In vitro factors affecting the concentration of gamma-LpE (γ-LpE) in human plasma

Larbi Krimbou, Michel Tremblay, Hélène Jacques, Jean Davignon, and Jeffrey S. Cohn

Abstract  Gamma-LpE (γ-LpE), a sphingomyelin-rich lipoprotein that contains apolipoprotein (apo) E as its only protein component, has been proposed to play a role in cellular cholesterol efflux by acting, like pre-β1-LpA-I, as an initial acceptor of cell-derived cholesterol. In order to further characterize the presence of γ-LpE in human plasma, we have separated γ-LpE by two-dimensional non-denaturing polyacrylamide-gradient gel electrophoresis and detected its presence by immunoblotting with 125I-labeled polyclonal anti-apoE antibody. Five species of γ-LpE were routinely detected in human plasma, ranging in mean particle diameter from 9.5 to 16.5 nm. The largest proportion of γ-migrating apoE was associated with γ-LpE having a diameter of 13.0 nm. Neither the amount of γ-LpE apoE (representing less than 1–2% of total plasma apoE) nor the number of γ-LpE subfractions was different in serum vs. plasma, or was affected by the presence of agents able to inhibit protein dimerization. γ-LpE subfractions were present in the plasma of patients having different apoE phenotypes (i.e., apoE 2/2, 3/3, or 4/4). Incubation of plasma at 37°C (90 min) caused a significant decrease in plasma γ-LpE (>80%) that was not dependent on LCAT or CETP activity. Storage (at −70°C) of hypertriglyceridemic plasma induced an increase in γ-LpE. Freezing of postprandial plasma samples, containing increased amounts of triglyceride-rich lipoproteins (TRL) enriched in apoE, also caused an increase in γ-LpE. Incubation of VLDL (d < 1.006 g/ml) with lipase resulted in the production of γ-migrating apoE. These results demonstrate that: 1) different γ-LpE subfractions exist in human plasma; 2) the amount of apoE associated with γ-LpE subfractions is dependent on in vitro conditions of plasma storage; and 3) TRL can act as a source of γ-LpE apoE in vitro.

Supplementary key words  HDL • cholesterol efflux • apoE

Apolipoprotein (apo) E is an arginine-rich glycoprotein (34,200 Da) that plays a pivotal role in lipoprotein metabolism (1). It mediates the delivery of lipids to cells by acting as a ligand for a number of cellular lipoprotein receptors, and also participates in the removal of cholesterol from cells by acting as a mediator or acceptor of excess cellular cholesterol (2). The importance of apoE in the pathogenesis of atherosclerosis has been strikingly demonstrated by the presence of spontaneous atherosclerosis in experimental animals made deficient in apoE (3, 4), and conversely by protection or regression of atherosclerosis in apoE-deficient animals supplemented with apoE (5–7).

In normolipidemic plasma, the majority of apoE is associated with apoB or apoA-I-containing lipoproteins (8). Recent studies have, however, demonstrated the existence of lipoproteins similar in size to high density lipoproteins (HDL), containing apoE as their only protein component (e.g., γ-LpE and pre-β1-LpE) (9–11). Gamma (γ)-LpE are spherical plasma lipoproteins, 12–16 nm in diameter, that have γ-mobility when separated by agarose gel electrophoresis (9, 10). γ-LpE are rich in sphingomyelin and apoE and have been proposed to play a role in reverse cholesterol transport by acting, together with pre-β1-LpA-I (small apoA-I-only-containing HDL), as initial acceptors of cell-derived cholesterol (9, 10). γ-LpE and pre-β1-LpA-I are thus regarded as antiatherogenic lipoproteins as they have the ability to potentiate the efflux of excess cholesterol from peripheral cells and to mediate the transport of this cholesterol back to the liver for eventual excretion into the bile (12, 13).

Abbreviations: apo, apolipoprotein; CAD, coronary artery disease; d, density; CETP, cholesteryl ester transfer protein; DTNB, 5,5-dithiobis-2-nitrobenzoic acid; ELISA, enzyme-linked immunosorbent assay; EC, esterified cholesterol; EDTA, ethylene-diamine-tetraacetate; FC, free cholesterol; FPLC, fast protein liquid chromatography; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; PBS, phosphate-buffered saline; RCT, reverse cholesterol transport; TRL, triglyceride-rich lipoprotein; VLDL, very low density lipoprotein.

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The original aim of work in our laboratory was to develop a quantitative assay for γ-LpE and to determine what physiological conditions caused the plasma level of γ-LpE to vary. During the course of this work however, it became evident that there were a number of in vitro factors that could effect the plasma concentration of γ-LpE and that could subsequently interfere with the accurate determination of in vivo levels of γ-LpE. They could also potentially affect the results of functional experiments in vitro. These factors have been investigated in the present study by characterizing the presence of γ-LpE in different plasma samples using a modified, more sensitive two-dimensional gel electrophoresis system to separate plasma apoE-containing lipoproteins with γ-mobility.

MATERIALS AND METHODS

Blood sampling

Blood samples were obtained from male and female subjects who had fasted overnight. The study was approved by the Clinical Research Institute of Montreal and informed consent was obtained from the subjects. Normolipidemic subjects were selected from our laboratory personnel. Hyperlipidemic subjects were from the Lipid Clinic of the Clinical Research Institute of Montreal. Blood was drawn from an arm 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). Plasma was separated from red blood cells by aspiration and was kept in ice until electrophoretic separation of γ-LpE or apoE-containing HDL, which was routinely carried out within 30 min of plasma isolation, except in those experiments where storage conditions were purposely modified. Plasma was stored at 4°C (3–5 days) until the analysis of lipids and apolipoproteins. Table 1 shows lipid and apolipoprotein concentrations of the study subjects. Blood treated with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) was prepared by drawing 2 ml of blood into a syringe containing 0.5 ml of DTNB (50 mg/ml in 0.1 m phosphate buffer, pH 7.4). DTNB plasma was prepared by microcentrifugation (40 sec, 3500 rpm, 4°C). Iodoacetate treatment of plasma was achieved by adding 0.5 ml EDTA plasma (prepared with a 30-sec spin in a microcentrifuge) to 100 ml of iodoacetic acid (5 mg/ml, in 2 m phosphate buffer, pH 8.0), as described previously (14).

Separation of lipoproteins by two-dimensional gel electrophoresis

Separation of plasma lipoproteins by two-dimensional non-denaturing gel electrophoresis was carried out as described previously (11). Briefly, plasma samples (200 μl) with 100 μl Tris-buffer were separated in the first dimension (according to their charge) by 0.75% agarose gel electrophoresis (100 V, 8 h, 4°C), and in a second dimension (according to their size) by 2–15% 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-3a,6a-diphenylglycuril, Pierce Chemical Co., Rockford, IL) (15), was run as a

<table>
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Mean values (±SD) are shown for lipid parameters for subjects A, B, and C, who were sampled on several occasions during a period of 2 years; nd, not determined.
standard on each gel. Electrophoretically separated proteins and lipoproteins were electrophoresed (30 V, 20 h, 4°C) onto nitrocellulose membranes (Hybond ECL, Amersham Life Science, Buckinghamshire, England), and apoE-containing lipoproteins were detected by incubating membranes with immunopurified polyclonal apoE antibody (Genzyme Corp., Cambridge, MA) labeled with 125I (15). The presence of labeled antibody was detected by autoradiography using XAR-2 Kodak film.

A second two-dimensional gel system was also used, which allowed for the \( \gamma \)-LpE of a number of samples to be analyzed together on the same gel. Plasma samples were separated by agarose gel electrophoresis, and the first cm (the \( \gamma \)-migrating region) was excised. Agarose gel slices (7 to 9 in number) were arranged at the top of 3–24% gradient gels, together with agarose gel slices containing radioactively labeled molecular-size standards. They were sealed in place with 0.75% agarose and were equilibrated by electrophoresis at 125 V (20 min, 4°C). Gradient gels were then pre-electrophoresed at 70 V for 1 h, followed by sample separation at 125 V (24 h, 4°C). Gels were transferred and blocked, and \( \gamma \)-LpE was immunolocalized as described before (11). Absence of Coomassie Blue-stained protein in gels after electrotransfer indicated that the efficiency of electrotransfer was close to 100%. In some experiments, films exposed to labeled anti-apoE antibody were scanned with an IS-1000 Digital Imaging System (Molecular Dynamics, Sunnyvale, CA).

Separation of plasma lipoproteins by automated gel filtration chromatography

Plasma lipoproteins were separated by automated gel filtration chromatography on a Pharmacia (Pharmacia LKB Biotechnology, Uppsala, Sweden) fast protein liquid chromatography (FPLC) system, as described previously (16). Plasma samples (1 ml) were manually transferred to a 2-ml sample loop with two washes of 0.5 ml saline solution. They were programmed (Liquid Chromatography Controller LCC-500 Plus) to be loaded and separated on a 50-cm column (16 mm internal diameter) packed with cross-linked agarose gel (Superose 6 prep grade, Pharmacia). The column was eluted with 0.15 mol/l NaCl (0.01% EDTA, 0.02% sodium azide, pH 7.2) at a rate of 1.0 ml/min, and 25 min after addition of sample, 90 × 1 ml fractions were collected sequentially. Sample elution was monitored spectrophotometrically at optical density 280 nm. FPLC fractions were concentrated before electrophoresis using Centricon-4206 concentrators (Amicon, Inc., Beverly, MA).

Measurement of lecithin:cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP) activity

LCAT activity was measured as relative cholesterol esterification achieved during a 16-h incubation of plasma at 37°C, as described previously (17), but with minor modifications. Free \( ^{3} \text{H} \) cholesterol (FC) (0.25 \( \mu \)Ci) in 100 \( \mu \)l ethanol was added to polystyrene tissue-culture wells and was evaporated by a stream of nitrogen. Plasma samples (100 \( \mu \)l) in 100 \( \mu \)l PBS were added to each well, and were incubated at 4°C for 16 h to allow for equilibration of \( ^{3} \text{H} \) FC with endogenous FC pools. \( ^{3} \text{H} \) FC-labeled plasma samples were then incubated at 37°C for 16 h. After stopping the reaction by immersing plates in an ice bath, lipids were extracted with methanol-chloroform 2:1 (v/v) and were spotted onto thin-layer chromatography plates (Merck). Plates were developed in n-hexane-diethyl ether–acetic acid 140:40:2 (v/v/v), and spots corresponding to FC and cholesteryl ester (CE) were cut from the plate and were assayed for radioactivity. Cholesterol esterification was expressed as the difference between the percentage of radioactive cholesterol in esterified form before and after incubation of plasma for 16 h at 37°C. CETP activity was measured as the percent of total tritiated CE \( (^{3} \text{H} \text{CE}) \) transferred from HDL\(_{3}\) to LDL in the presence of plasma aliquots, as described (18). At the conclusion of sample incubations, LDL were separated from HDL by precipitation with 13% polyethylene glycol 6000 1/1 (v/v). CETP activities were expressed as percentages of \( ^{3} \text{H} \text{CE} \) transferred per 16 h of incubation.

Lipid and lipoprotein analyses

Cholesterol and triglyceride concentrations were determined enzymatically on an autoanalyzer (Cobas Mira, Roche). HDL cholesterol concentration was determined by measuring cholesterol in the supernate after heparin-manganese precipitation of apoB-containing lipoproteins in the d > 1.006 g/ml fraction of plasma prepared by ultracentrifugation. Plasma apoB and apoA-I concentrations were measured by nephelometry (Behring Nephelometer 100 Analyzer), and apoE was determined by enzyme-linked immunosorbent assay (16). Plasma HDL apoE concentration was determined by measuring apoE in the supernate after precipitation of plasma apoB-containing lipoproteins with an equal volume of 13% (w/v) polyethylene glycol 6000 (19). ApoE phenotypes were determined by immunoblotting of plasma separated by minigel electrophoresis (20).
RESULTS

Two-dimensional gel electrophoresis was used to separate γ-LpE and other HDL-sized lipoproteins containing apoE from human plasma, as shown in Fig. 1. Lipoproteins from plasmas obtained after an overnight fast were separated according to their charge in a horizontal direction (negative to positive, left to right), and according to their size in a vertical direction (large to small, top to bottom). The separation of protein standards of known molecular diameter is shown on the left-hand side of each gel. Plasma samples were kept in ice and were separated by electrophoresis within 45 min of isolation, in order to avoid in vitro redistribution of apoE among different HDL subfractions (11). Triglyceride-rich lipoproteins (TRL), including chylomicrons and VLDL, were too large to enter the non-denaturing polyacrylamide gel and were therefore not detected. As shown by previous work in our laboratory, the majority of apoE associated with HDL-sized lipoproteins migrated with pre-β-mobility, and these lipoproteins were designated pre-β1- and pre-β2-LpE (Fig. 1) (11). A smaller proportion of HDL apoE migrated with α-mobility and these lipoproteins were designated α-LpE. γ-LpE (representing 5% or less of total HDL apoE) migrated between the origin and β-migrating LDL in the agarose gel run in the first dimension (indicated on the left of each gel). Because the quantity of apoE in γ-LpE compared to apoE in other HDL fractions was relatively small, membranes incubated with radioactive antibody had to be radiographically overexposed in order for γ-LpE lipoproteins to be detected. In general, 25–33% of plasma samples separated with this system did not have detectable amounts of γ-LpE.

For this reason, a more sensitive method was developed whereby only the first cm of each agarose gel was separated in the second dimension (see Methods). This approach had two advantages: 1) a larger number of samples could be run together on the same gel, and 2) membranes could be exposed to radiographic films for longer periods of time, allowing for optimal visualization of γ-LpE. This modified method for the separation of γ-LpE was used in all subsequent experiments.

γ-LpE lipoproteins have been shown by electron microscopy to be spherical particles ranging in size between 12 and 16 nm (9). In order to confirm this size distribution, plasma was separated by gel filtration chromatography on an FPLC system. γ-LpE in whole plasma and in pooled FPLC elution fractions was separated by the modified two-dimensional gel system (top panel, Fig. 2), and presence of TRL, LDL, and HDL in eluted fractions was determined by monitoring the elution profile of triglyceride, cholesterol and apoE (bottom panel of Fig. 2). Plasma used in this experiment was from the same normolipidemic subject (subject A), whose complete two-dimensional HDL apoE profile is shown in Fig. 1 (left-hand panel). The majority of γ-LpE eluted in fractions 31 to 42, which corresponded to fractions containing lipoproteins intermediate in size between LDL and HDL. These fractions also contained the bulk of plasma HDL apoE. In contrast to previous work showing that γ-LpE migrated in non-denaturing polyacrylamide gels as a disperse band with an apparent particle size of 14 ± 3 nm (9), five different bands or spots of γ-LpE were routinely observed when whole plasma was separated with our modified gel system. These γ-LpE subfractions were designated γ1-LpE, γ2-LpE, γ3-LpE, γ4-LpE, and γ5-LpE, as indicated in Fig. 2 (different subfractions can be more clearly seen for the same plasma sample in Fig. 3). The largest proportion of γ-migrating apoE was associated with γ2-LpE, which for subject A had an average particle size of 13.1 nm. Plasma IgG (γ-globulin) was found to migrate in exactly the same position as γ2-LpE. TheapoE migrated between the origin and LpE.

\[ \text{apoE} \] migrated between the origin and LpE.

- mobility and these lipoproteins were designated a\(^{11}\). A smaller proportion of HDL apoE migrated with 864 Journal of Lipid Research
presence of $\gamma_2$-LpE did not, however, depend on IgG, as removal of IgG from plasma samples by affinity chromatography did not reduce the amount of apoE detected in this position. A clear separation between $\gamma_2$-LpE and $\gamma_3$-LpE was not always achieved, although these subfractions were better resolved when films were exposed for shorter time periods (see Fig. 3). $\gamma_3$-LpE always appeared as a diffuse area of immunoreactivity above $\gamma_2$-LpE. It was difficult to ascertain whether this material represented a distinct subfraction, but as it made a significant contribution to total $\gamma$-LpE immunoreactivity, it was distinguished from $\gamma_2$-LpE. The two lighter spots with a particle diameter less than 12 nm (representing $\gamma_4$-LpE and $\gamma_5$-LpE) were always present, provided films were sufficiently exposed. $\gamma$-LpE subfractions were not clearly resolved after plasma was separated by gel filtration chromatography (Fig. 2), possibly due to the concentration procedure used to reduce the volume of pooled FPLC fractions.

Plasma from subject A was analyzed for $\gamma$-LpE on six different occasions (during a 24-month period). These gel separations were compared in order to determine the reproducibility of our gel system, and to determine the variability over time of $\gamma$-LpE subfraction distribution in the same individual. Four of these gels are shown in Figs. 2, 3, 5, and 6. At the same time, results from two normolipidemic subjects (subjects B and C, Table 1) were compared, in order to determine whether $\gamma$-LpE subfractions were similar in different individuals. The relative distribution of apoE among $\gamma$-LpE subfractions was measured by densitometric scanning of radiographic films and mean particle diameters were deter-
minded according to computer-generated regression curves derived by measuring the migratory distance of protein standards. Results shown in Table 2 demonstrate that five \( \gamma \)-LpE subfractions were consistently present in the plasma of the three subjects. The largest subfraction (\( \gamma_1 \)-LpE) had an average particle size of 16.4 nm and the smallest subfraction (\( \gamma_5 \)-LpE) had an average particle size of 9.9 nm. The largest proportion of \( \gamma \)-migrating apoE was consistently found associated with \( \gamma_2 \)-LpE (having an average particle diameter of 13.3 nm), and the remainder tended to be equally divided between the other subfractions.

The question was raised whether different \( \gamma \)-LpE subfractions were an in vitro phenomenon, or whether they were produced in vivo during the preparation of plasma samples. An experiment was therefore carried out in which \( \gamma \)-LpE was separated from blood processed in different ways. Although small differences were observed in the total amount of apoE associated with \( \gamma \)-LpE, a similar pattern of \( \gamma \)-LpE subfractions was observed: a) in EDTA-plasma compared to serum (lane a vs. lane e, Fig. 3), b) in plasma or serum centrifuged for a shorter time period than was customary (lane a vs. lane c, DTNB-treated plasma, prepared by drawing 2 ml of blood into a syringe containing the sulfhydryl reagent DTNB (0.5 ml containing 50 mg/ml DTNB in 0.1 M phosphate buffer, pH 7.4) and 2.5 mg EDTA; blood was centrifuged in a microfuge (40 sec, 3500 rpm, 4°C); lane d, iodoacetic acid-treated plasma, prepared by adding 0.5 ml EDTA plasma (prepared within 1 min) to 100 \( \mu l \) of iodoacetic acid (5 mg/ml in 2 m phosphate buffer, pH 8.0); lane e, serum, prepared by allowing blood kept on ice to clot for 30 min, followed by centrifugation for 15 min (3000 rpm, 4°C); lane f, serum, prepared by allowing blood kept on ice to clot for 30 min, followed by centrifugation for 40 sec in a microfuge (3500 rpm, 4°C). Molecular size markers were separated in the right-hand lane and correspond to those labeled in Fig. 2.

It has been suggested by Huang et al. (10) that \( \gamma \)-LpE is immunologically undetectable in plasma not containing the apoE3 isoform. Using conventional two-dimensional gels to separate plasma apoE-containing HDL-sized lipoproteins, we have, however, routinely observed \( \gamma \)-LpE in subjects with an apoE 2/2 phenotype (lacking apoE3) and also (though to a much lesser extent) in subjects with an apoE 4/4 phenotype (data not shown). With the more sensitive modified gel system, \( \gamma \)-LpE was detected (with few exceptions) in both normolipi-
emic subjects and hyperlipidemic patients, irrespective of apoE phenotype, as exemplified in Fig. 4 (subjects E to K, Table 1). The amount of apoE associated with γ-LpE of apoE 4/4 subjects was less than for other subjects, perhaps reflecting their significantly lower HDL apoE levels. An example of an apoE 4/4 subject with almost no detectable γ-LpE is shown in Fig. 4, lane a, right-hand panel. Particle size of γ-LpE subfractions was similar in patients with different apoE phenotypes; however, the distribution of apoE between subfractions was variable, often resulting in more apoE in smaller γ-LpE subfractions (e.g., increase of γ3-LpE and γ4-LpE in lane a of left-hand panel and γ4-LpE in lane b of right-hand panel).

During the course of the present study, we noticed that the presence of γ-LpE in isolated plasma samples was dependent on the time and temperature of sample storage. This is demonstrated by results in Fig. 5 for two separate experiments (panels A and B) carried out with the plasma of subjects A and B, respectively. When plasma was left at room temperature (22°C) for 90 min, concentration of γ-LpE (lane b) decreased more than 50% relative to γ-LpE in plasma kept on ice for the same time period (lane a). Incubation of plasma at 37°C for 90 min resulted in an even greater (>80%) decrease in γ-LpE (lane c). Longer periods of incubation up to 16 h resulted in a continued absence of γ-LpE that was evident even after only 1 min of incubation (~10% decrease). When plasma was incubated in the presence of cells (cultured fibroblasts), the disappearance of γ-LpE was prevented (data not shown). In order to determine whether the decrease in γ-LpE was due to conversion of γ-LpE into another lipoprotein as a consequence of cholesterol esterification or cholesteryl ester transfer, plasma was incubated with LCAT inhibitor (DTNB), CETP inhibitor (anti-CETP antibody, TP2) (21), or both (lanes e, f, and g, respectively). In each case, decrease in γ-LpE was not prevented, despite almost total inhibition of LCAT or CETP activity, as demonstrated by the data in Table 3.

The effect of freezing and plasma storage at −70°C on the presence in plasma of γ-LpE was also investigated. Freezing of normolipidemic plasmas had no effect on the size or relative amount of different γ-LpE subfractions. In contrast, freezing and storage of hypertriglyceridemic samples consistently resulted in a significant increase in the concentration of γ-LpE, particularly γ2-LpE, γ3-LpE, and γ5-LpE (data not shown). This was predominantly due to freezing and thawing of samples rather than time of storage, as hypertriglyceridemic samples stored in ice for 7 days displayed little change in γ-LpE concentration. These results demonstrated that freezing and thawing of plasma samples containing high concentrations of TRL (and hence increased amounts of TRL apoE (16)), led to the formation in vitro of γ-LpE similar in size to the γ-LpE found in fresh plasma.

Further evidence for the in vitro formation of γ-LpE

![Fig. 4. Presence of γ-LpE in the plasma of subjects with different apoE phenotypes. γ-LpE was prepared from freshly isolated plasma, as described in Methods. Plasma samples were from patients described in Table 1; left-hand panel, a and b: subjects E and F; middle panel, a and b: subjects G and H; right-hand panel, a, b, and c: subjects I, J, and K, respectively. Molecular size markers were separated in the right-hand lanes for two of the gels and correspond to those labeled in Fig. 2.](image-url)
plasma samples demonstrated that the plasma concentration of \( \gamma \)-LpE tended to decrease after the fat load. On the other hand, analysis of frozen samples for all three subjects showed an increase in the presence of \( \gamma \)-LpE, reflecting the in vitro formation of \( \gamma \)-LpE in plasma samples with increased levels of postprandial TRL.

In order to provide direct evidence that TRL could act as a source of \( \gamma \)-LpE in vitro, VLDL were isolated from plasma by ultracentrifugation (d < 1.006 g/ml) and \( \gamma \)-LpE was separated from fresh and frozen plasma and VLDL samples. Results of one experiment are shown in Fig. 7, panel A. The plasma of a very hypertriglyceridemic patient was analyzed (subject L, Table 1), who had almost undetectable levels of \( \gamma \)-LpE in fasting plasma. \( \gamma \)-LpE was separated from plasma stored in ice for 24 h (lane a) or stored at \(-70^\circ\)C for 24 h (lane b). Lipoprotein fractions were prepared by ultracentrifugation (d > 1.006 g/ml and d < 1.006 g/ml) and were run in lanes c and d, respectively. The VLDL (d < 1.006 g/ml) and d < 1.006 g/ml) and were run in lanes c and d, respectively. The VLDL (d < 1.006 g/ml) fraction was frozen (8 h at \(-70^\circ\)C) and was separated in lane e. \( \gamma \)-LpE was evident in frozen but not fresh plasma, and in this particular case, only \( \gamma_4 \)-LpE and \( \gamma_5 \)-LpE subfractions were generated by freezing. A similar pattern of \( \gamma \)-LpE bands was evident in frozen VLDL, but not in unfrozen VLDL. In a second experiment, plasma and VLDL samples were subjected to in vitro lipolysis. Plasma from subject M (Table 1) was kept on ice until separation (lane a); 2) incubated for 2 h at 37°C (lane b), or 3) incubated for 2 h at 37°C with lipase (lane c). VLDL (d < 1.006 g/ml) was similarly incubated for 2 h at 37°C (lane d), or incubated in the presence of lipase (lane e). As shown before (Fig. 5), incubation of plasma resulted in a considerable decrease in the presence of \( \gamma \)-LpE that was not reversed by the presence of lipase. In contrast, lipolysis of VLDL resulted in a significant increase in \( \gamma \)-LpE, which (in this particular patient) corresponded in size to \( \gamma_2 \)-LpE.

**DISCUSSION**

The results of the present study have demonstrated the existence of different subfractions of \( \gamma \)-LpE in the plasma of normolipidemic and hyperlipidemic subjects. These subfractions contained particles having hydrated diameters ranging from 9.5 to 16.5 nm and were intermediate in size between LDL and HDL (Fig. 2). Of the five subspecies of \( \gamma \)-LpE detected in normolipidemic plasma, \( \gamma_2 \)-LpE (having a mean particle diameter of 13.0 nm) accounted for the largest proportion of
γ-migrating apoE (Table 2). Although the existence of different γ-LpE subspecies has not been reported previously, the present data are consistent with previous work, which showed that the particle diameter of γ-LpE ranged from 12 to 16 nm when measured by electron microscopy and was 14 ± 3 nm when determined by non-denaturing polyacrylamide gel electrophoresis (9). The amount of apoE associated with γ-LpE is, however, very small, representing 5% or less of apoE in HDL-sized lipoproteins (Fig. 1) (11). By electroeluting γ-LpE from two-dimensional gels and quantitating apoE by ELISA, we estimated the plasma concentration of apoE in γ-LpE to be 0.1–1.0 μg/ml for normolipidemic subjects (n = 10, data not shown), representing only 1–2% of total plasma apoE. γ-LpE is thus, in quantitative terms, a very minor plasma lipoprotein fraction, which may explain why these particles have not always been detectable after separation by routine two-dimensional gel electrophoresis (10, 22).

Although it cannot be ruled out that different γ-LpE subfractions were produced in vitro during preparation of plasma from isolated blood or during separation of plasma by electrophoresis, the present results suggest that different-sized γ-LpE particles are a characteristic of human plasma in vivo. Neither the number nor the amount of apoE in γ-LpE subfractions was found to be different in serum versus plasma. The amount of apoE in different subfractions was also not affected by the length of time taken to centrifuge blood for the preparation of serum or plasma samples (Fig. 3). γ-LpE subfractions were not the result of apoE dimerization, as the presence of agents able to inhibit dimerization did

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<td>Anti-CETP (TP2)</td>
<td>33.5 ± 5.7</td>
<td>nd</td>
<td>37.3 ± 4.1</td>
<td>nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTNB + TP2</td>
<td>0.6 ± 0.1</td>
<td>nd</td>
<td>0.5 ± 0.1</td>
<td>nd</td>
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Values represent mean ± SD (n = 3); nd, not detectable. *Plasma was incubated in the presence and absence of reagents as described in Methods and in the Legend to Fig. 5.

**Fig. 6.** Effect of an oral fat load on the plasma concentration of γ-LpE. Subject A (Table 1) was given a liquid cream drink containing 1 g of fat per kg body weight. Blood samples were obtained in the fasting state (T0H) after a 12-h overnight fast, and at 2-h intervals after the fat load (T2H, T4H, T6H, T8H). Plasma samples were kept on ice until the end of the experiment and were then separated together on the same gel by electrophoresis (panel A). Samples were also frozen and stored at −70°C for 7 days before electrophoretic separation (panel B).
not affect the relative distribution of γ-LpE apoE (Fig. 3). This is consistent with the presence of γ-LpE subfractions in the plasma of subjects with an apoE 4/4 phenotype (Fig. 4), even though apoE4 cannot dimerize due to the absence of cysteine residues. Furthermore, smaller γ-LpE subfractions were not a product of enzymatic proteolysis, as plasma left at room temperature for 90 min or plasma incubated at 37°C for 90 min, caused a decrease rather than increase in the amount of apoE associated with γ-LpE (Fig. 5).

The apparent though very consistent disappearance of γ-LpE from plasma incubated at 37°C (Fig. 5 and also Fig. 7) is in contrast to the results of von Eckardstein et al. (23), who found that γ-LpE did not disappear from plasma during a 16-h incubation. The equal relative disappearance of apoE from all γ-LpE subfractions in the present study (Fig. 5) suggests that incubation of plasma did not result in conversion of one γ-LpE subfraction to another nor transfer of apoE from one subfraction to another. Disappearance of γ-LpE was prevented when plasma was incubated in the presence of cells (data not shown), suggesting a role of membrane phospholipid in maintaining plasma γ-LpE levels. The lack of effect of LCAT or CETP inhibition demonstrated that cholesterol esterification and cholesterol ester transfer were not responsible for the disappearance of γ-LpE. This is in contrast to the behavior of pre-βLpA-1, whose disappearance from human plasma during incubation at 37°C is prevented by inhibition of cholesterol esterification (24). The reason for disappearance of γ-LpE from incubated plasma is, therefore, not clear, although we favor the explanation that apoE is transferred to other plasma lipoproteins (with or without bound lipid, e.g., sphingomyelin), perhaps by the same mechanism that is responsible for apoE disappearance from pre-βLpA-1 during plasma incubation (11). The fact that more than 50% of γ-LpE in plasma can disappear even at room temperature (Fig. 5) points out that untreated plasma and stored at or near 0°C, in order to achieve a reproducible separation of γ-LpE.

In contrast to the consistent decrease in the amount of γ-LpE caused by incubation of plasma, γ-LpE increased (though not consistently) in plasma samples frozen and stored at −70°C. This inconsistency was found to be dependent on the type of plasma studied, such that normolipidemic samples frozen for either short or long periods of time had very similar concentrations of γ-LpE, in contrast to hypertriglyceridemic samples, in which considerably more apoE was associated with γ-LpE after freezing. In general, increase in γ-LpE was not a function of storage time at −70°C, but was rather a function of the freezing process itself or, alternatively, an effect of thawing, i.e., samples were brought to room temperature over a 40-min period by leaving them on the bench. This was particularly apparent for fresh and frozen plasma samples that contained increased amounts of postprandial TRL after the ingestion of a fat-rich meal (Fig. 6). The amount of γ-LpE in plasma was found to decrease after the fat load when fresh samples were analyzed. This is consistent with the decrease in plasma HDL apoE concentration after an oral fat load (25) and the postprandial decrease in plasma concentration of all apoE-containing HDL subtypes (11). Analysis of frozen samples, however, indicated an increase in the presence of γ-LpE. This was interpreted as evidence for the formation during freezing.
or thawing of γ-LpE from increased amounts of apoE-containing TRL in postprandial samples through conversion of TRL into γ-LpE or exchange of apoE from TRL to γ-LpE. As the relative amount of sialylated apoE is increased in the VLDL fraction of hypertriglyceridemic subjects and in the VLDL fraction of subjects fed a fat load (26), it is possible that this more negatively charged form of apoE has a greater tendency to associate with γ-LpE (this is currently being investigated). Significantly, freezing of postprandial samples did not result in a large number of nonspecific γ-migrating particles, but resulted instead in the formation of characteristic γ-LpE subspecies (e.g., γ2-LpE and γ3-LpE). Freezing of ultracentrifugally isolated VLDL fractions or in vitro lipolysis of VLDL (Fig. 7) provided additional support for the concept that TRL could act as a source of γ-LpE. The formation of HDL-sized lipoproteins containing apoE due to the in vitro lipolysis of VLDL has been demonstrated previously (27, 28). Whether formation of γ-LpE from TRL is of physiological significance or whether a relationship between TRL-apoE and γ-LpE exists in vivo remains, however, to be determined.

It has previously been shown that apoE isoforms are an important determinant of cholesterol efflux from cultured fibroblasts (10). γ-LpE of plasma containing only apoE3 isoform accumulated considerably more cellular free cholesterol during a 1-min incubation than did γ-LpE from plasma containing apoE2 or apoE4 isoforms. γ-LpE was, in fact, found to be immunologically undetectable in the plasma of apoE 2/2 or 4/4 subjects (10), leading to the suggestion that apoE4 is incapable of forming γ-LpE (29). In contrast, we have found γ-LpE particles of characteristic size in the plasma of both normolipidemic and hyperlipidemic apoE 4/4 individuals, as well as in the plasma of patients with an apoE 2/2 or 3/3 phenotype (Fig. 4). The γ-LpE of subjects or patients with an apoE 4/4 phenotype were, however, more difficult to detect than the γ-LpE of apoE 2/2 or 3/3 individuals, possibly due to the lower concentration of total plasma HDL apoE in apoE 4/4 individuals (Table 1), the preference of apoE4 to associate with TRL (30), or to the greater proportion of apoE found associated with smaller γ-LpE particles (i.e., γ4-LpE and γ5-LpE) in apoE 4/4 individuals.

Finally, it is important to note that although in vitro evidence has been presented demonstrating the involvement of γ-LpE in cellular cholesterol efflux (9, 10), it remains controversial whether these particles play a similar role in vivo. Recent studies using plasma from apoE-deficient mice and humans have questioned the quantitative importance of apoE-only-containing lipoproteins in mediating cellular cholesterol efflux, and have reiterated the significance of apoA-I- and apoA-II-containing lipoproteins (31). The present study was not designed to answer this controversy, but does provide evidence that γ-LpE has limited stability, and this may be an explanation for conflicting results from different laboratories. Future studies are thus required to determine: a) how factors affecting the plasma concentration of γ-LpE in vitro can alter the ability of plasma to mediate cholesterol efflux from cultured cells; b) the role of different γ-LpE subspecies in cellular cholesterol efflux, and c) the mechanisms responsible for the formation and degradation of γ-LpE.

In conclusion, the present study has provided evidence for the presence of different-sized γ-LpE particles in the plasma of subjects with different apoE phenotypes. Various in vitro factors have been found to affect the amount of γ-LpE in isolated plasma samples, which points out the importance of having controlled conditions of plasma preparation and storage in order to study the function of γ-LpE. In addition, our results suggest a link between triglyceride-rich lipoproteins and the formation of γ-LpE in human plasma, the significance of which deserves further investigation.

The technical assistance of Nancy Doyle is gratefully acknowledged. We would like to thank Genzyme Corporation for kindly providing immunopurified polyclonal human apoE antibody and also Dr. Ross Milne for his gift of TP2 anti-CETP antibody. We would like to thank the head nurse of the IRCM primary prevention lipid clinic, Denise Dubreuil, for her assistance in obtaining blood samples. This work was supported by a joint University-Industry grant (PA-14006) from the Medical Research Council of Canada and Parke-Davis, and by La Succeision J. A. De Sève. Dr. Cohn was supported by a grant from the Heart and Stroke Foundation of Quebec.

Manuscript received 21 July 1997 and in revised form 3 November 1997.

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