb., Vasc. Biol. 24*, 421–428.
27. Chau, P., Nakamura, Y., Fielding, C. J., and Fielding, P. E. (2006) Mechanism of preβ-HDL formation and activation. *Biochemistry 45*, 3981–3987.
28. Rocco, A. G., Mollica, L., Gianazza, E., Calabresi, L., Franceschini, G., Sirtori, C. R., and Eberini, I. (2006) A model structure for the heterodimer apoA-IMilano-apoA-II supports its peculiar susceptibility to proteolysis. *Biophys. J. 91*, 3043–3049.
29. Duong, P. T., Weibel, G. L., Lund-Katz, S., Rothblat, G. H., and Phillips, M. C. (2008) Characterization and properties of pre β-HDL particles formed by ABCA1-mediated cellular lipid efflux to apoA-I. *J. Lipid Res. 49*, 1006–1014.
30. Mendez, A. J., and Oram, J. F. (1997) Limited proteolysis of high density lipoprotein abolishes its interaction with cell-surface binding sites that promote cholesterol efflux. *Biochim. Biophys. Acta 1346*, 285–299.
31. Kunitake, S. T., Chen, G. C., Kung, S. F., Schilling, J. W., Hardman, D. A., and Kane, J. P. (1990) Pre-β high density lipoprotein. Unique disposition of apolipoprotein A-I increases susceptibility to proteolysis. *Arteriosclerosis 10*, 25–30.
32. Denis, M., Haidar, B., Marcil, M., Bouvier, M., Krimbou, L., and Genest, J.Jr. (2004) Molecular and cellular physiology of apolipoprotein A-I lipidation by the ATP-binding cassette transporter A1 (ABCA1). *J. Biol. Chem. 279*, 7384–7394.
33. Lorenzi, I., von Eckardstein, A., Radosavljevic, S., and Rohrer, L. (2008) Lipidation of apolipoprotein A-I by ATP-binding cassette transporter (ABC) A1 generates an interaction partner for ABCG1 but not for scavenger receptor BI. *Biochim. Biophys. Acta 1781*, 306–313.
34. Favari, E., Gomaraschi, M., Zanotti, I., Bernini, F., Lee-Rueckert, M., Kovanen, P. T., Sirtori, C. R., Franceschini, G., and Calabresi, L. (2007) A unique protease-sensitive high density lipoprotein particle containing the apolipoprotein A-I(Milano) dimer effectively promotes ATP-binding Cassette A1-mediated cell cholesterol efflux. *J. Biol. Chem. 282*, 5125–5132.
35. Asztalos, B. F., Schaefer, E. J., Horvath, K. V., Yamashita, S., Miller, M., Franceschini, G., and Calabresi, L. (2007) Role of LCAT in HDL remodeling: investigation of LCAT deficiency states. *J. Lipid Res. 48*, 592–599.
36. Calabresi, L., Favari, E., Moleri, E., Adorni, M. P., Pedrelli, M., Costa, S., Jessup, W., Gelissen, I. C., Kovanen, P. T., Bernini, F., and Franceschini, G. (2009) Functional LCAT is not required for macrophage cholesterol efflux to human serum. *Atherosclerosis 204*, 141–146.
37. Gomaraschi, M., Calabresi, L., and Franceschini, G. (2006) High-density lipoproteins: A therapeutic target for atherosclerotic cardiovascular disease. *Expert Opin. Ther. Targets 10*, 561–572.