Apolipoproteins Regulate the Kinetics of Endothelial Lipase-Mediated Hydrolysis of Phospholipids in Reconstituted High-Density Lipoproteins†

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ABSTRACT: Endothelial lipase (EL) is a newly identified member of the triglyceride lipase gene family that hydrolyzes high-density lipoprotein (HDL) phospholipids. This study investigates the ability of the major apolipoproteins of rHDL to regulate the kinetics of EL-mediated phospholipid hydrolysis in well-characterized, homogeneous preparations of spherical rHDL. The rHDL contained either apoA-I as the only apolipoprotein, (A-I)rHDL, apoA-II as the only apolipoprotein, (A-II)rHDL, or apoA-I as well as apoA-II, (A-I/A-II)rHDL. The rHDL were comparable in terms of size and lipid composition and contained cholesteryl esters (CE) as their sole core lipid. Phospholipid hydrolysis was quantitated as the mass of nonesterified fatty acids (NEFA) released from the rHDL during incubation with EL. The V_{max} of phospholipid hydrolysis for (A-I/A-II)rHDL [391.9 ± 12.9 nmol of NEFA formed (mL of EL)$^{-1}$ h^{-1}] was greater than (A-I)rHDL [152.8 ± 4.7 nmol of NEFA formed (mL of EL)$^{-1}$ h^{-1}]. The energy of activation (E_a) for the hydrolysis reactions was calculated to be 52.1 and 34.8 kJ mol^{-1} for (A-I)rHDL and (A-I/A-II)rHDL, respectively. Minimal phospholipid hydrolysis was observed for the (A-II)rHDL. Kinetic analysis showed that EL has a higher affinity for the phospholipids in (A-I)rHDL [K_m(app) = 0.10 ± 0.01 mM] than in (A-I/A-II)rHDL [K_m(app) = 0.27 ± 0.03 mM]. Furthermore, (A-I)rHDL is a competitive inhibitor of the EL-mediated phospholipid hydrolysis of (A-I/A-II)rHDL. These results establish that apolipoproteins are major determinants of the kinetics of EL-mediated phospholipid hydrolysis in rHDL.

Members of the triglyceride lipase gene family are of fundamental importance to biological processes. They regulate intestinal lipid absorption, energy homeostasis, and several aspects of plasma lipoprotein metabolism and atherosclerosis. Members of the family include lipoprotein lipase (LPL),[1] hepatic lipase (HL), and pancreatic lipase (PL). Endothelial lipase (EL) is one of the most recent members of the triglyceride lipase gene family to be identified. It is a 68 kDa glycoprotein that is synthesized in the placenta, liver, lung, steroid hormone-producing organs, macrophages, and endothelial cells (*1*−*3*). Importantly, expression of EL by endothelial cells is a unique feature of this member of the triglyceride lipase gene family. EL shares a 45%, 40%, and 27% identity with LPL, HL and PL, respectively (*1*, *2*). All members of the family exhibit varying degrees of triglyceride and phospholipase activity (*4*, *5*). EL has much greater phospholipase than triglyceride lipase activity (*2*, *3*, *5*).

High-density lipoproteins (HDL) are the preferred plasma substrate for EL (*5*). Moreover, EL has been found to play a major physiological role in HDL metabolism. Recent studies have established that the overexpression of EL in mice results in markedly reduced HDL−cholesterol (HDL-C) and apoA-I levels (*1*, *6*). Rader et al. have also shown that overexpression of EL in mice results in a dose-dependent increase in postheparin plasma phospholipase activity, increased catabolism of HDL−apolipoproteins, and increased uptake of apoA-I by both the liver and kidney (*7*). Antibody inhibition studies in wild-type, apoA-I transgenic, and HL knockout mice demonstrated that inhibition of mouse EL results in significantly increased HDL-C and apoA-I levels (*8*). In the EL knockout mouse model, HDL-C levels were also increased (*6*, *9*).

The aim of this study was to determine if the EL-mediated hydrolysis of HDL phospholipids is regulated by the apolipoprotein composition of the particles. This was achieved by determining the kinetics of EL-mediated phospholipid

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[1] Abbreviations: E_a, activation energy; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; BSA, bovine serum albumin; CE, cholesteryl esters; DPPC, 1,2-dipalmitoylphosphatidylcholine; EL, endothelial lipase; HL, hepatic lipase; HDL, high-density lipoprotein(s); HDL-C, high-density lipoprotein(s)−cholesterol; LCAT, lecithin:cholesterol acyltransferase; LPL, lipoprotein lipase; LDL, low-density lipoproteins; NEFA, nonesterified fatty acids; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PL, pancreatic lipase; PLTP, phospholipid transfer protein; rHDL, reconstituted high-density lipoprotein(s); TLC, thin-layer chromatography; TBS, Tris-buffered saline; UC, unesterified cholesterol.

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hydrolysis in well-defined, homogeneous preparations of spherical rHDL that were comparable in size and lipid composition and contained either apoA-I only, apoA-II only, or both apoA-I and apoA-II as the only apolipoprotein constituents. The results showed that EL has a higher binding affinity for (A-I)rHDL than for (A-I/A-II)rHDL but that the V_{max} of phospholipid hydrolysis was greater for (A-I/A-II)-rHDL than for (A-I)rHDL. Very little EL-mediated phospholipid hydrolysis was observed for (A-II)rHDL.

EXPERIMENTAL PROCEDURES

Isolation of ApoA-I and ApoA-II. ApoA-I and apo-AII were isolated from samples of pooled, autologously donated human plasma (Gribbles Pathology, Adelaide, Australia). HDL were isolated from the plasma by sequential ultracentrifugation and delipidated as described (*10*, *11*). ApoA-I and apoA-II were isolated from the resulting apoHDL by chromatography on a Q-Sepharose fast-flow column (Amersham Pharmacia Biotechnology, Uppsala, Sweden) attached to a FPLC system (Amersham Pharmacia) (*12*). The purity of the isolated apoA-I and apoA-II was confirmed by electrophoresis on a 20% homogeneous SDS−polyacrylamide PhastGel (Amersham Pharmacia) and Coomassie staining.

Isolation of Lecithin:Cholesterol Acyltransferase (LCAT) and Phospholipid Transfer Protein (PLTP). LCAT and PLTP were isolated from pooled samples of autologously donated human plasma as described (*13*, *14*).

LCAT activity was assessed using 1-palmitoyl-2-oleoylphosphatidylcholine (POPC)/unesterified cholesterol (UC)/apoA-I discoidal rHDL radiolabeled with [1α,2α-^{3}H]-cholesterol ([^{3}H]UC) as the substrate (*15*). The POPC and [^{3}H]UC were obtained from Sigma-Aldrich and Amersham Pharmacia Biotechnology (Uppsala, Sweden), respectively. The assay was linear as long as less than 30% of the [^{3}H]-UC was esterified. The preparation used in this study generated 2860 nmol of CE (mL of LCAT)$^{-1}$ h^{-1}.

PLTP activity was quantitated as the transfer of L-3-1,2-di[1-^{14}C]palmitoylphosphatidylcholine ([^{14}C]DPPC) (112 mCi/mmol) (Amersham Pharmacia) from [^{14}C]DPPC-labeled small unilamellar POPC vesicles to HDL (*16*). The preparation used in this study transferred 1035 nmol of phospholipid (mL of PLTP)$^{-1}$ h^{-1}.

Preparation of Unlabeled Spherical (A-I)rHDL and (A-II)-rHDL. Discoidal rHDL containing POPC, UC, and apoA-I were prepared using the cholate dialysis method (*17*). Spherical rHDL, containing apoA-I as the sole apolipoprotein and CE as the sole core lipid, were prepared by incubating the discoidal rHDL with low-density lipoproteins (LDL) and LCAT (*18*). Spherical rHDL containing apoA-II only, (A-II)rHDL), were prepared by displacing apoA-I from spherical (A-I)rHDL with lipid-free apoA-II (*18*). The (A-I)rHDL and (A-II)rHDL were dialyzed (3 × 1 L) against 10 mM Tris-buffered saline (TBS) (pH 7.4) containing 150 mM NaCl, 0.005% (w/v) EDTA-Na_2, and 0.006% (w/v) NaN_3 prior to use.

Preparation of Unlabeled Spherical (A-I/A-II)rHDL. Spherical rHDL containing both apoA-I and apoA-II on the same particle and CE as the sole core lipid were prepared as described previously (*19*). Briefly, discoidal (A-I)rHDL (84 mg of apoA-I) were incubated with discoidal (A-II)rHDL (28 mg of apoA-II) for 24 h at 37 °C with LDL (337 mg of apoB), fatty acid free bovine serum albumin (BSA) (final concentration 40 mg/mL), β-mercaptoethanol (final concentration 4.0 mM), and LCAT [8.5 mL of a preparation that generated 2860 nmol of CE (mL of LCAT)$^{-1}$ h^{-1}]. The final incubation volume was 116 mL. This generated both spherical (A-I)rHDL and spherical (A-I/A-II)rHDL.

The rHDL were isolated by ultracentrifugation (*19*) and dialyzed against TBS (3 × 1 L). The (A-I/A-II)rHDL were separated from the (A-I)rHDL by immunoaffinity chromatography on a column containing an anti-human apoA-II polyclonal antibody coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia). The unbound (A-I)rHDL were eluted from the column with TBS (5 × 10 mL). The bound (A-I/A-II)rHDL were recovered by elution with 0.1 M acetic acid (pH 2.7) (5 × 10 mL) and neutralized immediately with 1 M Tris (pH 11.0). The (A-I/A-II)rHDL were concentrated 6-fold using an Amicon Centriplus concentrator (Amicon, Millipore) that was maintained at 4 °C. The (A-I/A-II)rHDL were dialyzed against TBS (3 × 1 L) prior to use.

Preparation of 1,2-[^{14}C]DPPC-Labeled Spherical (A-I/A-II)-rHDL. Unlabeled spherical (A-I/A-II)rHDL (1.95 μmol of phospholipid) were mixed with 1,2-[^{14}C]DPPC-labeled POPC vesicles (0.195 μmol of phospholipid), PLTP [386 μL of a preparation that transferred 1035 nmol of phospholipid (mL of PLTP)$^{-1}$ h^{-1}], and fatty acid free BSA (final concentration of 20 mg/mL) and incubated for 3 h at 37 °C (*20*). The final volume of the incubation mixture was 989 μL. The resulting 1,2-[^{14}C]DPPC-labeled (A-I/A-II)rHDL were isolated by sequential ultracentrifugation ($1.063 < d < 1.21$ g/mL) as described previously (*20*). The isolated 1,2-[^{14}C]DPPC-labeled (A-I/A-II)rHDL (specific activity 3.1 × 10^5 cpm/mg of phospholipid) were dialyzed extensively against TBS (3 × 1 L) prior to use.

Expression of EL. COS cells were grown in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotic/antimyotic (A/A) at 37 °C and 5% CO_2. Prior to infection, cells were brought to 90% confluency in 150 mm plates. Growth medium was removed, and cells were washed with 10 mL of serum-free DMEM without phenol red and then incubated with recombinant adenovirus encoding EL in 5 mL of the same medium at a multiplicity of 3000 particles/cell. Two hours later, 9 mL of serum-free medium without phenol red containing 10 units/mL heparin was added to each of the plates. At 47.5 h postinfection, an additional 10 units/mL heparin (280 μL of 500 units/mL) was added, and cells were incubated for 30 min. Medium was collected and clarified by centrifugation at 2000 rpm for 10 min in 50 mL conical tubes and then frozen in 1 mL aliquots at −80 °C. The phospholipase activity of EL was determined by incubating the enzyme (50 μL) with spherical (A-I)rHDL (1.0 mM phospholipid) at 37 °C for 1.5 h. The preparation used in this study generated 105 nmol of NEFA (mL of EL)$^{-1}$ h^{-1}.

Determination of EL-Mediated Hydrolysis in Unlabeled Spherical (A-I)rHDL, (A-I/A-II)rHDL, and (A-II)rHDL. The (A-I)rHDL, (A-I/A-II)rHDL, and (A-II)rHDL (0.1−1.0 mM phospholipid) were incubated at 37 °C for 1.5 h with a constant amount of EL [20 μL of a preparation that hydrolyzed 105 nmol of NEFA (mL of EL)$^{-1}$ h^{-1}]. All of the incubations contained fatty acid free BSA (final concentration 20 mg/mL). The total volume of each incubation

was 100 μL. When the incubations were complete, the tubes were placed immediately on ice to terminate the hydrolysis reaction. EL-mediated phospholipid hydrolysis of (A-I)rHDL, (A-I/A-II)rHDL, and (A-II)rHDL was determined as the NEFA released from the rHDL, assuming that one molecule of NEFA is generated for each molecule of phospholipid hydrolyzed. NEFA mass was quantitated directly using a commercially available kit (Wako Pure Chemical Industries, Osaka, Japan).

Determination of EL-Mediated Hydrolysis in Mixtures of 1,2-[^{14}C]DPPC-Labeled Spherical (A-I/A-II)rHDL and Unlabeled Spherical (A-I)rHDL. 1,2-[^{14}C]DPPC−(A-I/A-II)-rHDL (0.15, 0.30, and 0.50 mM phospholipid) and unlabeled (A-I)rHDL (0.0, 0.2, or 0.4 mM phospholipid) were incubated at 37 °C for 1.5 h with a constant amount of EL [25 μL of a preparation that hydrolyzed 105 nmol of NEFA (mL of EL)$^{-1}$ h^{-1}]. All of the incubations contained fatty acid free BSA (final concentration of 20 mg/mL). The total volume of each incubation was 50 μL. Phospholipid hydrolysis was terminated by adding chloroform/methanol [1 mL, 2:1 (v/v)] to the incubation mixture. The lipids were extracted by the method of Folch et al. (*21*). NEFA was separated from the other lipids by thin-layer chromatography (TLC) using 20 × 20 cm silica gel 60 plastic sheets (Merck, Darmstadt, Germany) as described previously (*20*). The lipids were visualized with iodine; the areas corresponding to unhydrolyzed 1,2-[^{14}C]DPPC and radiolabeled NEFA were recovered and placed into 5 mL of Ready Safe liquid scintillation mixture (Beckman Instruments, Fullerton, CA). 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 efficiency. EL-mediated hydrolysis in the 1,2-[^{14}C]DPPC−(A-I/A-II)rHDL was determined as the amount of radiolabeled NEFA relative to the total radiolabel in the substrate.

EL-Mediated Phospholipid Hydrolysis in (A-I)rHDL and (A-I/A-II)rHDL as a Function of Temperature. (A-I)rHDL and (A-I/A-II)rHDL (final phospholipid concentration of 0.8 mM) were individually mixed with EL [20 μL of a preparation that hydrolyzed 105 nmol of NEFA (mL of EL)$^{-1}$ h^{-1}] and incubated for 1.5 h at either 19, 25, 37, or 41 °C. The incubations were performed in the presence of fatty acid free BSA (final concentration of 20 mg/mL). The total volume of each incubation was 100 μL. When the incubations were complete, the tubes were placed immediately on ice to terminate the hydrolysis reaction. EL-mediated phospholipid hydrolysis of (A-I)rHDL and (A-I/A-II)rHDL was determined as the NEFA released from the rHDL and quantitated directly using a commercially available kit (Wako Pure Chemical Industries, Osaka, Japan).

Kinetic Studies. The kinetic parameters, K_m(app) and V_{max}, were derived by nonlinear regression analysis using GraphPad Prism version 4.02a for the Macintosh (GraphPad Software Inc., San Diego, CA) and the equation $Y = V_{max}X/(K_m + X)$.

Calculations. The predicted rates of EL-mediated phospholipid hydrolysis for the competitive inhibition studies of 1,2-[^{14}C]DPPC−(A-I/A-II)rHDL and (A-I)rHDL were determined using the rate law for a simple competitive inhibition model (*22*). The predicted rates of phospholipid hydrolysis in 1,2-[^{14}C]DPPC−(A-I/A-II)rHDL in the presence of increasing amounts of (A-I)rHDL were determined using the equation:

$$\frac{V_{A-I/A-II}}{V_{max(A-I/A-II)}} = \frac{\frac{[(A\text{-}I/A\text{-}II)rHDL]}{K_{m(A-I/A-II)}}}{1 + \frac{[(A\text{-}I/A\text{-}II)rHDL]}{K_{m(A-I/A-II)}} + \frac{[(A\text{-}I)rHDL]}{K_{i(A-I)}}} \quad (1)$$

where $V_{A-I/A-II}$ is the predicted rate of phospholipid hydrolysis in 1,2-[^{14}C]DPPC−(A-I/A-II)rHDL [nmol of NEFA (mL of EL)$^{-1}$ h^{-1}], [(A-I/A-II)rHDL] and [(A-I)rHDL] are the respective phospholipid concentrations of the substrate [1,2-[^{14}C]DPPC−(A-I/A-II)rHDL] and the inhibitor [(A-I)-rHDL], $V_{max(A-I/A-II)}$ and $K_{m(A-I/A-II)}$ represent the V_{max} [nmol of NEFA formed (mL of EL)$^{-1}$ h^{-1}] and K_m (mM) determined respectively for (A-I/A-II)rHDL in the absence of inhibitor, and K_i is the inhibition constant determined for (A-I/A-II)rHDL in the presence of (A-I)rHDL.

Other Techniques. All chemical analyses were carried out on a Roche/Hitachi 902 analyzer (Roche Diagnostics, Zurich, Switzerland). Phospholipid and unesterified cholesterol concentrations were determined enzymatically (*23*, *24*). Total cholesterol concentrations were determined using a Roche Diagnostics kit. CE concentrations were calculated as the difference between the total and UC concentrations. ApoA-I and apoA-II concentrations were measured immunoturbidometrically (*25*). Spherical rHDL size was determined by electrophoresis on 3−40% nondenaturing polyacrylamide gradient gels that were prepared by the method of Rainwater et al. (*26*).

Statistical Methods. All statistical analyses were performed using GraphPad Prism version 4.02a for the Macintosh (GraphPad Software Inc., San Diego, CA). Two-way ANOVA methods, followed by Bonfferroni post-tests, were used to determine if there were significant differences between the substrates. Significance was set at $p < 0.05$.

Table 1: Physical Properties of (A-I)rHDL, (A-I/A-II)rHDL, and (A-II)rHDL[a]

spherical rHDL	composition[b] (% mass) PL	UC	CE	A-I	A-II	Stokes diameter[c] (nm)
(A-I)rHDL	28.6	1.3	16.2	53.9	0.0	8.9
(A-I/A-II)rHDL	36.6	0.8	17.6	30.7	14.3	9.4
(A-II)rHDL	40.3	2.3	12.8	1.8	42.8	9.4

[a] Spherical (A-I)rHDL, (A-I/A-II)rHDL, and (A-II)rHDL were prepared as described in Experimental Procedures. [b] Compositions of individual constituents were calculated from the means of triplicate determinations that varied by less than 10%. Abbreviations: PL, phospholipid; UC, unesterified cholesterol; CE, cholesteryl ester; A-I, apoA-I; A-II, apoA-II. [c] Nondenaturing gel electrophoresis and Coomassie staining were used to determine rHDL size.

RESULTS

Physical Properties of (A-I)rHDL, (A-I/A-II)rHDL, and (A-II)rHDL (Table 1). The composition and size of the (A-I)rHDL, (A-I/A-II)rHDL, and (A-II)rHDL are shown in Table 1. All of the rHDL were comparable in terms of size and lipid composition (Table 1). As judged by nondenaturing gradient gel electrophoresis, each rHDL preparation contained a single, monodisperse population of particles (results not shown). The slightly larger diameter of the (A-I/A-II)-

Table 2: Rate of EL-Mediated Phospholipid Hydrolysis in (A-I)rHDL, (A-I/A-II)rHDL, and (A-II)rHDL[a]

PL (mM)	rate of phospholipid hydrolysis [nmol of NEFA (mL of EL)$^{-1}$ h^{-1}][b]				
	(A-I)rHDL	(A-I/A-II)rHDL	(A-II)rHDL	(A-I/A-II)rHDL/ (A-I)rHDL	(A-II)rHDL/ (A-I)rHDL[c]
0.1	74.4 ± 6.0	104.4 ± 4.4	1.1 ± 1.7	1.40	0.01
0.2	111.1 ± 1.7	167.8 ± 4.4	18.9 ± 1.7	1.51	0.17
0.4	122.2 ± 8.8	231.1 ± 15.9	10.0 ± 2.9	1.90	0.08
0.8	143.3 ± 5.8	285.6 ± 13.0	21.1 ± 4.4	2.00	0.15
1.0	135.5 ± 7.3	313.3 ± 17.6	33.3 ± 2.9	2.31	0.25

[a] Spherical (A-I)rHDL, (A-I/A-II)rHDL, and (A-II)rHDL were prepared as described in Experimental Procedures. Spherical (A-I)rHDL, (A-I/A-II)rHDL, and (A-II)rHDL (final concentration 0.1−1.0 mM) were incubated with a constant amount of EL as described in Experimental Procedures, and the resulting NEFA formed were measured directly by mass assay. [b] NEFA values are the means of triplicate determinations that varied by less than ±SEM. [c] Abbreviations: PL, phospholipid; A-I, apoA-I; A-II, apoA-II.

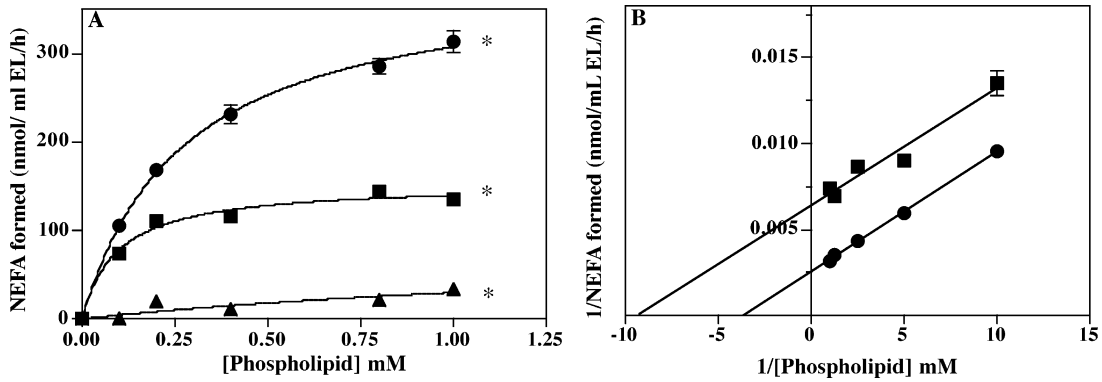

FIGURE 1: EL-mediated hydrolysis of (A-I)rHDL, (A-II)rHDL, and (A-I/A-II)rHDL. (A-I)rHDL (closed squares), (A-II)rHDL (closed triangles), and (A-I/A-II)rHDL (closed circles) were mixed with fatty acid free BSA and incubated at 37 °C for 1.5 h with EL as described under Experimental Procedures. The final rHDL phospholipid concentration varied from 0.1 to 1.0 mM. The rate of phospholipid hydrolysis was determined by mass assay of the NEFA generated in the incubation mixture as described. Panel A: The values are the means of triplicate determinations ± SEM. * = $p < 0.001$ for (A-I)rHDL versus (A-II)rHDL, (A-I)rHDL versus (A-I/A-II)rHDL, and (A-II)rHDL versus (A-I/A-II)rHDL. Panel B: A double reciprocal plot of the kinetic data from panel A is shown for (A-I)rHDL (closed squares) and (A-I/A-II)rHDL (closed circles).

rHDL and (A-II)rHDL, relative to the (A-I)rHDL (Table 1), is consistent with what has been reported previously (*18*, *19*).

Kinetics of EL-Mediated Hydrolysis of Phospholipids (A-I)rHDL, (A-I/A-II)rHDL, and (A-II)rHDL (Table 2, Figure 1). The kinetic studies were conducted under conditions that gave less than 30% phospholipid hydrolysis. The (A-I)rHDL, (A-I/A-II)rHDL, and (A-II)rHDL (0.1−1.0 mM phospholipid) were incubated with a constant amount of EL for 1.5 h. The rate of phospholipid hydrolysis was determined directly by mass assay of NEFA formed, assuming that one molecule of NEFA is generated for each molecule of phospholipid hydrolyzed (Table 2). EL-mediated phospholipid hydrolysis was greater in the (A-I/A-II)rHDL (closed circles) than in the (A-I)rHDL (closed squares) (Figure 1A). The phospholipid hydrolysis in (A-II)rHDL was, by comparison, very low (closed triangles). Lineweaver−Burk double reciprocal plots of the EL-mediated phospholipid hydrolysis for (A-I/A-II)rHDL and (A-I)rHDL are shown in Figure 1B. The kinetic parameters, V_{max} and K_m(app), were derived using nonlinear regression analysis. The results are shown in Table 3. The K_m(app) of EL for (A-I)rHDL and (A-I/A-II)rHDL was 0.10 ± 0.01 mM and 0.27 ± 0.03 mM, respectively. However, EL hydrolyzed the phospholipids in the (A-I/A-II)rHDL [V_{max} = 391.9 ± 12.9 nmol of NEFA (mL of EL)$^{-1}$ h^{-1}] more rapidly than in the (A-I)-rHDL [V_{max} = 152.8 ± 4.7 nmol of NEFA (mL of EL)$^{-1}$ h^{-1}].

Table 3: Kinetic Parameters of EL-Mediated Phospholipid Hydrolysis in rHDL[a]

spherical rHDL	K_m(app) (mM PL)[b]	V_{max} nmol of NEFA (mL of EL)$^{-1}$ h^{-1}	V_{max} (A-I/A-II)rHDL/ (A-I)rHDL
(A-I)rHDL	0.10 ± 0.01	152.8 ± 4.7	2.56
(A-I/A-II)rHDL	0.27 ± 0.03	391.9 ± 12.9	
(A-II)rHDL	nd[c]	nd[c]	

[a] Spherical (A-I)rHDL and (A-I/A-II)rHDL (final concentration 0.1−1.0 mM) were incubated with a constant amount of EL as described in Experimental Procedures. The resulting NEFA were measured by direct mass assay as described. The kinetic parameters for the EL-mediated phospholipid hydrolysis of (A-I)rHDL and (A-I/A-II)rHDL were derived by nonlinear regression analysis of the rate of phospholipid hydrolysis versus the concentration of substrate. [b] PL = phospholipid. [c] Not determined. The kinetic parameters for the EL-mediated phospholipid hydrolysis of (A-II)rHDL could not be determined due to the low hydrolysis observed.

EL-Mediated Phospholipid Hydrolysis of 1,2-[^{14}C]DPPC-Labeled (A-I/A-II)rHDL in the Presence of Increasing Amounts of Unlabeled (A-I)rHDL (Table 4, Figure 2). The preceding results raise the possibility that (A-I)rHDL may competitively inhibit the rate of phospholipid hydrolysis in (A-I/A-II)rHDL. To determine if this was the case, mixtures of 1,2-[^{14}C]DPPC-labeled (A-I/A-II)rHDL and unlabeled (A-I)rHDL were incubated with a constant amount of EL, and the formation of radiolabeled NEFA was determined. As the (A-I)rHDL were unlabeled, these results reflect the hydrolysis of the phospholipids in the (A-I/A-II)rHDL

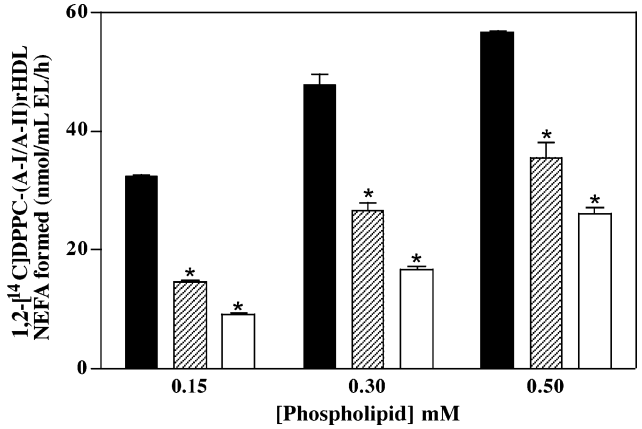

FIGURE 2: Competitive studies of the EL-mediated phospholipid hydrolysis in 1,2-[^{14}C]DPPC-labeled (A-I/A-II)rHDL by unlabeled (A-I)rHDL. (A-I/A-II)rHDL were radiolabeled with 1,2-[^{14}C]DPPC as described under Experimental Procedures. Mixtures of 1,2-[^{14}C]-DPPC-labeled (A-I/A-II)rHDL were incubated with EL in either the absence (closed bars) or presence of unlabeled (A-I)rHDL at a final phospholipid concentration of 0.2 mM (hatched bars) or 0.4 mM (open bars) as described. The rate of phospholipid hydrolysis of (A-I/A-II)rHDL (triplicate determinations ± SEM) [* = $p <$ 0.001, compared to (A-I/A-II)rHDL only] is shown.

only. Moreover, as the rate of exchange of phospholipids between reconstituted lipoproteins is very slow ($t_{1/2} > 10$ h), it is unlikely that any exchange of radiolabeled phospholipid between the substrates is of significance (*27*, *28*). It should also be noted that 1,2-[^{14}C]DPPC has been used extensively as a tracer to monitor phospholipid hydrolysis in a range of substrates, including spherical (A-I)rHDL and (A-II)rHDL (*29*−*31*). The composition (percent mass) of phospholipid/UC/CE/A-I/A-II in 1,2-[^{14}C]DPPC-labeled (A-I/A-II)rHDL and unlabeled (A-I)rHDL was 36.3/0.9/12.0/35.9/14.9 and 35.1/1.6/19.3/44.0/0.0, respectively.

The 1,2-[^{14}C]DPPC-labeled (A-I/A-II)rHDL (0.15, 0.30, and 0.50 mM phospholipid) were mixed with unlabeled (A-I)rHDL (either 0.0, 0.20, or 0.40 mM phospholipid) and incubated with a constant amount of EL. The radiolabeled NEFA generated during the incubation were isolated as described under Experimental Procedures. The rate of phospholipid hydrolysis in 1,2-[^{14}C]DPPC-labeled (A-I/A-II)-rHDL either in the absence (closed bars) or in the presence of unlabeled (A-I)rHDL [0.2 mM (hatched bars) or 0.4 mM phospholipid (open bars)] is shown in Figure 2. Phospholipid hydrolysis in the (A-I/A-II)rHDL decreased as the amount of unlabeled (A-I)rHDL in the incubation increased ($p <$ 0.001).

The predicted rates of EL-mediated phospholipid hydrolysis of 1,2-[^{14}C]DPPC-labeled (A-I/A-II)rHDL in the presence of (A-I)rHDL were calculated using eq 1 and the kinetic parameters determined for the EL-mediated phospholipid hydrolysis of (A-I/A-II)rHDL in the absence of any competitive inhibitor. For each incubation, the observed rate of phospholipid hydrolysis in (A-I/A-II)rHDL was comparable to the rate predicted by the model for a simple competitive inhibition (Table 4). Therefore, the data are consistent with the notion that (A-I)rHDL is a competitive inhibitor of the EL-mediated phospholipid hydrolysis of (A-I/A-II)rHDL.

EL-Mediated Phospholipid Hydrolysis in (A-I)rHDL and (A-I/A-II)rHDL as a Function of Temperature (Figure 3). These experiments were carried out in order to determine if

Table 4: EL-Mediated PL Hydrolysis in 1,2-[^{14}C]DPPC-Labeled (A-I/A-II)rHDL in the Presence of Unlabeled (A-I)rHDL[a]

[1,2-[^{14}C]DPPC-labeled (A-I/A-II)rHDL] (mM PL)[b]	phospholipid hydrolysis in 1,2-[^{14}C]DPPC-labeled (A-I/A-II)rHDL [nmol of NEFA formed (mL of EL)$^{-1}$ h^{-1}]			
	1,2-[^{14}C]DPPC−(A-I/A-II)rHDL + 0.2 mM (A-I)rHDL		1,2-[^{14}C]DPPC−(A-I/A-II)rHDL + 0.4 mM (A-I)rHDL	
	observed	predicted	observed	predicted
0.15	14.6 ± 0.25	14.5	10.5 ± 1.25	8.8
0.30	26.6 ± 1.47	24.7	16.7 ± 0.6	16.0
0.50	35.7 ± 2.33	34.3	26.2 ± 1.17	23.6

[a] (A-I/A-II)rHDL were labeled with 1,2-[^{14}C]DPPC as described in Experimental Procedures. The radiolabeled (A-I/A-II)rHDL and unlabeled (A-I)rHDL were incubated with a constant amount of EL as described. The values for the observed rates of hydrolysis of 1,2-[^{14}C]DPPC-labeled (A-I/A-II)rHDL are the means of triplicate determinations ± SEM. The predicted values for the rate of phospholipid hydrolysis in (A-I/A-II)rHDL were determined using eq 1 and the kinetic parameters determined for the EL-mediated phospholipid hydrolysis of [1,2-[^{14}C]DPPC-labeled (A-I/A-II)rHDL in the absence of (A-I)rHDL. [b] PL = phospholipid.

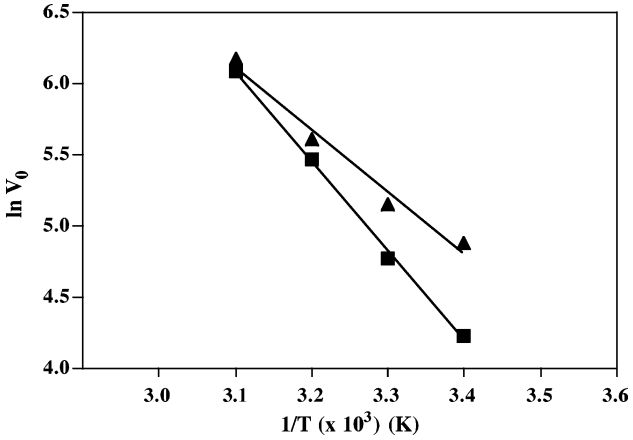

FIGURE 3: Arrhenius plots of EL-mediated phospholipid hydrolysis in (A-I)rHDL and (A-I/A-II)rHDL. (A-I)rHDL (closed squares) and (A-I/A-II)rHDL (closed triangles) (0.8 mM phospholipid) were incubated with EL at either 19, 25, 37, or 41 °C as described under Experimental Procedures. Activation energies (E_a) were calculated from the slope of the plots.

the differences in activation energy (E_a) for EL-mediated phospholipid hydrolysis reaction in (A-I)rHDL and (A-I/A-II)-rHDL reflected the differences in the binding affinity and hydrolysis rates in Table 2. (A-I)rHDL and (A-I/A-II)rHDL (0.8 mM phospholipid) were incubated with EL at either 19, 25, 37, or 41 °C. The Arrhenius plot of the natural logarithm of the initial rate [ln(V_0)] [nmol of NEFA formed (mL of EL)$^{-1}$ h^{-1}] versus the inverse temperature in kelvin (1/T (K)) was linear within this temperature range. The activation energies, calculated from the slope of the Arrhenius plots, were 52.1 and 34.8 kJ mol^{-1} for (A-I)rHDL (closed squares) and (A-I/A-II)rHDL (closed triangles), respectively (Figure 3).

DISCUSSION

EL is a newly identified member of the triglyceride lipase gene family that hydrolyzes HDL phospholipids (*1*−*5*). The HDL in human plasma have been classified on the basis of apolipoprotein composition into those containing apoA-I, but no apoA-II, (A-I)HDL, or apoA-I and apoA-II, (A-I/A-II)-

HDL (*32*). A minor HDL subpopulation containing apoA-II without apoA-I, (A-II)HDL, has also been reported (*33*). Recent studies have shown that the overexpression of EL in mice results in markedly reduced HDL-C and apoA-I levels (*1*, *6*, *7*); however, the influence of EL on HDL that contain apoA-II has not been reported.

The aim of this study was to determine how apoA-I and apoA-II influence the EL-mediated hydrolysis of HDL phospholipids. To achieve this aim, it was necessary to use HDL that were comparable in size and lipid composition and which varied only in their apolipoprotein content. The HDL in human plasma could not be used in a study of this type because they differ widely in size and lipid composition and contain apolipoproteins other than apoA-I and apoA-II (*34*, *35*). These problems were overcome in the present study by using preparations of well-characterized, homogeneous, spherical rHDL that contained either apoA-I or apoA-II as the sole apolipoprotein or both apoA-I and apoA-II (*13*, *19*). As these rHDL varied only in their apolipoprotein content, the differences that were observed for the kinetics of EL-mediated phospholipid hydrolysis could be attributed unequivocally to their apolipoprotein composition.

The results showed that while EL hydrolyzed the phospholipids in (A-I/A-II)rHDL with a greater maximal rate than in (A-I)rHDL, it had a higher binding affinity for (A-I)rHDL than for (A-I/A-II)rHDL. This trend is the opposite to what was observed previously for the reaction kinetics of HL with (A-I)rHDL and (A-I/A-II)rHDL (*31*). In those studies, the rate of HL-mediated phospholipid hydrolysis was greater in the (A-I)rHDL than in the (A-I/A-II)rHDL, but the binding affinity of HL for the (A-I/A-II)rHDL was greater than for (A-I)rHDL. This may reflect the free energy changes that occur for a tightly bound enzyme−substrate complex versus a more weakly bound complex.

It is well established that the greater the binding affinity of an enzyme for a substrate, the more tightly bound the resulting intermediate enzyme−substrate complex and the lower the subsequent free energy of the complex (*22*, *36*). A more tightly bound enzyme−substrate complex decreases the maximal rate of hydrolysis of the substrate as fewer molecules have the energy required to surmount the activation barrier associated with the reaction. In contrast, if the enzyme binds the substrate weakly, the greater free energy of the complexes allows for a greater proportion of the complexes to overcome the activation barrier of the reaction and results in a greater overall rate of hydrolysis of the substrate. Thus, the results of our studies which show that EL binds (A-I)rHDL more tightly than (A-I/A-II)rHDL, but the rate of EL-mediated phospholipid hydrolysis is greater in (A-I/A-II)rHDL than in (A-I)rHDL (Table 3), should be consistent with the energy of activation of EL-mediated phospholipid hydrolysis for (A-I)rHDL being greater than for (A-I/A-II)rHDL.

To determine if this was the case, the rates of reaction of EL with (A-I)rHDL and (A-I/A-II)rHDL were determined as a function of temperature, and the results are presented in the form of an Arrhenius plot (Figure 3). These results demonstrated that the lower rate of phospholipid hydrolysis observed for EL with (A-I)rHDL can be attributed to the higher activation barrier associated with the hydrolysis reaction, when compared to that observed for (A-I/A-II)-rHDL.

Moreover, in accordance with this trend, it follows that (A-I)rHDL may inhibit the EL-mediated phospholipid hydrolysis in (A-I/A-II)rHDL. Given that EL has a higher binding affinity for (A-I)rHDL than for (A-I/A-II)rHDL, it follows that EL should preferentially bind to the (A-I)rHDL and reduce the rate of phospholipid hydrolysis in the (A-I/A-II)rHDL. To determine if this was the case, the phospholipid hydrolysis of 1,2-[^{14}C]DPPC-labeled (A-I/A-II)rHDL was determined in incubation mixtures containing EL and unlabeled (A-I)rHDL. (A-I/A-II)rHDL phospholipid hydrolysis decreased as the amount of (A-I)rHDL in the incubation mixtures was increased (Figure 2). Moreover, if one models the rate of the EL-mediated phospholipid hydrolysis of (A-I/A-II)rHDL in the presence of (A-I)rHDL, it follows precisely a competitive inhibition model. These results support the notion that (A-I)rHDL is a competitive inhibitor of the EL-mediated phospholipid hydrolysis of (A-I/A-II)-rHDL.

It should be noted that apolipoprotein exchange between the rHDL substrates is not a relevant concern in the present study. Apolipoprotein A-II has an extremely high affinity for lipid and thus has a very low probability of dissociating from the (A-I/A-II)rHDL particles (*18*). Although apolipoprotein A-I does have a lower binding affinity for lipid than apolipoprotein A-II and may therefore exchange between the rHDL particles, it can only exchange with another molecule of apolipoprotein A-I in the present study. Therefore, even if exchange does occur, it would have no effect on the overall composition of the particles. It should also be emphasized that there is no evidence from any of the earlier studies carried out in our laboratory that exchange of apolipoproteins between the different rHDL particles occurs.

Our studies showed that very little EL-mediated phospholipid hydrolysis was observed for the (A-II)rHDL (Figure 1A). These results suggest that, under the conditions employed in our study, EL requires apoA-I to be incorporated into the lipoprotein particle for significant phospholipid hydrolysis to occur. This hypothesis is further supported by recent work from our laboratory showing that incubation with EL reduces the size and phospholipid concentration of (A-I)rHDL and (A-I/A-II)rHDL but not (A-II)rHDL (unpublished). The minor hydrolysis observed for (A-II)rHDL (Figure 1A) may in part be attributed to the presence of a trace amount (ca. 2% by mass) of apoA-I in the (A-II)rHDL. The presence of a trace amount of apoA-I in (A-II)rHDL is consistent with the results of previous studies and indicates that the displacement of apoA-I from spherical (A-I)rHDL by lipid-free apoA-II is not always quantitative (*18*, *20*, *31*).

One of the most interesting findings to come out of the present project is that apoA-I must be present in a rHDL particle for significant EL-mediated phospholipid hydrolysis to occur. Moreover, the incorporation of apoA-II into a rHDL particle that already contains apoA-I more than doubles the rate of EL-mediated phospholipid hydrolysis. Recent work from this laboratory has established that the presence of apoA-II on the surface of (A-I/A-II)rHDL promotes a major conformational change in the C-terminal domain of apoA-I (*37*). This was shown to be due to the formation of salt bridges between apoA-II and the C-terminal domain of apoA-I. These findings raise the possibility that the differences in the binding affinity of EL for (A-I/A-II)rHDL and (A-I)-rHDL may be related to the conformational change in apoA-I

that is induced by apoA-II. If we assume that the activation of EL by apoA-I is the result of a protein−protein interaction, as has been reported for HL with apoE (*38*), it follows that the presence of apoA-II may disrupt the binding between apoA-I and EL in (A-I/A-II)rHDL. This decreases the binding affinity of EL for (A-I/A-II)rHDL and increases the maximal rate of EL-mediated phospholipid hydrolysis for (A-I/A-II)rHDL compared to (A-I)rHDL.

These results provide valuable insights into the physiological role of EL in the regulation of HDL metabolism. Previous studies have shown that HL-mediated phospholipid hydrolysis is greater in (A-I)rHDL than in (A-I/A-II)rHDL (*31*). In contrast, this present study has established that EL preferentially hydrolyzes the phospholipids in rHDL substrates containing both apoA-I and apoA-II than those that contain apoA-I as their sole apolipoprotein. When taken together, these findings support the notion that HL and EL may have complementary roles in the metabolism of HDL particles in plasma.

In conclusion, this study shows that apoA-I and apoA-II regulate the binding affinity of EL for HDL and that they have a major influence on the rate at which EL hydrolyzes HDL phospholipids. The results also establish that the presence of apoA-I in rHDL is essential for significant EL-mediated phospholipid hydrolysis to be observed. The finding that EL hydrolyzes the phospholipids in (A-I/A-II)rHDL more rapidly than in (A-I)rHDL suggests that EL may preferentially decrease plasma levels of (A-I/A-II)HDL.

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