Apolipoprotein E Enhances Hepatic Lipase-Mediated Hydrolysis of Reconstituted High-Density Lipoprotein Phospholipid and Triacylglycerol in an Isoform-Dependent Manner†

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ABSTRACT: This study compares the kinetics of hepatic lipase (HL)-mediated phospholipid and triacylglycerol hydrolysis in spherical, reconstituted high-density lipoproteins (rHDL) that contain either apolipoprotein E2 (apoE2), apoE3, apoE4, or apoA-I as the sole apolipoprotein. HL-mediated phospholipid hydrolysis was assessed by incubating various concentrations of rHDL that contained only cholesteryl esters (CE) in their core, (E2/CE)rHDL, (E3/CE)rHDL, (E4/CE)rHDL, and (A-I/CE)rHDL, with a constant amount of HL. The rate of phospholipid hydrolysis was determined as the formation of nonesterified fatty acid mass. HL-mediated triacylglycerol hydrolysis was assessed in rHDL containing CE, unlabeled triacylglycerol, and [^{3}H]triacylglycerol in their core, (E2/TG)rHDL, (E3/TG)rHDL, (E4/TG)rHDL, and (A-I/TG)rHDL. Triacylglycerol hydrolysis was determined as the ratio of ^{3}H-labeled hydrolysis products to ^{3}H-labeled unhydrolyzed triacylglycerol. The rates of phospholipid hydrolysis in the (E2/CE)rHDL, (E3/CE)rHDL, and (E4/CE)rHDL were significantly greater than that in the (A-I/CE)rHDL. The rates of triacylglycerol hydrolysis were also greater in the (E2/TG)rHDL, (E3/TG)rHDL, and (E4/TG)rHDL compared to the (A-I/TG)rHDL, although to a lesser degree than observed with phospholipid hydrolysis. Furthermore, the rates of both phospholipid and triacylglycerol hydrolyses were greater in the (E2)rHDL than in either the (E3)rHDL or the (E4)rHDL. These results show that apoE increases the rate of HL-mediated phospholipid and triacylglycerol hydrolysis in rHDL and that this influence is isoform dependent.

Hepatic lipase (HL)[1] is a 66 kDa glycoprotein that is synthesized by the liver (*1*) and macrophages (*2*). HL is also found in the ovaries and adrenals; however, there is no evidence of synthesis at these sites (*3*, *4*). HL hydrolyzes acyl−ester bonds of triacylglycerols and the *sn*-1 acyl−ester bond of phospholipids. The substrates of HL include chylomicron remnants (*5*, *6*), very low-density lipoproteins (*7*), intermediate-density lipoproteins (*8*, *9*), low-density lipoproteins (LDL) (*9*, *10*), and high-density lipoproteins (HDL) (*11*−*15*). Inverse relationships between HL activity and plasma HDL concentrations in transgenic animal studies (*16*, *17*) and in humans (*18*−*20*) demonstrate that HL has a major role in HDL metabolism.

We have shown previously that the interaction of HL with HDL is influenced by apolipoprotein A-I (apoA-I) and apoA-II, the main apolipoproteins in HDL (*21*, *22*). This was achieved by using spherical reconstituted HDL (rHDL) of defined size and apolipoprotein content. The results of those studies showed that the V_{max} for phospholipid and triacylglycerol hydrolysis was greater in rHDL containing apoA-I as the sole apolipoprotein, (A-I)rHDL, than in (A-II)rHDL. The rate of phospholipid hydrolysis was intermediate in rHDL containing both apoA-I and apoA-II, (A-I/A-II)rHDL. Those studies also established that apolipoproteins regulate the affinity of HL for HDL, with the affinity of HL for the phospholipids and triacylglycerol in (A-II)rHDL being greater than for the phospholipids and triacylglycerol in (A-I)rHDL. The affinity of HL for the phospholipids in (A-I/A-II)rHDL was intermediate between those of (A-I)rHDL and (A-II)rHDL.

ApoE is polymorphic and exists as three different isoforms (apoE2, apoE3, and apoE4), which are products of a single gene (*23*). ApoE is involved in HDL metabolism as demonstrated by decreased plasma clearance of HDL-cholesteryl ester (CE) in apoE knock-out mice (*24*). Further evidence for a role of apoE in HDL metabolism is provided

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[1] Abbreviations: HL, hepatic lipase; LDL, low-density lipoproteins; HDL, high-density lipoproteins; rHDL, reconstituted high-density lipoproteins; apo, apolipoprotein; CE, cholesteryl esters; LCAT, lecithin: cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; UC, unesterified cholesterol; [^{3}H]UC, [1α, 2α-^{3}H]cholesterol; IPTG, isopropyl β-D-thiogalactopyranoside; DMPC, dimyristoylphosphatidylcholine; BSA, bovine serum albumin; NEFA, nonesterified fatty acids; TBS, Tris-buffered saline; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate; SEM, standard error of the mean.

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by antibodies directed against the receptor-binding domain of apoE, which reduce selective uptake of HDL-CE by HepG2 cells (*25*). ApoE also regulates lecithin:cholesterol acyltransferase (LCAT)-mediated cholesterol esterification in HDL (*26*).

In normolipidemic humans, the majority of plasma apoE is transported as a component of HDL (*27*, *28*). A number of HDL subpopulations that contain apoE, but not apoA-I, have been identified in human plasma (*29*−*32*). These HDL exhibit either γ-, pre-β-, or α-mobility when subjected to agarose gel electrophoresis. The γ-migrating apoE-containing HDL are large, spherical particles that mediate cholesterol efflux from cells as efficiently as apoA-I-containing HDL (*29*).

The aim of this study was to determine how apoE influences the ability of HL to hydrolyze HDL phospholipids and triacylglycerol. To this end we compared HL-mediated phospholipid and triacylglycerol hydrolysis in spherical rHDL containing either apoE2, apoE3, apoE4, or apoA-I as the sole apolipoprotein. The results show that the rates of HL-mediated phospholipid and triacylglycerol hydrolysis were greater in apoE-containing rHDL compared to (A-I)-rHDL and that apoE influenced the hydrolysis rates in an isoform-dependent manner.

EXPERIMENTAL PROCEDURES

Isolation of ApoA-I and ApoA-II. HDL were obtained from expired, autologously donated human plasma (Gribbles Pathology, Adelaide, Australia) by sequential ultracentrifugation ($1.07 < d < 1.21$ g/mL) (*33*). The HDL were delipidated (*34*), and apoA-I and apoA-II were isolated from the resulting apoHDL by anion exchange chromatography on a column of Q-Sepharose Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) attached to an FPLC system (*35*). The isolated apoA-I and apoA-II appeared as single bands following electrophoresis on a homogeneous 20% SDS−polyacrylamide PhastGel (Amersham Pharmacia Biotech) and Coomassie staining.

Preparation of LCAT and Cholesteryl Ester Transfer Protein (CETP). LCAT was isolated from pooled samples of expired, autologously donated human plasma (*36*). Activity was assessed using discoidal rHDL containing 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) (Avanti Polar Lipids, Alabaster, AL), unesterified cholesterol (UC) (Sigma), tracer amounts of [1α,2α-^{3}H]cholesterol ([^{3}H]UC) (Amersham Pharmacia Biotech), and apoA-I as the substrate (*37*). The assay was linear as long as <30% [^{3}H]UC was esterified. The activity of the LCAT preparations used in this study ranged from 1866 to 2860 nmol of CE generated/mL of LCAT/h. The isolated LCAT appeared as a single band on a silver-stained homogeneous 20% SDS gel.

CETP was also isolated from pooled samples of human plasma (*38*). Activity was determined as the transfer of [^{3}H]-CE from [^{3}H]CE-HDL_3 to ultracentrifugally isolated LDL (*39*, *40*). The assay was linear as long as <30% [^{3}H]CE was transferred from HDL_3 to LDL. The activity of the preparation used in this study (expressed in units per milliliter, with 1 unit being the transfer activity of 1 mL of a preparation of pooled human lipoprotein-deficient plasma) was 12.9 units/mL.

Production of Recombinant ApoE2, ApoE3, and ApoE4. ApoE2, apoE3, and apoE4 were obtained by bacterial overexpression as previously described (*41*, *42*). Briefly, apoE-containing pET32a vectors (Novagen, Madison, WI) with thioredoxin as the fusion partner were transformed into *Escherichia coli* strain BL21-CodonPlus (Stratagene, La Jolla, CA). Vectors containing human cDNA for either apoE2, apoE3, or apoE4 were kindly provided by Dr. Karl Weisgraber, Gladstone Institute of Cardiovascular Disease, University of California, San Francisco, CA. Protein expression in bacterial cultures was induced with isopropyl β-D-thioglactopyranoside (IPTG). The cells were harvested by centrifugation (*41*) and the pellet resuspended in 30 mL of ice-cold buffer (10 mM Tris-HCl, 0.25 M NaCl, 1 mM EDTA-Na_2, pH 7.6). The apoE−thioredoxin complex was obtained by sonicating the cells for 4×2 min and removing the cell debris by centrifugation at 32 000 rpm for 20 min using a TLA-100.4 rotor (Beckman Instruments, Fullerton, CA). Small unilamellar dimyristoylphosphatidylcholine (DMPC) vesicles (600 mg at 50 mg/mL in 10 mM Tris-HCl buffer, pH 7.6) were combined with the apoE−thioredoxin complex at 24 °C. The mixture was incubated for 16 h at 24 °C. The density of the apoE−thioredoxin/DMPC mixture was raised to 1.21 g/mL and overlayed with 1.125 and 1.006 g/mL density solutions prior to centrifugation at 55 000 rpm for 16 h at 15 °C in a 55.2 Ti rotor (Beckman Instruments). The apoE−thioredoxin/DMPC complexes were collected and dialyzed against 0.1 M ammonium bicarbonate (3×1 L). Thioredoxin was cleaved from the apoE with thrombin (1:10, thrombin/apoE, w/w). The apoE/DMPC complexes were lyophilized and delipidated. ApoE was isolated by gel permeation chromatography on a column of Sephacryl S-300 (Amersham Pharmacia Biotech) (*42*). The isolated apoE appeared as a single band following electrophoresis on a homogeneous 20% SDS−polyacrylamide PhastGel.

Purification of HL. HL was purified from the blood of patients injected with a bolus of 25 000 IU of heparin prior to undergoing angioplasty (Cardiovascular Investigational Unit, Royal Adelaide Hospital) (*21*, *22*). The purified HL appeared as a single band following electrophoresis on a homogeneous 20% SDS−polyacrylamide PhastGel and staining with Coomassie Blue.

HL activity was assessed as the nanomoles of nonesterified fatty acids (NEFA) generated per milliliter of HL per hour using 20% triacylglycerol Intralipid (Fresenius Kabi, Graz, Austria) as a substrate. Purified HL (40 μL) was incubated at 37 °C for 1 h with Intralipid (final triacylglycerol concentration of 0.1 mmol/L) and fatty acid-free bovine serum albumin (BSA) (final concentration of 20 mg/mL) in the presence or absence of 1 M NaCl. The final incubation volume was 200 μL. NEFA mass generated during the incubation was measured by an enzymatic colorimetric assay (Wako Pure Chemical Industries, Osaka, Japan). Because the NEFA generated in the presence and absence of 1 M NaCl were identical, the HL was judged to be free of lipoprotein lipase activity.

Preparation of (E2/CE)rHDL, (E3/CE)rHDL, (E4/CE)-rHDL, and (A-I/CE)rHDL. Discoidal rHDL containing POPC, UC, and either apoE2, apoE3, apoE4, or apoA-I were prepared according to the cholate dialysis method (*43*). Spherical rHDL containing CE as the only core lipid were prepared by incubating the discoidal rHDL with LDL and isolated LCAT (*44*). The spherical rHDL containing CE as the only core lipid, designated (E2/CE)rHDL, (E3/CE)rHDL,

(E4/CE)rHDL, and (A-I/CE)rHDL, were dialyzed extensively (3 × 1 L) against 0.01 M Tris-buffered saline (TBS) (pH 7.4) containing 0.15 M NaCl, 0.005% (w/v) EDTA-Na_2, and 0.006% (w/v) NaN_3 prior to use.

Preparation of [^{3}H]Triolein-Labeled (E2/TG)rHDL, (E3/TG)rHDL, (E4/TG)rHDL, and (A-I/TG)rHDL. Spherical, [^{3}H]triolein-labeled rHDL were prepared by incubating (E2/CE)rHDL, (E3/CE)rHDL, (E4/CE)rHDL, and (A-I/CE)rHDL individually with [^{3}H]triolein-labeled Intralipid and CETP (*21*, *38*). The resulting spherical rHDL containing triacylglycerol, [^{3}H]triolein, and CE in their core, designated (E2/TG)rHDL, (E3/TG)rHDL, (E4/TG)rHDL, and (A-I/TG)-rHDL, were dialyzed extensively against TBS (3 × 1 L) prior to use. The specific activities of the (E2/TG)rHDL, (E3/TG)-rHDL, (E4/TG)rHDL, and (A-I/TG)rHDL were 4.6 × 10^6, 5.4 × 10^6, 4.8 × 10^6, and 1.8 × 10^6 dpm/mg of triacylglycerol, respectively.

Determination of HL-Mediated Phospholipid Hydrolysis in (E2/CE)rHDL, (E3/CE)rHDL, (E4/CE)rHDL, and (A-I/CE)rHDL. To examine the kinetics of HL-mediated phospholipid hydrolysis, (E2/CE)rHDL, (E3/CE)rHDL, (E4/CE)rHDL, and (A-I/CE)rHDL were incubated individually with HL. All incubations were carried out in stoppered plastic tubes in a shaking water bath maintained at 37 °C. Details of the incubations are described in the legend to Figure 1. The mixtures were placed on ice when the incubations were complete. The extent of phospholipid hydrolysis was determined by quantitating the amount of NEFA mass in the incubation mixtures.

Determination of HL-Mediated Triacylglycerol Hydrolysis in (E2/TG)rHDL, (E3/TG)rHDL, (E4/TG)rHDL, and (A-I/TG)rHDL. To examine the kinetics of HL-mediated triacylglycerol hydrolysis, (E2/TG)rHDL, (E3/TG)rHDL, (E4/TG)rHDL, and (A-I/TG)rHDL were incubated individually with HL. These incubations were carried out as described above and in the legend to Figure 2. Hydrolysis was stopped by the addition of chloroform/methanol [1 mL, 2:1 (v/v)] to the incubation mixtures. The lipids were extracted according to the method of Folch et al. (*45*). Hydrolysis products were separated from the other rHDL lipids by thin-layer chromatography and visualized with iodine (*21*). The areas corresponding to unhydrolyzed triacylglycerol and the triacylglycerol hydrolysis products (diacylglycerol, monoacylglycerol, glycerol, and NEFA) were cut from the sheets and placed directly into 10 mL of Ready Safe liquid scintillation mixture (Beckman Instruments). Radioactivity was determined using a Beckman LS 6000TA liquid scintillation counter with automatic quenching correction (Beckman Instruments). The silica gel had a negligible effect on the counting.

Determination of Phospholipid Acyl Chain and Headgroup Packing Order in (E2/CE)rHDL, (E3/CE)rHDL, and (E4/CE)rHDL. Phospholipid acyl chain and headgroup packing order was determined in the (E2/CE)rHDL, (E3/CE)rHDL, and (E4/CE)rHDL by measuring the steady-state fluorescence polarization of samples labeled respectively with 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) (44). Polarization measurements were made at 5 °C intervals, from 5 to 50 °C. The molar ratio of rHDL phospholipid/probe was 500:1. The final phospholipid concentration was 0.5 mM.

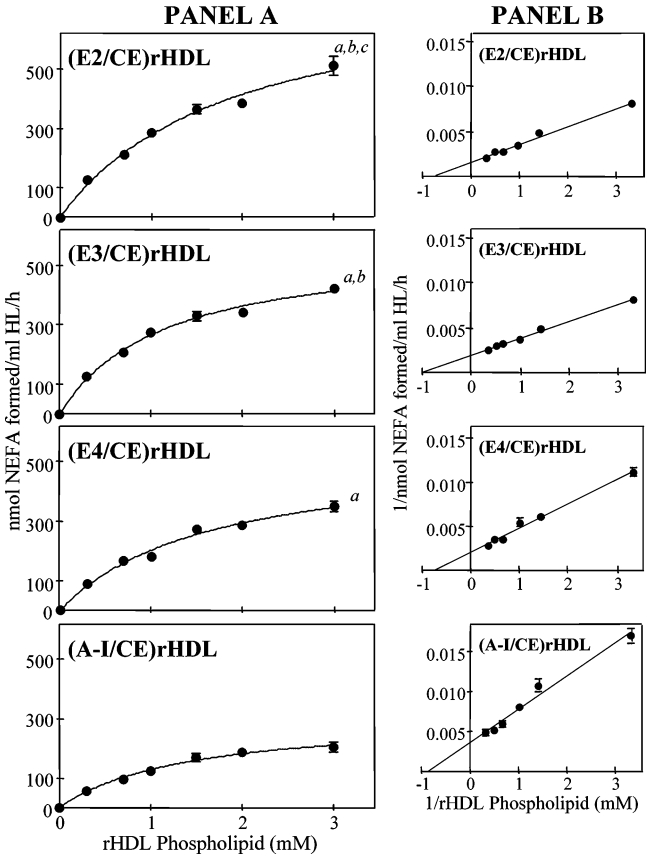

FIGURE 1: Kinetics of phospholipid hydrolysis in (E2/CE)rHDL, (E3/CE)rHDL, (E4/CE)rHDL, and (A-I/CE)rHDL. (E2/CE)rHDL, (E3/CE)rHDL, (E4/CE)rHDL, and (A-I/CE)rHDL (0.3−3.0 mM phospholipid) were incubated at 37 °C for 1 h with HL (40 μL of a preparation that generated 1100 nmol of NEFA/mL of HL/h). The incubation mixtures also contained BSA (final concentration of 20 mg/mL) in a final incubation volume of 100 μL. The rate of phospholipid hydrolysis in (E2/CE)rHDL, (E3/CE)rHDL, (E4/CE)-rHDL, and (A-I/CE)rHDL as a function of substrate concentration is shown in panel A. The values are the means ± SEM of triplicate determinations [*a*, $p < 0.0001$ for (E2/CE)rHDL versus (A-I/CE)-rHDL, (E3/CE)rHDL versus (A-I/CE)rHDL and (E4/CE)rHDL versus (A-I/CE)rHDL; *b*, $p < 0.0001$ for (E2/CE)rHDL versus (E4/CE)rHDL and (E3/CE)rHDL versus (E4/CE)rHDL; *c*, $p < 0.0005$ for (E2/CE)rHDL versus (E3/CE)rHDL]. Panel B shows Lineweaver−Burk double-reciprocal plots of the kinetic data.

Calculations. HL-mediated phospholipid hydrolysis in the unlabeled (E2/CE)rHDL, (E3/CE)rHDL, (E4/CE)rHDL, and (A-I/CE)rHDL was ascertained by direct enzymatic assay of NEFA mass. HL-mediated triacylglycerol hydrolysis in the radiolabeled (E2/TG)rHDL, (E3/TG)rHDL, (E4/TG)-rHDL, and (A-I/TG)rHDL was determined as the amount of radiolabel in the hydrolysis products relative to the total radiolabel in the substrate. The kinetic parameters K_m(app) and V_{max} were determined by nonlinear regression using GraphPad Prism 4.0 computer software (GraphPad Software Inc.). The data were fitted directly to the Michaelis−Menten equation: $V = (V_{max} \times [S])/(K_m + [S])$, where V is the velocity of HL-mediated hydrolysis and [S] is the concentration of rHDL. The Lineweaver−Burk linear transformations shown in Figures 1 and 2 are for display purposes only.

Other Techniques. All chemical analyses were carried out on a Roche Diagnostics/Hitachi 902 automatic analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Phospholipid concentrations were determined by enzymatic assay

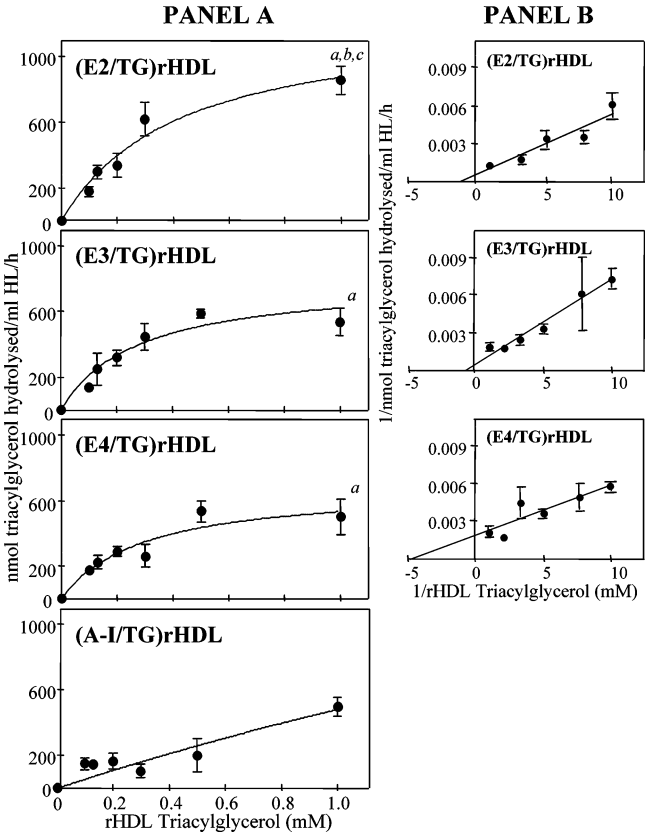

FIGURE 2: Kinetics of triacylglycerol hydrolysis in (E2/TG)rHDL, (E3/TG)rHDL, (E4/TG)rHDL, and (A-I/TG)rHDL. (E2/TG)rHDL, (E3/TG)rHDL, (E4/TG)rHDL, and (A-I/TG)rHDL (0.1−1.0 mM triacylglycerol) containing [^{3}H]triolein were incubated at 37 °C for 45 min with HL (8 μL of a preparation that formed 902 nmol of NEFA/mL of HL/h). The incubation mixtures contained BSA (final concentration of 20 mg/mL), and the final incubation volume was 50 μL. The rate of triacylglycerol hydrolysis in (E2/TG)rHDL, (E3/TG)rHDL, (E4/TG)rHDL, and (A-I/TG)rHDL as a function of substrate concentration is shown in panel A. The values are the means ± SEM of triplicate determinations [*a*, $p < 0.0001$ for (E2/TG)rHDL versus (A-I/TG)rHDL, $p < 0.0005$ for (E3/TG)rHDL versus (A-I/TG)rHDL, and $p < 0.005$ for (E4/TG)rHDL versus (A-I/TG)rHDL; *b*, $p < 0.001$ for (E2/TG)rHDL versus (E4/TG)rHDL; *c*, $p < 0.05$ for (E2/TG)rHDL versus (E3/TG)rHDL]. Panel B shows Lineweaver−Burk double-reciprocal plots of the kinetic data.

of phosphatidylcholine (*46*). A Roche Diagnostics Kit was used for total cholesterol assays. UC concentrations were determined enzymatically (*47*). CE concentrations were calculated as the difference between the total and UC concentrations.

A protein assay using bicinchoninic acid was used to determine apoA-I concentrations as described previously (*48*). Triacylglycerol concentrations were determined enzymatically (*49*). Spherical rHDL size was determined by electrophoresis on 3−40% non-denaturing polyacrylamide gradient gels prepared according to the method of Rainwater et al. (*50*).

Statistical Methods. To determine whether differences in hydrolysis rates between substrates were significant, two-way ANOVA was performed using GraphPad Prism 4.0. Bonferroni post-tests were conducted on the triacylglycerol hydrolysis data to determine at which concentrations of rHDL the hydrolysis rates differed between substrates. Two-way ANOVA was also used to determine if the differences between the steady-state fluorescence polarization data sets were significant. In all cases significance was set at $p < 0.05$.

Table 1: Physical Properties of Spherical rHDL Substrates for HL

spherical rHDL	composition,[a] % mass PL	UC	CE	TG	protein	Stokes' diameter,[b] nm
(E2/CE)rHDL[c]	39	3	23	0	35	11.0
(E3/CE)rHDL[c]	36	3	25	0	36	11.1
(E4/CE)rHDLc	43	3	21	0	34	11.1
(A-I/CE)rHDL[c]	34	2	18	0	46	8.9
(E2/TG)rHDL[d]	48	17	5	7	22	10.4
(E3/TG)rHDL[d]	48	15	6	7	24	10.1
(E4/TG)rHDL[d]	48	15	5	8	24	10.3
(A-I/TG)rHDL[d]	35	3	12	11	38	9.2

[a] The compositions were calculated from the means of triplicate determinations, which varied by <6%. PL, phospholipid; UC, unesterified cholesterol; CE, cholesteryl ester; TG, triacylglycerol. [b] All rHDL preparations contained a single, homogeneous population of particles as judged by non-denaturing gradient gel electrophoresis. [c] Spherical rHDL containing CE as the sole core lipid were prepared by incubating discoidal rHDL with LDL and LCAT as described under Experimental Procedures. These rHDL preparations were used to determine the kinetics of HL-mediated phospholipid hydrolysis. [d] Spherical rHDL containing [^{3}H]triolein in their core were prepared as described under Experimental Procedures. These rHDL preparations were used to determine the kinetics of HL-mediated triacylglycerol hydrolysis.

RESULTS

Kinetics of HL-Mediated Phospholipid Hydrolysis in (E2/CE)rHDL, (E3/CE)rHDL, (E4/CE)rHDL, and (A-I/CE)rHDL (Figure 1; Tables 1 and 2). (E2/CE)rHDL, (E3/CE)rHDL, (E4/CE)rHDL, and (A-I/CE)rHDL were prepared as described under Experimental Procedures. All of the preparations were comparable in terms of percent mass of phospholipid, CE, and protein and differed only in the type of apolipoprotein they contained (Table 1). The proportions of lipid and apolipoprotein in the rHDL were comparable to what has been reported for the spherical HDL in normal human plasma (*51*). The (E2/CE)rHDL, (E3/CE)rHDL, and (E4/CE)rHDL were all similar in size and ∼25% larger in diameter than the (A-I/CE)rHDL.

To compare the kinetics of HL-mediated phospholipid hydrolysis in the (E2/CE)rHDL, (E3/CE)rHDL, (E4/CE)-rHDL, and (A-I/CE)rHDL, various concentrations of each rHDL preparation (final concentrations of 0.3−3.0 mM phospholipid) were incubated individually with a constant amount of HL. The rate of phospholipid hydrolysis (Figure 1) was greater in the (E2/CE)rHDL, (E3/CE)rHDL, and the (E4/CE)rHDL compared to that in the (A-I/CE)rHDL [$p < 0.0001$ for (E2/CE)rHDL versus (A-I/CE)rHDL, (E3/CE)-rHDL versus (A-I/CE)rHDL, and (E4/CE)rHDL versus (A-I/CE)rHDL]. The rate of phospholipid hydrolysis also varied according to the isoform of apoE in the rHDL, with (E2/CE)rHDL > (E3/CE)rHDL > (E4/CE)rHDL [$p < 0.0005$ for (E2/CE)rHDL versus (E3/CE)rHDL, $p < 0.0001$ for (E2/CE)rHDL versus (E4/CE)rHDL, and $p < 0.0001$ for (E3/CE)rHDL versus (E4/CE)rHDL].

Nonlinear regression analysis was used to determine the kinetic parameters for phospholipid hydrolysis. The parameters from two representative and independent experiments are shown in Table 2. In both experiments the V_{max} for HL-mediated phospholipid hydrolysis in the (E2/CE)rHDL, (E3/CE)rHDL, and (E4/CE)rHDL was greater than in the (A-I/

Table 2: Kinetic Parameters of HL-Mediated Phospholipid Hydrolysis in (E2/CE)rHDL, (E3/CE)rHDL, (E4/CE)rHDL, and (A-I/CE)rHDL[a]

	experiment 1			experiment 2		
spherical rHDL	V_{max}, nmol of NEFA formed/mL of HL/h	K_m(app), mM PL	catalytic efficiency, V_{max}/K_m(app)	V_{max}, nmol of NEFA formed/mL of HL/h	K_m(app), mM PL	catalytic efficiency, V_{max}/K_m(app)
(E2/CE)rHDL	808 ± 65	1.9 ± 0.3	425	905 ± 45	2.0 ± 0.2	453
(E3/CE)rHDL	570 ± 24	1.1 ± 0.1	518	567 ± 19	1.0 ± 0.1	567
(E4/CE)rHDL	538 ± 37	1.7 ± 0.2	316	677 ± 33	1.7 ± 0.2	398
(A-I/CE)rHDL	435 ± 50	2.4 ± 0.5	181	439 ± 32	2.7 ± 0.3	163

[a] Spherical rHDL (0.3−3.0 mM phospholipid) were incubated with a constant amount of HL as described in the legend to Figure 1. Phospholipid hydrolysis was determined by measuring the generation of NEFA mass in the incubation mixture. Kinetic parameters from triplicate determinations of two representative and independent experiments are shown. Kinetic parameters for experiment 1 were determined by nonlinear regression analysis of the data in Figure 1 as described under Experimental Procedures. Nonlinear regression analysis was also used to determine the kinetic parameters for experiment 2 (plots not shown).

Table 3: Kinetics of Triacylglycerol Hydrolysis in (E2/TG)rHDL, (E3/TG)rHDL, (E4/TG)rHDL, and (A-I/TG)rHDL[a]

rHDL triacylglycerol, mM	HL-mediated triacylglycerol hydrolysis, nmol of triacylglycerol hydrolyzed/mL of HL/h			
	(A-I/TG)rHDL	(E2/TG)rHDL	(E3/TG)rHDL	(E4/TG)rHDL
		Experiment 1[b]		
0.1	148.7 ± 36.0	178.0 ± 29.4	140.6 ± 15.3	177.2 ± 12.9
0.13	147.9 ± 0.4	298.5 ± 42.1	251.1 ± 96.3	223.1 ± 42.3
0.2	165.4 ± 45.9	338.9 ± 71.1	319.4 ± 48.8	287.1 ± 29.6
0.3	106.1 ± 41.3	618.2 ± 101.1***	447.2 ± 78.9***	261.1 ± 68.0§§§
0.5	201.9 ± 100.0	nd	588.0 ± 26.0***	533.5 ± 63.5**
1.0	497.9 ± 58.2	853.8 ± 84.6***	535.9 ± 82.3§§	497.8 ± 110.7§§§
		Experiment 2[c]		
0.08	72.7 ± 4.1	201.1 ± 28.8	267.0 ± 6.2	299.6 ± 38.2
0.12	119.9 ± 29.7	490.7 ± 149.1	285.4 ± 73.5	404.4 ± 45.3
0.18	152.0 ± 17.6	788.1 ± 169.2***	605.6 ± 171.6*	633.4 ± 3.7**
0.3	510.4 ± 93.4	761.4 ± 40.9	493.3 ± 110.4	763.5 ± 18.0
0.5	426.1 ± 122.4	1114.2 ± 86.2***	973.3 ± 116.5**	939.7 ± 39.3***
0.8	812.4 ± 169.2	1581.6 ± 10.7***	1083.0 ± 175.4§	nd

[a] The rates of HL-mediated triacylglycerol hydrolysis in [^{3}H]triolein-labeled spherical rHDL at various concentrations of rHDL triacylglycerol are shown. All values are the mean ± SEM of triplicate determinations. Results from two representative and independent experiments are shown. [b] Experiment 1: ***, $p < 0.001$ for (E2/TG)rHDL vs (A-I/TG)rHDL and (E3/TG)rHDL vs (A-I/TG)rHDL; **, $p < 0.01$ for (E4/TG)rHDL vs (A-I/TG)rHDL; §§§, $p < 0.001$ for (E4/TG)rHDL vs (E2/TG)rHDL; §§, $p < 0.01$ for (E3/TG)rHDL vs (E2/TG)rHDL; nd, not determined. [c] Experiment 2: ***, $p < 0.001$ for (E2/TG)rHDL vs (A-I/TG)rHDL and (E4/TG)rHDL vs (A-I/TG)rHDL; **, $p < 0.01$ for (E3/TG)rHDL vs (A-I/TG)rHDL and (E4/TG)rHDL vs (A-I/TG)rHDL; *, $p < 0.05$ for (E3/TG)rHDL vs (A-I/TG)rHDL; §, $p < 0.05$ for (E3/TG)rHDL vs (E2/TG)rHDL; nd, not determined.

Table 4: Kinetic Parameters of HL-Mediated Triacylglycerol Hydrolysis in (E2/TG)rHDL, (E3/TG)rHDL, and (E4/TG)rHDL[a]

	experiment 1			experiment 2		
sperhical rHDL	V_{max}, nmol of triacylglycerol hydrolyzed/mL of HL/h	K_m(app), mM TG	catalytic efficiency, V_{max}/K_m(app)	V_{max}, nmol of triacylglycerol hydrolyzed/mL of HL/h	K_m(app), mM TG	catalytic efficiency, V_{max}/K_m(app)
(E2/TG)rHDL	1245 ± 185	0.4 ± 0.1	3113	2697 ± 571	0.6 ± 0.2	4495
(E3/TG)rHDL	792 ± 130	0.3 ± 0.1	2640	1801 ± 495	0.5 ± 0.3	3602
(E4/TG)rHDL	684 ± 121	0.3 ± 0.1	2280	1541 ± 14	0.3 ± 0.1	5137
(A-I/TG)rHDL	nd[b]	nd	nd	nd	nd	nd

[a] [^{3}H]Triolein-labeled spherical rHDL (0.1−1.0 mM triacylglycerol for experiment 1 and 0.08-0.8 mM triacylglycerol for experiment 2) were incubated with a constant amount of HL as described in the legend to Figure 2. The extent of triacylglycerol hydrolysis was determined by separating the hydrolysis products from the unhydrolyzed triacylglycerol by thin-layer chromatography and liquid scintillation counting. Kinetic parameters from triplicate determinations of two representative and independent experiments are shown. Kinetic parameters for experiment 1 were determined by nonlinear regression analysis of the data in Figure 2 as described under Experimental Procedures. Nonlinear regression analysis was also used to determine the kinetic parameters for experiment 2 (plots not shown). [b] nd, not determined; kinetic parameters were not determined for (A-I/TG)rHDL as the rate of triacylglycerol increased linearly from 0.08 to 1.0 mM rHDL triacylglycerol.

CE)rHDL. Variations in the absolute V_{max} values are the result of using different HL preparations for each experiment. In both of the experiments HL had a higher affinity (lower K_m) for the phospholipids in (E3/CE)rHDL compared to (E2/CE)rHDL and (E4/CE)rHDL. The catalytic efficiency of phospholipid hydrolysis was greater in (E2/CE)rHDL, (E3/CE)rHDL, and (E4/CE)rHDL compared with (A-I/CE)rHDL for both experiments.

Kinetics of HL-Mediated Hydrolysis of Triacylglycerol in (E2/TG)rHDL, (E3/TG)rHDL, (E4/TG)rHDL, and (A-I/TG)-rHDL (Figure 2; Tables 1, 3, and 4). The physical properties of [^{3}H]triolein-labeled (E2/TG)rHDL, (E3/TG)rHDL, (E4/TG)rHDL, and (A-I/TG)rHDL are shown in Table 1. All of the rHDL preparations were comparable in terms of percent mass of phospholipid. Their triacylglycerol contents ranged from 48 to 62% of the total core lipids. The apoE-containing

rHDL were all comparable in size, and their diameters were 10−13% larger than that of the (A-I/TG)rHDL.

Various concentrations of the (E2/TG)rHDL, (E3/TG)-rHDL, (E4/TG)rHDL, and (A-I/TG)rHDL (final concentrations of 0.1−1.0 mM triacylglycerol) were incubated individually with a constant amount of HL. The rates of triacylglycerol hydrolysis (Figure 2) in the (E2/TG)rHDL, (E3/TG)rHDL, and (E4/TG)rHDL were significantly different from that of the (A-I/TG)rHDL [$p < 0.0001$ for (E2/TG)rHDL versus (A-I/TG)rHDL, $p < 0.0005$ for (E3/TG)rHDL versus (A-I/TG)rHDL, and $p < 0.005$ for (E4/TG)rHDL versus (A-I/TG)rHDL]. In addition, the rate of triacylglycerol hydrolysis in the (E2/TG)rHDL was significantly different from the rate of triacylglycerol hydrolysis in either the (E3/TG)rHDL or the (E4/TG)rHDL [$p < 0.05$ for (E2/TG)rHDL versus (E3/TG)rHDL and $p < 0.001$ for (E2/TG)rHDL versus (E4/TG)rHDL].

Table 3 shows the rates of HL-mediated triacylglycerol hydrolysis at the various concentrations of rHDL for two representative and independent experiments. At 0.3 mM rHDL triacylglycerol in experiment 1, the rate of triacylglycerol hydrolysis was greater in the (E2/TG)rHDL and the (E3/TG)rHDL than in the (A-I/TG)rHDL ($p < 0.001$). At 0.5 mM triacylglycerol, the rate of hydrolysis was greater in the (E3/TG)rHDL than in the (A-I/TG)rHDL ($p < 0.001$) and greater in the (E4/TG)rHDL than in the (A-I/TG)rHDL ($p < 0.01$). At 1.0 mM rHDL triacylglycerol the rate of hydrolysis was greater in the (E2/TG)rHDL compared to that in the (A-I/TG)rHDL ($p < 0.001$). Significant differences were also apparent at 1.0 mM triacylglycerol for the rate of triacylglycerol hydrolysis in (E2/TG)rHDL versus (E4/TG)-rHDL ($p < 0.001$) and in (E2/TG)rHDL versus (E3/TG)-rHDL ($p < 0.01$).

Similar results were obtained in experiment 2. At 0.18 mM rHDL triacylglycerol the rate of triacylglycerol hydrolysis was greater in the (E2/TG)rHDL than in the (A-I/TG)rHDL ($p < 0.001$), greater in the (E3/TG)rHDL than in the (A-I/TG)rHDL ($p < 0.05$), and greater in the (E4/TG)rHDL than in the (A-I/TG)rHDL ($p < 0.01$). At 0.5 mM triacylglycerol, the rate of hydrolysis was greater in the (E2/TG)rHDL and (E4/TG)rHDL than in the (A-I/TG)rHDL ($p < 0.001$) and greater in the (E3/TG)rHDL than in the (A-I/TG)rHDL ($p < 0.01$). At 0.8 mM triacylglycerol, the rate of hydrolysis was greater in the (E2/TG)rHDL than in the (A−I/TG)rHDL ($p < 0.001$) and greater in the (E2/TG)rHDL than in the (E3/TG)rHDL ($p < 0.05$).

The kinetic parameters of HL-mediated triacylglycerol hydrolysis in the (E2/TG)rHDL, (E3/TG)rHDL, and (E4/TG)rHDL from two representative and independent experiments are shown in Table 4. In both cases kinetic parameters of triacylglycerol hydrolysis in the (A-I/TG)rHDL could not be determined as the rate of hydrolysis was linear up to the highest concentration of triacylglycerol. Given that the extent of rHDL triacylglycerol enrichment in both of the experiments was greater than what has been reported for the HDL of triglyceridemic subjects (*52*), this result suggests that the HL-mediated hydrolysis of HDL triacylglycerol in human plasma may not be saturable. In both experiments 1 and 2 the V_{max} for triacylglycerol hydrolysis was greater in the (E2/TG)rHDL than in either the (E3/TG)rHDL or the (E4/TG)-rHDL. As was the case for phospholipid hydrolysis, the difference in the V_{max} from experiments 1 and 2 is a result of using different HL preparations for each experiment. The K_m(app) values were comparable for the (E2/TG)rHDL, (E3/TG)rHDL, and (E4/TG)rHDL, indicating that apoE isoforms do not affect the affinity of HL for rHDL triacylglycerol.

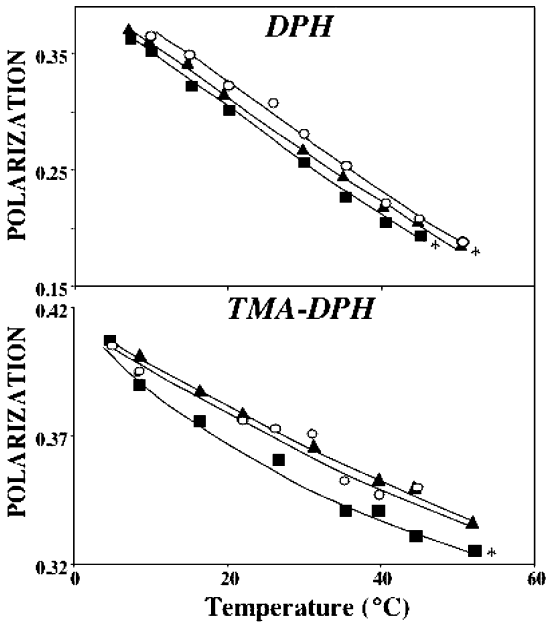

FIGURE 3: Phospholipid acyl chain and headgroup packing in (E2/CE)rHDL, (E3/CE)rHDL, and (E4/CE)rHDL. (E2/CE)rHDL (▲), (E3/CE)rHDL (■), and (E4/CE)rHDL (○) were labeled with either DPH or TMA-DPH. Steady-state fluorescence polarization was determined as a function of temperature. The values represent the mean ± SEM of at least three determinations. *, $p < 0.0001$, (E2)-rHDL versus (E3)rHDL, (E2)rHDL versus (E4)rHDL, and (E3)-rHDL versus (E4)rHDL for the DPH-labeled rHDL; *, $p < 0.0001$, (E2)rHDL versus (E3)rHDL and (E3)rHDL versus (E4)rHDL for the TMA-DPH-labeled rHDL.

Comparison of Phospholipid Acyl Chain and Headgroup Packing Order in (E2/CE)rHDL, (E3/CE)rHDL, and (E4/CE)rHDL (Figure 3). Phospholipid packing order was assessed in (E2/CE)rHDL, (E3/CE)rHDL, and (E4/CE)rHDL labeled with either DPH or TMA-DPH. The steady-state fluorescence polarization of the samples was determined as a function of temperature (Figure 3).

The DPH polarization values varied depending on the apoE isoform content of the rHDL, with the values for (E3/CE)-rHDL (■) < (E2/CE)rHDL (▲) < (E4/CE)rHDL (○) [$p < 0.0001$, (E2/CE)rHDL versus (E3/CE)rHDL, (E2/CE)rHDL versus (E4/CE)rHDL, and (E3/CE)rHDL versus (E4/CE)-rHDL]. This indicates that the phospholipid acyl chains in (E3/CE)rHDL are less ordered than in either (E2/CE)rHDL or (E4/CE)rHDL and that the phospholipid acyl chains in the (E2/CE)rHDL are less ordered than in the (E4/CE)rHDL.

The packing order of the phospholipid headgroups also varied according to the apoE isoform content of the particles, with the TMA-DPH polarization values for spherical (E3/CE)rHDL (■) being lower than for either the (E2/CE)rHDL (▲) or the (E4/CE)rHDL (○) [$p < 0.0001$, (E2/CE)rHDL versus (E3/CE)rHDL and (E3/CE)rHDL versus (E4/CE)-rHDL]. This indicates that the phospholipid headgroups in (E3/CE)rHDL are less ordered than in any of the other spherical rHDL. This difference was more apparent at higher than at lower temperatures. The polarization values for the (E2/CE)rHDL and (E4/CE)rHDL were not significantly different.

DISCUSSION

Previous studies from this laboratory have established that the two major apolipoproteins in HDL, apoA-I and apoA-II, influence the interaction of HL with HDL (*21*, *22*). In those studies, rHDL that varied only in their apolipoprotein composition were used as substrates for HL to show that apoA-I and apoA-II influence the kinetics of HL-mediated hydrolysis of lipoprotein phospholipids and triacylglycerol. The aim of the present study was to determine if apoE also regulates the kinetics of HL-mediated phospholipid and triacylglycerol hydrolysis in rHDL. This was achieved by comparing the ability of HL to hydrolyze phospholipids and triacylglycerols in rHDL containing either apoE2, apoE3, apoE4, or apoA-I as the sole apolipoprotein. The results show that the rate of HL-mediated phospholipid hydrolysis is greater in (E2/CE)rHDL, (E3/CE)rHDL, and (E4/CE)rHDL compared to in (A-I/CE)rHDL. Triacylglycerol hydrolysis was also greater in (E2/TG)rHDL, (E3/TG)rHDL, and (E4/TG)rHDL compared to in (A-I/TG)rHDL, although not to the same extent as was observed for phospholipid hydrolysis.

The greater rate of phospholipid hydrolysis in the apoE-containing rHDL supports previous findings that HL preferentially hydrolyzes the phospholipids in apoE-rich HDL compared to apoE-poor HDL isolated from human plasma (*53*). In that study apoE-rich and apoE-poor HDL were separated by heparin−Sepharose chromatography and used as substrates for HL. However, as those preparations were heterogeneous in terms of their phospholipid and apolipoprotein contents, it was not possible to establish unequivocally that the greater hydrolysis in the apoE-rich HDL was due solely to the presence of apoE in the particles. The advantage of the present study is that it shows definitively that the apoE in HDL enhances HL-mediated phospholipid hydrolysis.

The increased rate of phospholipid and triacylglycerol hydrolysis in the apoE-containing rHDL compared to the (A-I)rHDL can be explained in a number of ways. It is well established that members of the triglyceride lipase gene family have two domains that are structurally and functionally distinct. The C-terminal domain, which contains several charged residues, has lipid binding and anchoring functions, whereas the N-terminal domain contains the catalytic site (*54*). In the case of hepatic lipase and HDL, the HDL must interact with the charged residues in the HL lipid binding domain before their phospholipid and triacylglycerol acyl chains can access the active site of the enzyme (*55*). As the surface of apo-E containing HDL is less negatively charged than that of (A-I)HDL (*30*), it follows that the interaction of apo-E-containing HDL and (A-I)HDL with charged residues in the lipid binding domain of HL may differ. This, in turn, could affect access of the HDL phospholipids and triacylglycerol molecules to the catalytic site of HL and alter the rate at which they are hydrolyzed.

Changes in HL surface charge that occur during interfacial activation may also contribute to the different rates of phospholipid and triacylglycerol hydrolysis. The catalytic site of HL contains a Ser^{152}, His^{263}, and Asp^{176} catalytic triad. This triad is conserved in pancreatic lipase. The catalytic domains of HL and pancreatic lipase also have similar three-dimensional structures (*54*). Studies of pancreatic lipase indicate that the area surrounding the active site becomes negatively charged upon interfacial activation (*56*). If this is also the case for HL, it follows that phospholipid and triacylglycerol molecules from apoE-containing HDL may be able to access the active site of HL more readily than is the case for the phospholipid and triacylglycerol molecules in the more negatively charged (A-I)HDL,. This is consistent with the rate of phospholipid and triacylglycerol hydrolysis in (E2)rHDL, (E3)rHDL, and (E4)rHDL being greater than that in (A-I)rHDL.

It is also possible that the increased phospholipid and triacylglycerol hydrolysis in apoE-containing rHDL relative to (A-I)rHDL may simply be due to the larger surface area of the apoE-containing particles increasing the availability of phospholipid and triacylglycerol molecules for hydrolysis by HL.

One of the most interesting findings to emerge from this study was that the greater rate of phospholipid and triacylglycerol hydrolysis in the apoE-containing rHDL is isoform dependent. The human apoE gene is polymorphic, with the three isoforms arising from cysteine−arginine interchanges at residues 112 and 158 in the N-terminal domain. ApoE2 has cysteines at these two residues, whereas apoE3 has a cysteine and an arginine, respectively, and apoE4 has two arginines. In the present study the finding that the rates of phospholipid and triacylglycerol hydrolysis differed in the (E2)rHDL, (E3)rHDL, and (E4)rHDL indicates that HL may play a part in determining how apoE isoforms regulate plasma lipoprotein concentrations.

In this study the V_{max} for phospholipid and triacylglycerol hydrolysis was considerably greater in the (E2)rHDL compared to that in either the (E3)rHDL or the (E4)rHDL. The difference between apoE2 and both apoE3 and apoE4 is that apoE2 has an uncharged cysteine at residue 158, whereas apoE3 and apoE4 have a positively charged arginine at this position. It is believed that the charge of residue 158 may have a role in determining the conformation of the receptor-binding region of apoE. ApoE2 possesses only 1% of the receptor-binding activity of apoE, whereas modification of apoE2 to give a positively charged residue at 158 activates receptor binding ~13-fold (*57*). The present results raise the possibility that the charge of residue 158 in apoE may also influence the interaction of HL with apoE-containing rHDL.

It was also observed in this study that HL has a higher affinity for the phospholipids in the (E3/CE)rHDL compared to either the (E2/CE)rHDL or the (E4/CE)rHDL. This may be related to the ability of apoE isoforms to influence the packing order of phospholipid molecules in rHDL. The results in Figure 3 show that phospholipid headgroup and acyl chain packing is more disordered in (E3/CE)rHDL than in either (E2/CE)rHDL or (E4/CE)rHDL. This may enhance the binding affinity of HL for (E3)rHDL phospholipid molecules by increasing the access of the HL binding site to the surface monolayer of the particles, as well as by regulating accessibility of the phospholipid acyl chains to the active site of HL.

Although there is evidence that apoE isoforms modulate HDL levels in vivo, the basis for this observation, and the precise role of HL in regulating HDL levels, is not entirely clear. Epidemiological studies have shown that the $\epsilon 2$ allele is associated with high HDL levels (*58*, *59*). This suggests that apoE2-containing HDL are less efficient HL substrates than either apoE3- or apoE4-containing HDL and is therefore

at odds with the present data. This discrepancy may reflect the inability of the LDL receptor to remove apoE2-containing HDL from the plasma compartment and/or the reduced selective uptake of these particles by SR-B1. It is also noteworthy that an earlier meta analysis failed to establish any relationship between the $\epsilon 2$ allele and HDL levels (*60*). This highlights the fact that the relationship between apoE genotype and HDL levels is complex. Although hepatic lipase undoubtedly contributes to this relationship, other plasma factors and receptors are also likely to be involved.

In conclusion, this study shows for the first time that HL-mediated phospholipid hydrolysis, and to a lesser extent triacylglycerol hydrolysis, is greater in apoE-containing rHDL compared to (A-I)rHDL. The results also establish that apoE isoforms influence phospholipid and triacylglycerol hydrolysis in rHDL. These results provide the first insight into the effects of apoE isoforms on this aspect of HDL metabolism.

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