Structural and functional properties of human plasma high density-sized lipoprotein containing only apoE particles

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Abstract **To investigate the metabolism of HDL-apolipoprotein E (apoE) particles in human plasma, we isolated a fraction of plasma HDL-apoEs that lack apoA-I (HDL-LpE) from subjects with apoE3/3 phenotype by immunoaffinity. Plasma HDL-LpE had a particle size ranging from 9 nm to 18.5 nm in diameter and was characterized by two-dimensional nondenaturing gradient gel electrophoresis as having either γ-, preβ$_1$-, preβ$_2$-, or α-electrophoretic mobility. HDL-LpE was also present in the medium of cultured human hepatoma cell lines and monocyte-derived macrophages. The majority of apoE3 was found as a monomeric form in HDL-LpE and floated at density d > 1.21 g/ml. Plasma levels of HDL-LpE in normolipidemic, CETP-deficient, and ABCA1-deficient subjects were 0.72 ± 0.15 mg/dl (n = 12), 1.77 ± 0.75 mg/dl (n = 3), and 0.55 ± 0.11 mg/dl (n = 3), respectively. The ratio of HDL-apoE containing apoA-I to HDL-LpE was significantly higher 4 h after a fat load, representing a 35 ± 9% increase (n = 3). Isolated plasma HDL-LpE3 was as effective as apoE3, reconstituted HDL particles, or apoA-I in promoting cellular cholesterol efflux. These results demonstrate that *1*) plasma HDL-LpE may have hepatogenous and macrophagic origins; *2*) HDL-LpE was preserved even with large reductions in apoA-I-containing lipoproteins; *3*) HDL-LpE was active in the transfer of apoE to triglyceride-rich lipoproteins, and *4*) HDL-LpEs efficiently take up cell-derived cholesterol.**—Krimbou, L., M. Marcil, H. Chiba, and J. Genest, Jr. **Structural and functional properties of human plasma high density-sized lipoprotein containing only apoE particles. *J. Lipid Res.* 2003.** 44: **884–892.**

Supplementary key words HDL-apoE • hypoalphalipoproteinemia • postprandial lipemia • cholesterol efflux • reverse cholesterol transport

The importance of apolipoprotein E (apoE), a multifunctional protein, in the onset and development of atherosclerosis and in the pathophysiology of Alzheimer's disease, has been recognized for a number of years. Increasing evidence from both animal and human studies suggests that apoE is able to protect against atherosclerosis by *a*) promoting efficient uptake of triglyceride-rich lipoproteins (TRLs) from the circulation (1), *b*) maintaining normal macrophage lipid homeostasis (2), *c*) playing a role in cellular cholesterol efflux and reverse cholesterol transport (RCT) (3), *d*) preventing oxidation (4), *e*) modulating inflammatory response by suppressing lymphocyte activation (5), and *f*) restricting platelet aggregation (6) and suppressing growth factor-induced smooth muscle cell migration and proliferation (7). ApoE is believed to play a significant role in the pathophysiology of Alzheimer's disease by controlling neuronal growth (8). Importantly, both immunosuppression and stimulation of neurite outgrowth by apoE are unrelated to its cholesterol-transporting properties because minimally lipidated or delipidated apoEs are equally active in regulating these lymphocyte and neuronal cell functions (9). In contrast, apoE inhibition of platelet aggregation requires its presence in a lipoprotein form (6). Taken together, these studies suggest that minimally lipidated or delipidated apoE in circulation may have a direct impact on vascular occlusive diseases independent of its cholesterol transport function.

In human plasma, apoE is almost entirely associated with lipoproteins containing apoB or apoA-I (10), though several studies have demonstrated the existence of minor lipoprotein subfractions similar in size to HDL and containing apoE as their only protein component (11–13). It has been suggested that HDL-apoE is involved in several aspects of plasma lipoprotein metabolism, including *1*) receptor-mediated delivery of HDL cholesterol to the liver

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Abbreviations: ABCA1, ATP binding cassette A1; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; LpE, lipoprotein containing only apoE; PEG, polyethylene glycol; RCT, reverse cholesterol transport; TD, Tangier disease; TRL, triglyceride-rich lipoprotein; 2D-PAGGE, two-dimensional nondenaturing gradient gel electrophoresis.

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(14), *2*) hepatic lipase-catalyzed hydrolysis of HDL phospholipid (15), *3*) plasma cholesterol esterification (16), *4*) plasma cholesteryl ester (CE) transfer (17), *5*) efflux of cell-derived cholesterol (11, 18), and *6*) stimulation of endothelial production of heparin sulfate (19).

The present study aims to provide evidence for the physiological presence of HDL-lipoprotein containing only apoE (LpE) in human plasma and the role of these particles in the RCT process. Our results demonstrated the presence of different-sized HDL containing only apoE particles in normolipidemic and hypoalphalipoproteinemic human plasma. HDL-LpE particles participate in a dynamic traffic of apoE between HDL and TRLs and were efficient acceptors of cell-derived cholesterol.

MATERIALS AND METHODS

Samples

Blood samples were obtained from male and female subjects with apoE3/3 phenotype after an overnight fast. Blood was drawn from the antecubital vein into evacuated tubes containing ethylenediamine-tetraacetate (EDTA, final concentration: 1.5 mg/ml). Collection tubes were immediately placed in ice before being centrifuged (3,000 rpm, 15 min, 4°C). Plasma was separated from red blood cells by aspiration and was kept in ice until electrophoretic separation of apoE-containing HDL and precipitation of apoB-containing particles by polyethylene glycol (PEG) 6000 or immunoaffinity techniques. This study was approved by the ethics committees of the institutions. Plasma from patients with cholesteryl ester transfer protein (CETP) deficiency was provided by Dr. Hitoshi Chiba from Hokkaido University School of Medicine, Sapporo, Japan; plasma from subjects with Tangier disease (TD) [ATP binding cassette A1 (ABCA1)-deficient homozygote] and apoA-I $(L141R)_{Pisa}$ was provided by Dr. Arnold von Eckardstein from the Institute of Arteriosclerosis Research, Münster, Germany (20); and plasma from subjects with familial HDL deficiency (ABCA1-deficient heterozygote) was from our laboratory and was previously described (21, 22).

Isolation of HDL-LpE from plasma

Plasma was first depleted of apoB-containing lipoprotein by precipitation with PEG, as described previously (23). Briefly, the plasma sample was mixed with an equal volume of aqueous 13% (w/v) PEG, and the solution was allowed to stand for 10 min at room temperature. After centrifugation (2,000 *g*, 15 min, room temperature), supernate-containing HDL was kept in ice until immunoaffinity separation. HDL-LpE fractions were isolated from fresh plasma depleted of apoB-containing lipoproteins using RLP-Cholesterol Assay Kits (Jimro-II, Japan Immunoresearch Laboratories, Japan). According to the manufacturer's instructions, 10 μl of plasma-depleted apoB was added to 300 μl of gel suspension consisting of anti-human apoA-I and apoB-100 mouse monoclonal antibodies bound to Sepharose (24). The suspension was gently mixed for 2 h at room temperature with a vertical magnetic-bead oscillator (RLP Mixer J-100A, Photal, Otsuka Electronics, Japan). The mixture was allowed to settle for 15 min. The supernatant (230 μl) containing HDL-LpE was aspirated and stored at 4°C until time of analysis. The recovery of apoE after removal of apoB- and apoA-I-containing lipoproteins by precipitation and immunoaffinity in normolipidemic subjects with an apoE3/3 phenotype was 78.7 ± 7.5 (n = 10).

Affinity purification of plasma HDL-LpE particles

An affinity chromatography isolation procedure utilizing the human immunopurified anti-apoA-I and anti-apoE antibodies (12171-21A and 12171-21E, Genzyme Corp., Cambridge, MA) was employed as described previously (9). Briefly, plasma (20 ml) from a normolipidemic subject with an apoE3/3 phenotype was first depleted of apoB-containing lipoprotein by precipitation with PEG; supernate-containing HDL was depleted of apoA-I-containing lipoproteins by immunopurifed anti-apoA-I coupled CNBr-activated Sepharose; and then HDL-LpE particles were isolated by immunopurifed anti-apoE coupled CNBr-activated Sepharose. Immunoaffinity isolated plasma HDL-LpE3 particles were used for cellular cholesterol efflux experiments.

Isolation of HDL-apoE with apoA-I

Plasma was depleted of apoB-containing lipoprotein by precipitation with PEG, and then apoA-I-containing particles were separated by using anti-apoA-I latex (Genzyme Corp.), as we have previously described (25). Briefly, plasma depleted of apoB (100 μl) was added to 300 μl of anti-apoA-I latex suspension, gently mixed for 45 min at room temperature, and then centrifuged at 12,000 rpm for 10 min. The supernatant, which contained apoA-I-bound latex was washed with PBS, and apoA-I-containing particles were eluted in 100 mM triethylamine (pH 11.5) and immediately neutralized with 1 M sodium phosphate (pH 6.8) (9). The apoA-I-containing particles were dialyzed and concentrated using Centricon Centrifugal Filter, MWCO 10000 (Millipore, Billerica, MA). Isolated HDL-apoE with apoA-I particles were used for analysis of apoE forms. The concentration of HDL-apoEs with apoA-I was calculated as the difference between total HDL-apoE and HDL-LpE, both determined by ELISA.

Separation of lipoproteins by two-dimensional nondenaturing gradient gel electrophoresis

Lipoprotein in the HDL size range in total plasma and HDL-LpE fractions were separated by two-dimensional nondenaturing gradient gel electrophoresis (2D-PAGGE), as previously described (12, 26). Briefly, plasma samples (30–100 μl) and HDL-LpE fractions (isolated from 30–100 μl of the same plasma) were separated in the first dimension (according to their charge) by 0.75% agarose gel electrophoresis (100 V, 8 h, 4°C), and in the second dimension (according to the size) by 2–15% or 3–24% polyacrylamide concave gradient gel electrophoresis (80 V, 20 h, 4°C). A high-molecular-weight protein mixture (7.1 nm to 17.0 nm, Pharmacia, Piscataway, NJ) iodinated using IODO-GEN® Iodination Reagent (1,3,4,6-tetrachloro-3α-6α-diphenylglycouril, Pierce Chemical Co., Rockford, IL) (27) was run as a standard on each gel. Electrophoretically separated proteins and lipoproteins were electrotransferred (30 V, 20 h, 4°C) onto nitrocellulose membranes (Hybond ECL, Amersham Life Science, Buckinghamshire, England), and apoE or apoA-I-containing lipoproteins were detected by incubating the membranes with immunopurified polyclonal apoE antibody (Genzyme Corp.) or anti-apoA-I antibody (Biodesign, Kennebunk, ME) labeled with ^{125}I. The presence of labeled antibody was detected by autoradiography using XAR-2 Kodak film.

Cell culture

Human hepatoma cell lines (HepG2) were cultured under standard conditions as we have previously described (26). Briefly, HepG2 (ATCC HB 8065) were grown in Eagle's minimum essential medium with nonessential amino acids, sodium pyruvate, and 10% fetal bovine serum. After a 24 h incubation, serum-free medium from HepG2 (3 ml) was collected in the presence of 1 mmol/l phenylmethylsulfonyl fluoride, concentrated by using Centricon filters (Amicon) to a final volume of 200 μl, and sepa-

rated by 2D-PAGGE. Culture of human monocyte-derived macrophages was performed as previously described (28). Briefly, mononuclear cells were obtained by monocytopheresis from a normolipidemic subject with an apoE3/3 phenotype. Ten $\times$ 10^6 mononuclear cells were seeded into individual 35 mm diameter wells and incubated for 2 h at 37°C to allow the monocytes to attach. Each well was then rinsed twice with 2 ml of RPMI 1640 to remove unattached cells and fed with 2 ml of RPMI 1640 containing 10% autologous serum. After a 24 h incubation, serum-free medium from macrophages was collected, concentrated, and separated by 2D-PAGGE.

Lipid and lipoprotein analyses

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 phenotypes were determined by immunoblotting of plasma separated by IEF minigel electrophoresis (29). ApoE in total plasma, HDL, and HDL-LpE were assayed by ELISA. Plasma fraction d $>$ 1.21 mg/dl was isolated from normolipidemic plasma by sequential utracentrifugation using a Beckman ultracentrifuge. Reconstituted HDL containing only apoE r(LpE) particles were prepared as previously described (30). Acetyl-LDL was prepared by reaction with acetic anhydride (31) and labeled with [^{3}H]cholesteryl oleate as described (32). Briefly, label was transferred from [^{3}H]CE-containing liposomes to HDL_3 by CETP, followed by CETP-mediated transfer of the [^{3}H]CE from HDL_3 to acetyl-LDLs, which were then separated from each other by density ultracentrifugation. The final specific activity of the labeled acetyl-LDL was 20 cpm/ng of protein.

Statistical analysis

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

RESULTS

In order to establish the relationship of HDL-apoE particles with lipoproteins containing apoA-I, plasma was depleted of apoB-containing lipoproteins by precipitation with PEG, and then depleted of apoA-I-containing lipoproteins by immunoaffinity precipitation using anti-apoA-I antibody, as described in Materials and Methods. 2D-PAGGE separation of isolated plasma HDL-LpE fraction from a normolipidemic subject is shown in **Fig. 1** (left panel). The majority of HDL-LpE had a particle size range from 9 nm to 18.5 nm in diameter, and could be characterized as having either γ-, preβ_1-, preβ_2- or α-electrophoretic mobility. We subsequently classified HDL-LpE subpopulations as γ-LpE, pre-β_1-LpE, pre-β_2-LpE, and α-LpE, corresponding to their electrophoretic migration in the first dimension. HDL-LpE did not contain or comigrate with apoB-100, apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III, or apoJ as determined by 2D-PAGGE followed by immunoblotting with different antibodies (data not shown). We have previously documented that the presence of different HDL-LpE fractions after 2D-PAGGE separation is an accurate reflection of the presence of different subpopulations in vivo (12, 13).

The question was raised whether different HDL-LpE particles were produced by hepatocytes and/or macrophages, or whether they were a product of plasma apoE-containing lipoproteins remodeling. An experiment was therefore carried out in which HDL-LpE was isolated from the medium of cultured HepG2 and monocyte-derived macrophages. The medium of cultured HepG2 was depleted of apoB-containing lipoprotein by precipitation with PEG, and then depleted of apoA-I-containing lipoproteins by immunoaffinity precipitation using anti-apoA-I antibody. As shown in Fig. 1 (middle panel), HepG2 secreted apoE-containing lipoprotein without apoA-I in the HDL size range, with a particle diameter ranging from 9.5 nm to 18.5 nm, and had pre-β electrophoretic mobility on agarose gel. At the same time, separation of apoE-containing lipoproteins secreted from cultured monocyte-derived macrophages from a normolipidemic subject with an apoE3/3 phenotype by 2D-PAGGE (Fig. 1, right panel) shows that a significant proportion of HDL-sized LpE particles secreted by human macrophages have pre-β electrophoretic mobility and particle size ranging from 7.5 nm to 18.5 nm. Furthermore, the majority of LpE particles secreted by macrophages were distributed in the d $>$ 1.21 g/ml. By contrast, cholesterol enrichment of macrophages generates large apoE-containing particles with a density similar to HDL and LDL particles (data not shown).

The nature of apoE forms within HDL-apoE subpopulations was assessed by SDS-PAGE under nonreducing conditions followed by immunoblotting with apoE antibody, as shown in **Fig. 2**. The three forms of apoE3 were found in HDL-apoE subpopulations (Fig. 2A). In addition to the monomeric apoE3 form, both homodimer and apoE3-A-II complex were present in HDL-apoE with apoA-I isolated by immunoaffinity, as described in Materials and Methods (Fig. 2B); however, most of the apoE3 was found as a monomeric form in HDL-LpE (Fig. 2C).

To further define density distribution and lipidation of HDL-LpE particles, isolated HDL-LpE subjected to ultracentrifugation demonstrated that most of the HDL-LpE was distributed in the bottom fraction of the density gradient (d $>$ 1.21) as shown in **Fig. 3A** (right panel). No apoE was detected in the density d $<$ 1.21 (data not shown). Plasma apoE and apoA-I (d $>$ 1.21) were used as control in this experiment, as shown in Fig. 3B (left panel). ApoE-containing particles in d $>$ 1.21 are similar to those found in ultracentrifuged HDL-LpE (d $>$ 1.21) (Fig. 3A, left panel). Pre-β-LpA-I particles were also distributed in the bottom fraction of the density gradient (d $>$ 1.21) (Fig. 3B, right panel).

In order to determine whether the plasma concentration of HDL-LpE was dependent on plasma HDL levels, HDL-LpE was quantitated in plasma from CETP-deficient subjects (Int 14A homozygote) and ABCA1-deficient subjects. As shown in **Table 1**, the concentration of HDL-LpE was significantly higher in CETP-deficient subjects, 1.77 $\pm$

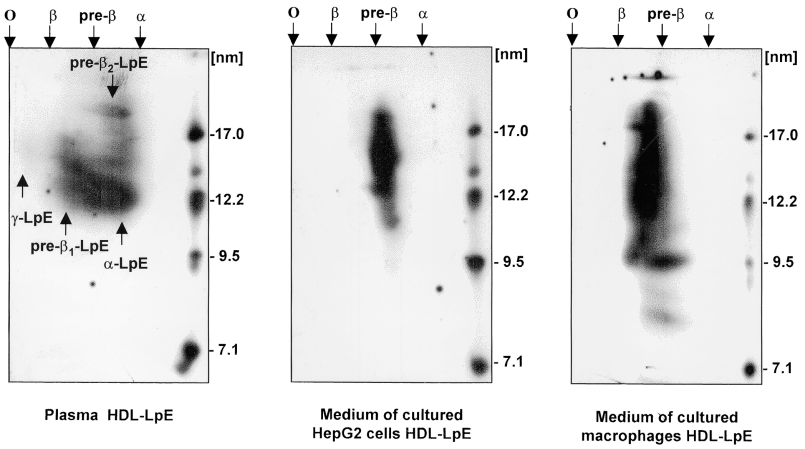

Fig. 1. Separation of HDL-sized lipoprotein containing only apolipoprotein E (LpE) from plasma, medium of cultured human hepatoma cell lines (HepG2), and monocyte-derived macrophages by two-dimensional nondenaturing gradient gel electrophoresis. HDL-LpE particles isolated from plasma, medium of cultured HepG2 cells, and macrophages were separated according to charge in the first dimension (from left to right) by agarose gel electrophoresis (top of figure), and then according to size in the second dimension (top to bottom) by polyacrylamide gradient (3–24%) gel electrophoresis. ApoE was detected with ^{125}I-labeled human anti-apoE antibody. Molecular size markers are indicated on the right. Different HDL-LpE subpopulations are indicated with vertical arrows.

0.75 mg/dl (n = 3), compared with normal controls, 0.72 ± 0.15 mg/dl (n = 12); however, in ABCA1-deficient subjects, despite the severe reduction in, or absence of, apoA-I-containing mature HDL particles (**Fig. 4**, familial HDL deficiency and TD subjects), the level of plasma HDL-LpE was preserved 0.55 ± 0.11 mg/dl (n = 3) (Table 1). HDL-apoE was also found in other forms of severe hypoalphalipoproteinemia, LCAT deficiency, and apoA-I gene mutation $(L141R)_{Pisa}$ (Fig. 4).

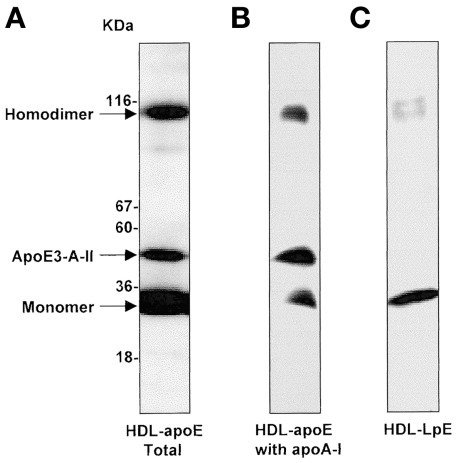

Fig. 2. Analysis of apoE3 forms present in HDL-apoE subpopulations. A: Total HDL was obtained by polyethylene glycol (PEG) precipitation of plasma apoB-containing lipoproteins. B: HDL-apoE with apoA-I was isolated by immunoaffinity. C: HDL-LpE was isolated by PEG precipitation followed by immunoaffinity. All procedures are described in Materials and Methods. Samples were added to SDS incubation buffer without a reducing agent and separated by SDS-polyacrylamide gradient gel (4–22.5%). ApoE was detected by immunoblotting with ^{125}I-labeled apoE antibody.

It is well known that the distribution of apoE between plasma lipoproteins changes after the ingestion of a fat-rich meal (12, 33). In order to study HDL-LpE particles in a more physiological context, the plasma apoE concentration in HDL-apoE subpopulations was determined in three subjects after an overnight fast, and then at 2 h intervals for 4 h or 8 h after the ingestion of an oral fat load (liquid cream). The aim of these experiments was to determine which subfractions of apoE-containing HDL were most active in donating and/or accepting apoE during postprandial plasma triglyceride lipolysis. Results for one subject is shown in **Fig. 5A**. Although no change in the plasma concentration of apoE was observed during the postprandial period, a major redistribution of apoE occurred. The plasma concentration of HDL-apoE (total), HDL-apoE with apoA-I, and HDL-LpE decreased significantly after the fat load and returned to the fasting levels within 8 h. HDL-LpE particles decreased particularly 4 h after the fat load.

To evaluate the quantitative importance of HDL-LpE to mediate transfer of apoE during the postprandial period, the ratio of HDL-apoE with apoA-I to HDL-LpE was determined in three normolipidemic subjects after an oral fat load. As shown in Fig. 5B, this ratio was significantly higher 4 h after a fat load, representing a 35.2 ± 8.6% increase (n = 3), compared with the fasting state (t = 0 h).

It is well accepted that HDL-apoE particles play an important role in the RCT process. In order to provide direct evidence that HDL-LpE particles could act as an acceptor of cellular cholesterol, murine J744 macrophages were incubated with [^{3}H]CE-labeled acetyl-LDL for 6 h and then chased in serum-free medium containing 10 μg of either human plasma purified apoE3, reconstituted HDL particles r(LpE3), immunoaffinity isolated plasma HDL-LpE particles from a normolipidemic subject with an

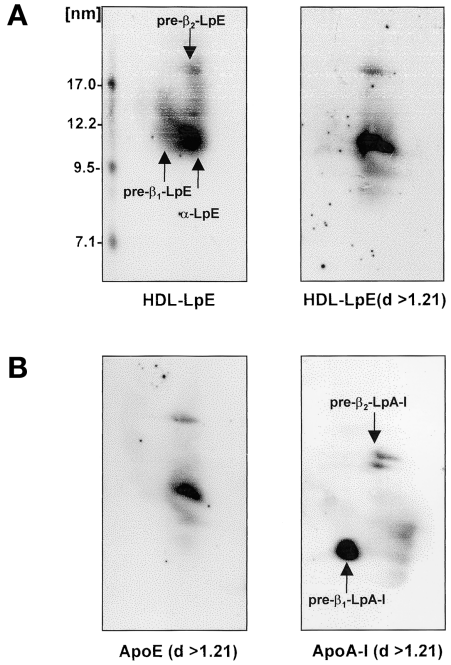

Fig. 3. Density distribution of isolated HDL-LpE particles. A: After isolation of plasma HDL-LpE, as described in Materials and Methods, isolated HDL-LpE was adjusted to 1.24 g/ml with solid KBr, then d = 1.21 g/ml KBr solution was overlaid onto HDL-LpE fraction and ultracentrifuged. The d < 1.21 g/ml and d > 1.21 g/ml fractions were collected. After dialysis, the fractions were subjected to two-dimensional gel electrophoresis. The left panel shows the separation of native plasma HDL-LpE. The right panel shows the distribution of isolated HDL-LpE in the d > 1.21. No apoE was detected in the d < 1.21. B: Plasma from the same subject was ultracentrifuged, and d > 1.21 g/ml bottom fraction was subjected to two-dimensional gel electrophoresis. ApoE and apoA-I were detected by immunoblotting with ^{125}I-labeled antibodies (left and right panels, respectively).

apoE3/3 phenotype, or lipid-free apoA-I. As shown in **Fig. 6**, isolated plasma HDL-LpE3 particles were as effective as apoE3, r(LpE3), or apoA-I in promoting efflux of acetyl-LDL-derived [^{3}H]cholesterol from J744 macrophages.

DISCUSSION

The results of the present study demonstrate the presence of HDL-LpE in the plasma of normolipidemic subjects. HDL-LpE had a particle size ranging from 9 nm to 18.5 nm in diameter, and was intermediate in size between LDL and HDL. HDL-LpE particles were characterized by 2D-PAGGE as having either γ-, preβ$_1$-, preβ$_2$-, or α-electrophoretic mobility (designated γ-LpE, preβ$_1$-LpE, preβ$_2$-LpE, and α-LpE, respectively). The physiological existence of plasma HDL-LpE particles was supported by the presence of HDL-sized lipoproteins containing only apoE in the medium of cultured HepG2 and monocyte-derived macrophages (Fig. 1), suggesting that plasma HDL-LpE may have hepatogenous and macrophagic origins. Fazio and Yao (34) had documented that apoE-containing lipoprotein particles secreted by HepG2 cells have pre-β and -α electrophoretic mobility on agarose gels. On the other hand, it has been shown that without cholesterol enrichment, human monocyte-macrophages secreted lipid-poor apoE with a density >1.21 g/ml. By contrast, cholesterol enrichment of monocyte-macrophages induced the association of apoE with phospholipid and cholesterol to form discoidal particles that floated at densities of 1.08–1.10 g/ml (35).

It was previously documented that the majority of apoE3 in the plasma exists in disulfide-linked structures, either as the homodimer or as the heterodimer apoE3-A-II (36). In this study, we demonstrate that the three forms of apoE3 are present in HDL-apoE subpopulations (Fig. 2A); however, most of apoE3 was found as a monomeric form in HDL-LpE (Fig. 2C). Weisgraber and Shinto (36) had previously documented that apoE3 homodimer migrated abnormally on SDS gels (M_r ~100,000) and was higher than the expected molecular mass (M_r = 68,400) for a dimeric structure. It is possible that the reported homodimeric form of apoE3 might represent apoE3 complexed with some other plasma protein. In support of this, we have previously documented that apoE was found associated with α$_2$-macroglobulin in human plasma (37).

TABLE 1. Levels of cholesterol, triglyceride, apolipoprotein, and plasma HDL-lipoprotein containing only apoE in normolipidemic, cholesteryl ester transfer protein deficiency (Int 14A homozygote), and ATP binding cassette A1 deficiency subjects with apoE3/3 phenotype

	Plasma Concentration							
Subjects	Cholesterol	Triglycerides	HDL Cholesterol	HDL-ApoE Total	HDL-LpE[a]	ApoB	ApoA-I	ApoE
	mmol/l				*mg/dl*			
Controls (n = 12)	4.59 ± 0.51	1.28 ± 0.56	1.36 ± 0.28	1.62 ± 0.22	0.72 ± 0.15	84 ± 12	145 ± 16	3.8 ± 0.7
CETP deficiency								
1	5.33	0.72	3.26	6.06	2.40	70	182	8.7
2	5.07	3.69	3.00	2.80	0.93	77	193	6.1
3	7.96	1.67	5.84	5.89	1.99	71	248	8.7
Mean ± SD					1.77 ± 0.75			
ABCA1 deficiency								
Familial HDL deficiency	4.14	2.54	0.19	1.14	0.68	177	17	2.9
TD1	3.50	2.10	0.09	1.07	0.47	87	3	2.4
TD2	3.20	1.67	0.10	1.00	0.52	79	5	2.7
Mean ± SD					0.55 ± 0.11			

ABCA1, ATP binding cassette A1; CETP, cholesteryl ester transfer protein; LpE, lipoprotein containing only apoE; TD, Tangier disease.
[a] Fraction of HDL-apoE without apoA-I.

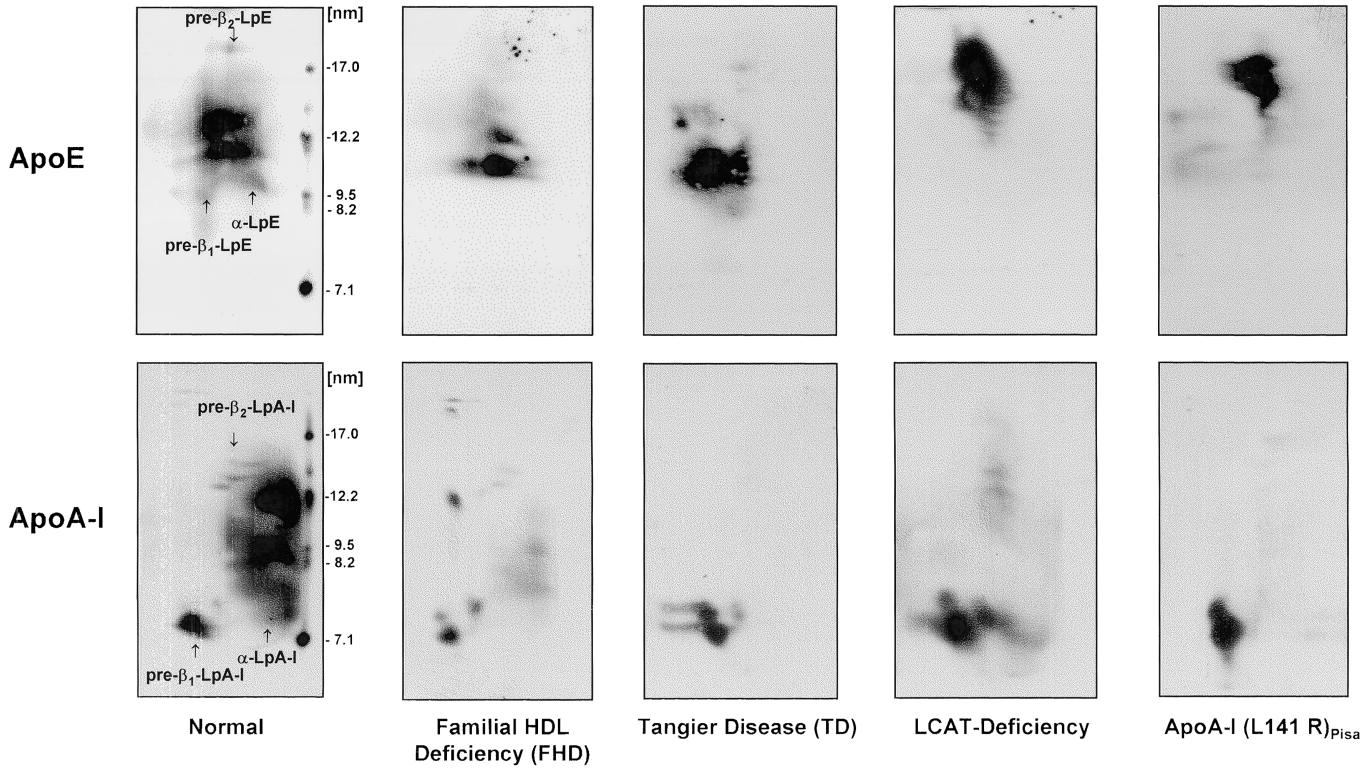

Fig. 4. Two-dimensional gradient gel electrophoresis separation of apoE-containing HDL-sized particles in subjects with hypoalphalipoproteinemia. Plasma (100 μl) from a normolipidemic subject, ATP binding cassette A1 (ABCA1) deficiency (familial HDL deficiency and Tangier disease), LCAT deficiency, and apoA-I $(L\text{-}141R)_{Pisa}$ subjects were separated by two-dimensional gel electrophoresis. ApoE and apoA-I were detected by immunoblotting with ^{125}I-labeled antibodies (top and bottom panels, respectively). Migration of ^{125}I-labeled molecular mass markers is indicated on the left.

In normolipidemic subjects, the concentration of HDL-LpE was 0.72 ± 0.15 mg/dl, representing ~45% of total HDL-apoE (Table 1). Previous studies have reported the presence of increased concentrations of apoE-rich HDL particles in CETP-deficient subjects (Int14A homozygote) (23, 38). In the present investigation, CETP-deficient subjects were found to have a 2.5-fold greater plasma HDL-LpE concentration compared with normal subjects (Table 1). In addition, most of HDL-LpE from CETP-deficient subjects was larger with pre-β_2 electrophoretic mobility (data not shown).

We found that HDL-LpE particles were preserved even with large reductions in apoA-I-containing lipoproteins. This conclusion is supported by the finding that *1*) in ABCA1-deficient subjects (21, 22), despite the severe reduction in, or absence of, apoA-I-containing mature HDL particles (Fig. 4, familial HDL deficiency and TD subjects), the levels of plasma HDL-LpE were preserved (0.55 ± 0.11 mg/dl, n = 3) (Table 1); and *2*) HDL-apoE was present in other forms of hypoalphalipoproteinemia (apoA-I gene mutation, LCAT deficiency) (Fig. 4).

In previous work, we demonstrated that in normal plasma, HDL-apoE subpopulations participate in the plasma transfer of apoE during the postprandial period (12). Results in Fig. 5A show that HDL-apoE has similar kinetics in donating and/or accepting apoE postprandially; however, at 4 h after the fat load, we demonstrate that HDL-LpE particles were more active in donating apoE than HDL-apoE with apoA-I (+35.2 ± 8.6%, n = 3; Fig. 5B). we postulate that apoE3 dimerization in HDL-apoE subpopulations might affect the transfer of apoE to TRL during the postprandial period or, alternatively, the lipid state of apoE itself in HDL-apoE subpopulations can directly affect its transfer. In the present study, evidence was in fact obtained to support both of these possibilities. First, most of apoE3 was found as a monomeric form in HDL-LpE (Fig. 2C), while the majority of apoE3 in HDL-apoE with apoA-I exists as both homodimer and apoE3-A-II forms (Fig. 2B). At the same time, the majority of HDL-LpE was recovered at d > 1.21 (Fig. 3A), indicating that these particles were poorly lipidated. Dimerization or lipid state of apoE in HDL-apoE might thus regulate its transfer between lipoproteins in the postprandial period in the same way that apoE3 dimerization reduces its binding to LDL receptor (36). Thus, the monomeric form of apoE might represent the biologically active form of exchangeable apoE.

Additional evidence for the importance of HDL-LpE in the transfer of apoE to TRL during the early stages of lipolysis was provided by the observation that *1*) most of apoE associated with apoB-containing lipoproteins is found as monomeric form, and *2*) 4 h after the fat load and 15 min after the administration of heparin, HDL-LpE concentration returned to its fasting level (0.80 ± 0.04,

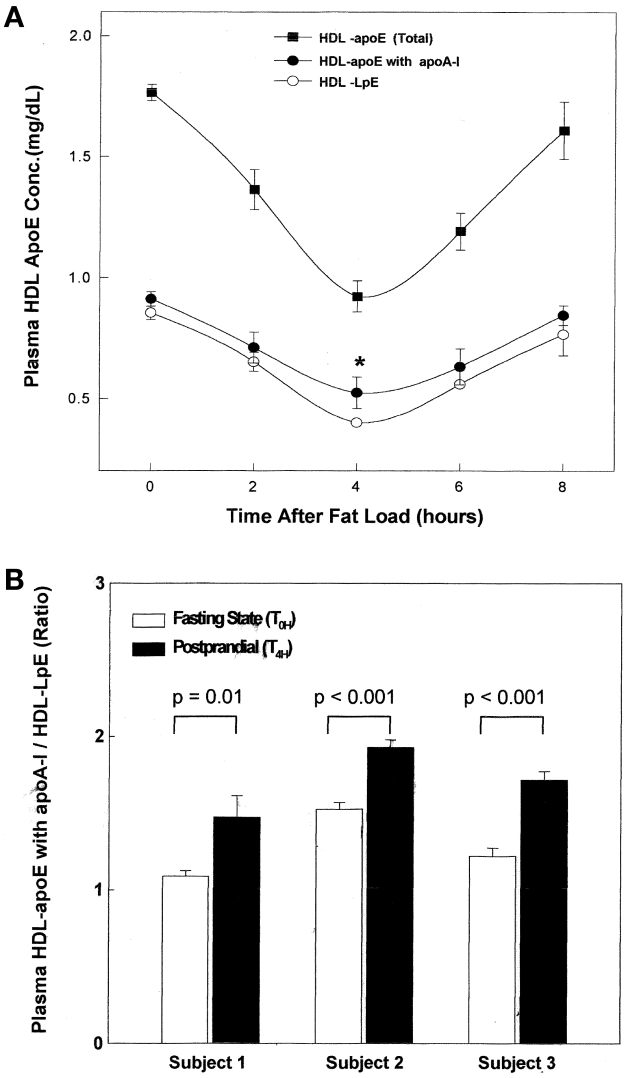

Fig. 5. Plasma concentration of apoE associated with HDL-apoE subpopulations in a normolipidemic subject fed an oral fat load. After an overnight fast, subject was given a liquid-cream drink containing 1 g fat per kg body weight. Blood samples were obtained in the fasting state (0 h) and then at 2 h intervals for 8 h. A: Plasma was depleted of apoB by precipitation with PEG (HDL), and then depleted of apoA-I-containing lipoproteins by immunoaffinity at each time point. Plasma apoE concentration in total HDL-apoE and HDL-LpE was determined by ELISA (results for one subject are shown; *, statistical difference at $P = 0.01$). B: The ratio of HDL-apoE with apoA-I to HDL-LpE was determined in the fasting state (0 h) and 4 h after fat load in the three subjects. Plotted values in A and B are mean ± SD of triplicate measures. Subject 1 was sampled in A.

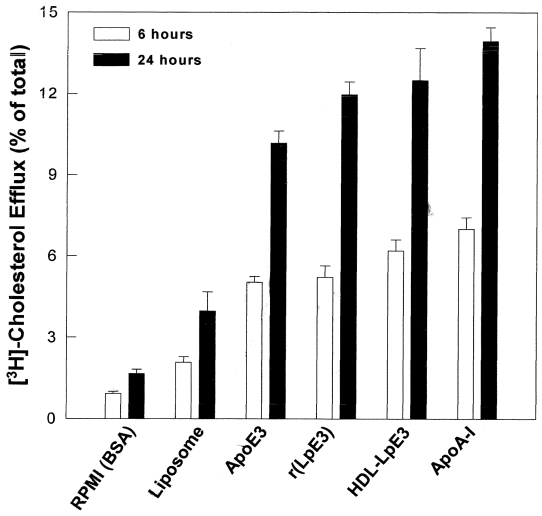

Fig. 6. HDL-LpE-mediated cellular cholesterol efflux from J744 macrophages. J744 cells were incubated with 15 μg/ml [^{3}H]cholesteryl ester-labeled acetyl-LDL in RPMI, 0.2% BSA for 6 h. The cells were then rinsed and incubated with fresh medium containing either 10 μg/ml human plasma purified apoE3, r(LpE3), apoA-I, or immunoaffinity-purified plasma HDL-LpE particles from a normolipidemic apoE3/3 subject for the indicated times. ^{3}H-labeled cpm in the media and cells was measured to calculate percent [^{3}H]cholesterol in the medium.

0.50 ± 0.03, and 0.77 ± 0.05 mg/dl; t_{0H}, t_{4H}, and $t_{4H+heparin}$, respectively). At the same time, in vitro lipolysis of VLDL resulted in a significant formation of HDL-LpE particles, which have preβ electrophoretic mobility on agarose gel corresponding in size to plasma HDL-LpE (data not shown), consistent with the concept that in vivo lipolysis of TRL may generate HDL-LpE particles (13).

The mechanisms by which HDL protects against atherosclerosis are not fully established. Despite the severe reduction of HDL, many patients with hypoalphalipoproteinemia appear not to be at increased risk for coronary artery disease (39, 40). This observation raises the hypothesis that the maintenance of RCT in both HDL-deficient and normal plasma does not depend on the majority of HDL particles but on the presence of effective subfractions involved in the RCT process (41). This concept is supported by the finding that *1*) HDL-LpE particles were present in patients with hypoalphalipoproteinemia (Fig. 4) and *2*) isolated plasma HDL-LpE3 particles efficiently take up cell-derived cholesterol (Fig. 6). Interestingly, γ-LpE, a sphingomyelin-rich lipoprotein that contains apoE as its sole protein component present in HDL-LpE, has been proposed to play a role in cellular cholesterol efflux by acting, like pre-β_1-LpA-I, as initial acceptor of cell-derived cholesterol in normal and HDL-deficient plasma subjects (41). Moreover, it has been documented that plasma of mice lacking apoE has a reduced capacity to promote cholesterol efflux from macrophages; however, low-dose expression of apoE in macrophages nearly restored the cholesterol efflux capacity of apoE-deficient plasma through the formation of apoE-containing HDL particles, which promote cellular cholesterol efflux (42).

It is well established that several pleiotropic biological effects of apoE have been attributed to its ability to modulate cell-signaling events through cell membrane heparan sulfate proteoglycans and/or LDL receptor-related proteins (LRPs) (7, 43, 43). Of interest, the observation of Fagan et al. (44) demonstrating that apoE-containing HDL is a ligand for the LRP receptor, which facilitates cholesterol esterification stimulated by HDL-apoE. Moreover, it was documented that efflux of both cholesterol and phosphatidylcholine promoted by apoE from astrocytes and neurons was abolished following treatment with hepa-

rinase or lactoferrin, which block the interaction of apoE heparan sulfate proteoglycans or LRP (45). On the other hand, a recent study by Li et al. (46) had documented that reconstituted discoidal apoE-phospholipid particles are ligands for the scavenger receptor BI (SR-BI), which mediates both selective CE uptake from the lipoprotein to cells and cellular cholesterol efflux (47).

Our findings demonstrate that HDL-LpE particles were present in plasma of normolipidemic and hypoalphalipoproteinemic subjects. In addition, HDL-LpE participates in plasma transfer of apoE during the postprandial period, and is an efficient acceptor of cell-derived cholesterol. Further study of HDL-LpE interactions with ABCA1, SR-BI, and LRP receptors may provide new insights into the mechanism of cholesterol transport in the central nervous system and in peripheral cells, and the pleiotropic biological effects of apoE in preventing or treating atherosclerotic vascular disease.

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