Evidence That Apolipoprotein A-I Facilitates Hepatic Lipase-Mediated Phospholipid Hydrolysis in Reconstituted HDL Containing Apolipoprotein A-II†

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ABSTRACT: This study examines hepatic lipase (HL) mediated phospholipid hydrolysis in mixtures of apolipoprotein-specific, spherical reconstituted high-density lipoproteins (rHDL). We have shown previously that apolipoprotein A-I (apoA-I) and apoA-II have a major influence on the kinetics of HL-mediated phospholipid and triacylglycerol hydrolysis in well-characterized, homogeneous preparations of spherical rHDL [Hime, N. J., Barter, P. J., and Rye, K.-A. (1998) *J. Biol. Chem. 273*, 27191−27198]. In the present study, phospholipid hydrolysis was assessed in mixtures of rHDL containing either apoA-I only, (A-I)-rHDL, apoA-II only, (A-II)rHDL, or both apoA-I and apoA-II, (A-I/A-II)rHDL. The rHDL contained trace amounts of radiolabeled phospholipid, and hydrolysis was measured as the formation of radiolabeled nonesterified fatty acids (NEFA). As predicted from our previous kinetic studies, the (A-II)rHDL acted as competitive inhibitors of HL-mediated phospholipid hydrolysis in (A-I)rHDL. Less expected was the observation that the rate of phospholipid hydrolysis in (A-II)rHDL was enhanced when (A-I)rHDL were also present in the incubation mixture. The rate of phospholipid hydrolysis in (A-I/A-II)rHDL was also greater than in (A-II)rHDL, indicating that apoA-I enhances phospholipid hydrolysis when it is present as a component of (A-I/A-II)rHDL. It is concluded that apoA-I enhances HL-mediated phospholipid hydrolysis in apoA-II containing rHDL, irrespective of whether the apoA-I is present in the same particle as the apoA-II [as in (A-I/A-II)rHDL] or whether it is present as a component of a different particle, such as when (A-I)rHDL are added to incubations of (A-II)rHDL.

Hepatic lipase (HL)[1] is a lipolytic enzyme found predominantly on the surface of hepatic sinusoids (*1*). HL possesses both triacylglycerol hydrolase and phospholipase A_1 activity and is involved in the metabolism of chylomicron remnants, very low density lipoproteins, intermediate density lipoproteins, low-density lipoproteins, and high-density lipoproteins (HDL) (*2*, *3*). The role of HL in HDL metabolism is of particular importance, as shown by strong negative associations between HL lipolytic activity and plasma HDL_2 levels (*4*−*6*) and the dramatic reduction in the HDL levels of rabbits transgenic for human HL (*7*). There is evidence that the phospholipase A_1 activity of HL is important in the uptake of HDL cholesteryl esters (CE) by the liver (*8*, *9*). HL is also involved in the selective uptake of HDL-CE via the scavenger receptor BI in a process that is independent of its lipolytic activity (*10*). HL also mediates the dissociation of lipid-poor apolipoprotein (apo) A-I from HDL (*11*).

Many investigators have shown that apolipoproteins influence HL activity. Thuren et al. demonstrated that while apoA-I, A-II, C-I, C-II, C-III, and E all inhibit HL-mediated hydrolysis of phospholipids in monolayers maintained at high surface pressure, apoA-I, C-II, C-III, and E activate HL-mediated phospholipid hydrolysis in monolayers at low surface pressure (*12*). A number of studies have focused on the role of apoA-II in HL-mediated phospholipid and triacylglycerol hydrolysis. These studies have produced conflicting results. For example, lipid-free apoA-II has been reported to both increase (*13*) and decrease (*14*) HL-mediated triacylglycerol hydrolysis in lipid emulsions. Shinomiya et al. showed that when apoA-II is incorporated into HDL_2, the ability of HL to hydrolyze the triacylglycerol in the particles is inhibited (*15*). There is also evidence that HL-mediated liberation of nonesterified fatty acids (NEFA) from HDL containing both apoA-I and apoA-II, (A-I/A-II)HDL, is greater than from HDL containing only apoA-I, (A-I)-HDL (*16*, *17*). Studies of mice that are either transgenic for human apoA-II (*18*, *19*) or deficient in apoA-II (*20*) also suggest that apoA-II inhibits HL activity.

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[1] Abbreviations: HL, hepatic lipase; HDL, high-density lipoproteins; HDL_2, high-density lipoproteins, subfraction 2; CE, cholesteryl esters; apo, apolipoprotein; rHDL, reconstituted high-density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; PLTP, phospholipid transfer protein; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; UC, unesterified cholesterol; DPPC, 1,2-dipalmitoylphosphatidylcholine; TBS, Tris-buffered saline; BSA, bovine serum albumin; NEFA, nonesterified fatty acids.

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In a previous study we examined the underlying reasons for these conflicting results (*21*). In that study we used well-defined, homogeneous preparations of spherical reconstituted HDL (rHDL) containing either apoA-I or apoA-II as the sole apolipoprotein constituent to show that, while the V_{max} of phospholipid hydrolysis is greater for (A-I)rHDL than for (A-II)rHDL, the affinity of HL for the phospholipids in (A-II)rHDL is greater than for the phospholipids in (A-I)rHDL (*21*). Those results indicated that apoA-II-containing HDL may act as competitive inhibitors of HL-mediated phospholipid and triacylglycerol hydrolysis in HDL that contain apoA-I.

In the present study we have shown that apoA-II-containing HDL are indeed competitive inhibitors of HL-mediated phospholipid hydrolysis in (A-I)rHDL. This was achieved by incubating mixtures of (A-I)rHDL and (A-II)-rHDL with HL. The results also unexpectedly show that the rate of HL-mediated phospholipid hydrolysis in apoA-II containing rHDL is markedly enhanced by the presence of apoA-I.

EXPERIMENTAL PROCEDURES

Isolation of ApoA-I and ApoA-II. HDL were ultracentrifugally isolated from samples of pooled expired, autologously donated human plasma (Gribbles Pathology, Adelaide) and delipidated as described (*22*, *23*). ApoA-I and apoA-II were isolated from the resulting apoHDL by chromatography on a Q-Sepharose Fast Flow column (Amersham Pharmacia Biotech, Uppsala, Sweden) attached to an FPLC system (Amersham Pharmacia Biotech) (*24*). 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.

Purification of Lecithin:Cholesterol Acyltransferase (LCAT) and Phospholipid Transfer Protein (PLTP). LCAT and PLTP were purified from pooled samples of autologously donated human plasma. The purified LCAT, prepared as described previously (*25*), appeared as a single band following electrophoresis on a homogeneous 20% SDS-gel and silver staining. LCAT activity was assessed as described by Piran and Morin (*26*) using 1-palmitoyl-2-oleoylphosphatidylcholine (POPC)/unesterified cholesterol (UC)/apoA-I discoidal rHDL labeled with [1α,2α-^{3}H]cholesterol ([^{3}H]UC) as a substrate. The POPC and [^{3}H]UC were obtained respectively from Sigma and Amersham Pharmacia Biotech. The assay was linear as long as less than 30% of the [^{3}H]UC was esterified. The preparation used in this study generated 1080 nmol of CE (mL of LCAT)$^{-1}$ h^{-1}.

The purification of PLTP has been described previously (*27*). 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 Biotech) from [^{14}C]-DPPC-labeled small unilamellar POPC vesicles to ultracentrifugally isolated HDL (*28*). The PLTP used in this study transferred 3150 nmol of phospholipid (mL of PLTP)$^{-1}$ h^{-1}.

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). The method of purification is described elsewhere except that a linear 0.4−1.3 M NaCl gradient was used to elute HL from the heparin−Sepharose Fast Flow column (*21*). The purified HL appeared as a single band following SDS−polyacrylamide gel electrophoresis on a 20% homogeneous PhastGel (Amersham Pharmacia Biotech) and staining with Coomassie Blue. HL activity was assessed as the nmol of NEFA generated (mL of HL)$^{-1}$ h^{-1} using spherical rHDL containing triacylglycerol in their core as a substrate (*21*). NEFA mass was assayed using a commercially available kit (Wako Pure Chemical Industries, Osaka, Japan). The HL activity in the individual experiments is presented in the figure legends.

Preparation of Unlabeled Spherical (A-I)rHDL and (A-II)rHDL. Discoidal rHDL containing POPC, UC, and apoA-I were prepared by the cholate dialysis method (*29*). Spherical rHDL containing CE as the only core lipid and apoA-I as the sole apolipoprotein constituent, (A-I)rHDL, were prepared by incubating the discoidal rHDL with low-density lipoproteins and LCAT (*30*). Spherical (A-II)rHDL were prepared by displacing all of the apoA-I from the spherical (A-I)rHDL with lipid-free apoA-II (*30*). The (A-I)rHDL and (A-II)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 [^{14}C]DPPC-Labeled (A-I)rHDL and [^{14}C]-DPPC-Labeled (A-II)rHDL. [^{14}C]DPPC-labeled (A-I)rHDL were prepared by incubating unlabeled (A-I)rHDL (21.5 μmol of phospholipid) with [^{14}C]DPPC-labeled POPC vesicles (2.15 μmol of phospholipid) for 3 h at 37 °C with purified PLTP [final transfer activity 367 nmol of phospholipid transferred (mL of PLTP)$^{-1}$ h^{-1}] and fatty acid free bovine serum albumin (BSA) (final concentration 20 mg/mL) (*21*). The volume of the incubation mixture was 12 mL. The resulting [^{14}C]DPPC-labeled (A-I)rHDL were isolated by sequential ultracentrifugation as described previously (*21*). [^{14}C]DPPC-labeled (A-II)rHDL were prepared by displacing all of the apoA-I from [^{14}C]DPPC-labeled (A-I)rHDL with lipid-free apoA-II as described (*30*). The specific activities of the [^{14}C]DPPC-labeled (A-I)rHDL and [^{14}C]DPPC-labeled (A-II)rHDL were 3.0 × 10^5 dpm/mg of phospholipid and 2.8 × 10^5 dpm/mg of phospholipid, respectively. The [^{14}C]-DPPC-labeled (A-I)rHDL and (A-II)rHDL were dialyzed extensively (3 × 1 L) against TBS prior to use.

Preparation of Unlabeled (A-I/A-II)rHDL. Spherical rHDL containing both apoA-I and apoA-II on the same particle were prepared as described (*31*). Briefly, discoidal (A-I)-rHDL and discoidal (A-II)rHDL were prepared by the cholate dialysis method (*29*). Discoidal (A-I)rHDL (final concentration apoA-I 143 μg/mL) were incubated with discoidal (A-II)rHDL (final concentration apoA-II 43 μg/mL) at 37 °C for 24 h with low-density lipoproteins (final concentration apoB 581 μg/mL), fatty acid free BSA (final concentration 57 mg/mL), β-mercaptoethanol (final concentration 4.0 mM), and LCAT [final activity, 162 nmol of CE generated (mL of incubation mixture)$^{-1}$ h^{-1}]. The final incubation volume was 35.7 mL. These conditions generated both spherical (A-I)rHDL and spherical (A-I/A-II)rHDL. When the incubation was complete, the rHDL were isolated by ultracentrifugation at 100 000 rpm, at 4 °C, in the density range $1.07 < d < 1.21$ g/mL using a TLA-100.4 rotor (Beckman Instruments, Fullarton, CA) with two 16 h spins at the lower density and a single 16 h spin at the higher density. These procedures

were carried out in a Beckman TL-100 tabletop ultracentrifuge (Beckman Instruments). The rHDL were dialyzed against TBS (3 × 1 L) and subjected to immunoaffinity chromatography using a column of anti-human apoA-II polyclonal antibody coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech). The (A-I)rHDL, which did not bind to the column, were washed off with TBS. The (A-I/A-II)rHDL that bound to the column were eluted with 0.1 M acetic acid (pH 2.7) and neutralized immediately with 1 M Tris, pH 11.0 (final concentration 0.1 M).

Preparation of [^{14}C]DPPC-Labeled (A-I/A-II)rHDL. [^{14}C]-DPPC-labeled (A-I/A-II)rHDL were prepared by incubating unlabeled (A-I/A-II)rHDL with [^{14}C]DPPC-labeled POPC vesicles, purified PLTP, and fatty acid free BSA as described for (A-I)rHDL. The specific activity of the [^{14}C]DPPC-labeled (A-I/A-II)rHDL was 2.3×10^5 dpm/mg of phospholipid. The [^{14}C]DPPC-labeled (A-I/A-II)rHDL was dialyzed extensively (3 × 1 L) against TBS prior to use.

Determination of HL-Mediated Phospholipid Hydrolysis in [^{14}C]DPPC-Labeled (A-I)rHDL, [^{14}C]DPPC-Labeled (A-I/A-II)rHDL, and [^{14}C]DPPC-Labeled (A-II)rHDL. All incubations were carried out in stoppered plastic tubes in a shaking water bath maintained at 37 °C. Details of individual incubations are described in the figure legends. Hydrolysis was stopped by the addition of chloroform/methanol [1 mL, 2:1 (v/v)]. The lipids were extracted by the method of Folch et al. (*32*). NEFA were separated from the other rHDL lipids by thin-layer chromatography on 20 × 20 cm silica gel 60 plastic sheets (Merck, Darmstadt, Germany) as described elsewhere (*21*) and visualized with I_2. The areas corresponding to phosphatidylcholine 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 HL-Mediated Phospholipid Hydrolysis in Unlabeled (A-I)rHDL, Unlabeled (A-II)rHDL, and Unlabeled (A-I/A-II)rHDL. These incubations were carried out exactly as described above for the radiolabeled rHDL. After the incubation the mixtures were placed on ice. Phospholipid hydrolysis was determined directly by assaying the NEFA mass in the incubation mixtures.

Immunoaffinity Chromatography of Mixtures of (A-I)rHDL and (A-II)rHDL after Incubation with HL. (A-I)rHDL (final concentration 0.1 mM phospholipid) and (A-II)rHDL (final concentration 0.4 mM phospholipid) were incubated at 37 °C for 3 h with HL [1.12 mL of a preparation which generated 682 nmol of NEFA (mL of HL)$^{-1}$ h^{-1}]. The incubation also contained fatty acid free BSA (final concentration 20 mg/mL). The final incubation volume was 4.49 mL. When the incubation was complete, the mixture was placed on ice and then added to a 1 mL sealed Poly-Prep column (Bio-Rad Laboratories, Hercules, CA) containing a human anti-apoA-I polyclonal antibody covalently coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech). The rHDL which did not bind to the column were eluted with TBS (5 column volumes). The rHDL which bound to the column were eluted with 0.1 M acetic acid (7 column volumes) and neutralized immediately with 1 M Tris, pH 11.0 (final concentration 0.1 M). The eluted fractions (1.1 mL) were assayed for apoA-I and apoA-II.

Calculations. Phospholipid hydrolysis in the radiolabeled (A-I)rHDL and (A-II)rHDL was determined as the amount of radiolabeled NEFA relative to the total substrate radiolabel. Phospholipid hydrolysis in the unlabeled (A-I)rHDL, (A-II)rHDL, and (A-I/A-II)rHDL was determined directly by mass assay. The kinetic parameters K_m(app) and V_{max} were estimated from the line of best fit by linear regression analysis of a Lineweaver−Burk double-reciprocal plot of the rate of hydrolysis versus the concentration of substrate. In all cases, the regression coefficients (r) were >0.94. V_{max} was determined as the reciprocal of the intercept on the y axis. K_m(app) was calculated as the product of the slope and V_{max}.

The predicted rates of HL-mediated phospholipid hydrolysis were determined using a variation of the model described by Barter et al. (*33*). The rates of phospholipid hydrolysis in incubations containing mixtures of (A-I)rHDL and (A-II)rHDL are expressed mathematically as

$$V = \frac{\dfrac{V_{A-Imax}[\text{(A-I)rHDL PL}]}{K'_{A-I}} + \dfrac{V_{A-IImax}[\text{(A-II)rHDL PL}]}{K'_{A-II}}}{1 + \dfrac{[\text{(A-I)rHDL PL}]}{K'_{A-I}} + \dfrac{[\text{(A-II)rHDL PL}]}{K'_{A-II}}} \quad (1)$$

where V is the predicted rate of phospholipid hydrolysis in the mixture [nmol of NEFA formed (mL of HL)$^{-1}$ h^{-1}] and [(A-I)rHDL PL] and [(A-II)rHDL PL] are the concentrations of (A-I)rHDL and (A-II)rHDL (millimolar phospholipid), respectively. V_{A-Imax} and $V_{A-IImax}$ represent the values of V_{max} [nmol of NEFA formed (mL of HL)$^{-1}$ h^{-1}] for (A-I)rHDL and (A-II)rHDL, respectively. K'_{A-I} and K'_{A-II} represent the K_m(app) (millimolar phospholipid) for (A-I)rHDL and (A-II)rHDL, respectively, for a given amount of HL.

The predicted rates of HL-mediated phospholipid hydrolysis in (A-I/A-II)rHDL in incubations containing mixtures of (A-I/A-II)rHDL and (A-I)rHDL were determined using the equation:

$$V_{A-I/A-II} = \frac{\dfrac{[\text{(A-I/A-II)rHDL PL}]}{K'_{A-I/A-II}}}{1 + \dfrac{[\text{(A-I/A-II)rHDL PL}]}{K'_{A-I/A-II}} + \dfrac{[\text{(A-I)rHDL PL}]}{K'_{A-I}}} V_{A-I/A-IImax} \quad (2)$$

where $V_{A-I/A-II}$ is the predicted rate of phospholipid hydrolysis in (A-I/A-II)rHDL [nmol of NEFA formed (mL of HL)$^{-1}$ h^{-1}] and [(A-I/A-II)rHDL PL] is the concentration of (A-I/A-II)rHDL (millimolar phospholipid). $V_{A-I/A-IImax}$ represents the V_{max} [nmol of NEFA formed (mL of HL)$^{-1}$ h^{-1}] for (A-I/A-II)rHDL. $K'_{A-I/A-II}$ represents the K_m(app) (millimolar phospholipid) for (A-I/A-II)rHDL for a given amount of HL.

Other Techniques. All chemical analyses were carried out on a Cobas Fara centrifugal analyzer (Roche Diagnostics, Zurich, Switzerland). Boehringer Mannheim kits were used for phospholipid, UC, and total cholesterol assays (Boehringer Mannheim GmbH, Mannheim, Germany). CE con-

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

spherical rHDL	stoichiometry[b] (mol/mol) PL	UC	CE	A-I	A-II	Stokes diameter[c] (nm)
Section A						
[^{14}C]DPPC−(A-I)rHDL[d]	73.9	6.0	73.9	3.0	0.0	8.6
[^{14}C]DPPC−(A-II)rHDL[d]	65.4	5.3	68.6	0.0	6.0	9.4
Section B						
[^{14}C]DPPC−(A-I)rHDL[e]	116.7	11.6	76.7	3.0	0.0	9.3
unlabeled (A-II)rHDL[e]	89.5	8.6	62.5	0.1	6.0	10.0
Section C						
unlabeled (A-I)rHDL[f]	109.0	10.6	74.9	3.0	0.0	9.3
[^{14}C]DPPC−(A-II)rHDL[f]	96.0	9.5	64.0	0.1	6.0	10.1

[a] (A-I)rHDL were prepared by incubating discoidal rHDL with low-density lipoproteins and LCAT. [^{14}C]DPPC−(A-I)rHDL were prepared as described under Experimental Procedures. Unlabeled (A-II)rHDL and [^{14}C]DPPC−(A-II)rHDL were prepared by displacing all of the apoA-I, respectively, from unlabeled (A-I)rHDL and [^{14}C]DPPC−(A-I)rHDL with lipid-free apoA-II. [b] Stoichiometries were calculated from means of triplicate determinations which varied by less than 6%. Abbreviations: PL, phospholipid; UC, unesterified cholesterol; CE, cholesteryl ester; A-I, apoA-I; A-II, apoA-II. [c] All of the rHDL preparations contained a single, homogeneous population of particles as judged by nondenaturing gradient gel electrophoresis. [d] These rHDL preparations were used to examine total phospholipid hydrolysis in incubations containing mixtures of (A-I)rHDL and (A-II)rHDL. [e] These rHDL preparations were used to examine the effect of (A-II)rHDL on phospholipid hydrolysis in (A-I)rHDL. [f] These rHDL preparations were used to examine the effect of (A-I)rHDL on phospholipid hydrolysis in (A-II)rHDL.

centrations were calculated as the difference between the total and UC concentrations. ApoA-I and apoA-II concentrations were measured immunoturbidometrically (*34*), using sheep anti-human apoA-I (*35*) and sheep anti-human apoA-II immunoglobulin (Boehringer Mannheim GmbH). Spherical rHDL size was determined by electrophoresis on 3−40% nondenaturing polyacrylamide gradient gels prepared according to the method of Rainwater et al. (*36*).

Statistical Methods. The two-tailed, Student's *t* test for two samples with equal variance was used to determine whether differences between values were significant.

RESULTS

HL-Mediated Hydrolysis of Phospholipids in (A-I)rHDL Only, (A-II)rHDL Only, and in Mixtures of (A-I)rHDL and (A-II)rHDL (Tables 1−4). We have shown previously that the kinetics of HL-mediated phospholipid and triacylglycerol hydrolysis in spherical rHDL varies according to their apolipoprotein content (*21*). In that study the V_{max} for phospholipid and triacylglycerol hydrolysis was greater in (A-I)rHDL than in (A-II)rHDL, while the affinity of HL for the phospholipids and triacylglycerol in (A-II)rHDL was greater than for the phospholipids and triacylglycerol in (A-I)rHDL.

In the present study we have investigated the HL-mediated hydrolysis of phospholipids in incubations containing mixtures of spherical [^{14}C]DPPC-labeled (A-I)rHDL and [^{14}C]-DPPC-labeled (A-II)rHDL. We have demonstrated previously that the hydrolysis of trace amounts of [^{14}C]DPPC reflects the hydrolysis of the bulk rHDL phospholipid (*21*). The physical properties of the [^{14}C]DPPC-labeled (A-I)rHDL and (A-II)rHDL are presented in Table 1A. As earlier cross-linking studies have shown that the (A-I)rHDL and (A-II)-rHDL contain three and six apolipoprotein molecules/particle, respectively (*37*), the ratios of constituents in (A-I)rHDL and (A-II)rHDL are expressed relative to three molecules of apoA-I and six molecules of apoA-II. As is seen in Table 1A, displacement of apoA-I from (A-I)rHDL by lipid-free apoA-II does not displace any of the lipid constituents (*38*). As such, the (A-I)rHDL and (A-II)rHDL differed only in their apolipoprotein composition. The slightly larger diameter of the (A-II)rHDL relative to the (A-I)rHDL is consistent with what has been reported previously from this laboratory (*30*). As these rHDL did not contain triacylglycerol, the only substrate for HL was phospholipid.

In the first series of experiments varying concentrations of [^{14}C]DPPC-labeled (A-I)rHDL (final concentration 0.1−1.1 mM phospholipid) and [^{14}C]DPPC-labeled (A-II)rHDL (final concentration 0.1−1.1 mM phospholipid) were incubated individually with a constant amount of HL. In both cases the rate of phospholipid hydrolysis increased as the concentration of the substrate increased (Table 2). As shown previously (*21*), the rate of phospholipid hydrolysis was greater in the (A-I)rHDL than in the (A-II)rHDL. Kinetic parameters for the phospholipid hydrolysis were obtained as described under Experimental Procedures. The V_{max} for (A-I)rHDL and (A-II)rHDL was 193.2 and 59.1 nmol of NEFA formed (mL of HL)$^{-1}$ h^{-1}, respectively. HL had a greater affinity for the phospholipids in (A-II)rHDL (apparent K_m = 0.27 mM) than for the phospholipids in (A-I)rHDL (apparent K_m = 0.76 mM). These kinetic parameters are consistent with our previous results (*21*).

Mixtures of the same preparations of [^{14}C]DPPC-labeled (A-I)rHDL and [^{14}C]DPPC-labeled (A-II)rHDL were then incubated with HL. These incubation mixtures contained varying concentrations of (A-I)rHDL (final concentration 0.1−0.9 mM phospholipid) plus (A-II)rHDL at a final phospholipid concentration of either 0.2 or 0.4 mM. The rate of phospholipid hydrolysis in these mixtures is shown in Table 2.

The values for the kinetic parameters for phospholipid hydrolysis in incubations containing either (A-I)rHDL alone or (A-II)rHDL alone were used to calculate from eq 1 the predicted rates of phospholipid hydrolysis in incubations containing mixtures of (A-I)rHDL and (A-II)rHDL. In all cases, the observed rate of phospholipid hydrolysis in incubations containing mixtures of (A-I)rHDL and (A-II)-rHDL was greater than the rate predicted by the mathematical model (Table 3).

To determine whether this was also the case when phospholipid hydrolysis was measured directly using a mass assay to measure NEFA formation, mixtures of unlabeled (A-I)rHDL (final concentration 0.1−0.7 mM phospholipid) and unlabeled (A-II)rHDL (final concentration 0.4 mM phospholipid) were incubated with HL. The phospholipid hydrolysis that was observed in these mixtures is shown in Table 4. Kinetic parameters for the phospholipid hydrolysis in incubations containing either unlabeled (A-I)rHDL alone or unlabeled (A-II)rHDL alone were determined by measuring NEFA mass. The V_{max} for (A-I)rHDL and (A-II)rHDL was 739.6 and 262.4 nmol of NEFA formed (mL of HL)$^{-1}$ h^{-1}, respectively. HL had a greater affinity for the phospholipids in (A-II)rHDL (apparent K_m = 0.23 mM) than for the phospholipids in (A-I)rHDL (apparent K_m = 0.97 mM). These kinetic parameters were used to calculate from eq 1

Table 2: Rate of HL-Mediated Phospholipid Hydrolysis in Incubations Containing [^{14}C]DPPC-Labeled (A-I)rHDL Only, [^{14}C]DPPC-Labeled (A-II)rHDL Only, and Mixtures of [^{14}C]DPPC-Labeled (A-I)rHDL and [^{14}C]DPPC-Labeled (A-II)rHDL[a]

(A-I)rHDL only		(A-II)rHDL only		(A-I)rHDL + (A-II)rHDL (0.2 mM PL)				(A-I)rHDL + (A-II)rHDL (0.4 mM PL)			
(A-I) rHDL (mM PL)	PL hydrolysis[b] [nmol of NEFA (mL of HL)$^{-1}$ h^{-1}]	(A-II) rHDL (mM PL)	PL hydrolysis[c] [nmol of NEFA (mL of HL)$^{-1}$ h^{-1}]	total rHDL (mM PL)	(A-I) rHDL (mM PL)	(A-II) rHDL (mM PL)	PL hydrolysis[d] [nmol of NEFA (mL of HL)$^{-1}$ h^{-1}]	total rHDL (mM PL)	(A-I) rHDL (mM PL)	(A-II) rHDL (mM PL)	PL hydrolysis[d] [nmol of NEFA (mL of HL)$^{-1}$ h^{-1}]
0.1	22.7 ± 3.6	0.1	16.7 ± 1.3								
0.3	48.9 ± 2.5	0.3	26.6 ± 1.2	0.3	0.1	0.2	90.6 ± 3.5		-		
0.5	68.6 ± 2.9	0.5	30.5 ± 3.1	0.5	0.3	0.2	105.4 ± 4.9	0.5	0.1	0.4	155.7 ± 2.5
0.7	89.5 ± 13.4	0.7	44.6 ± 4.3	0.7	0.5	0.2	126.1 ± 5.6	0.7	0.3	0.4	173.2 ± 13.6
0.9	117.1 ± 15.5	0.9	49.7 ± 8.7	0.9	0.7	0.2	151.1 ± 10.7	0.9	0.5	0.4	162.8 ± 19.9
1.1	148.8 ± 23.9	1.1	73.4 ± 15.3	1.1	0.9	0.2	164.7 ± 10.6	1.1	0.7	0.4	190.7 ± 28.3

[a] (A-I)rHDL and (A-II)rHDL were labeled with [^{14}C]DPPC as described under Experimental Procedures. Aliquots of either (A-I)rHDL or (A-II)rHDL (final concentration 0.1−1.1 mM phospholipid) were incubated individually at 37 °C for 3 h with HL [20 μL of a preparation which generated 876 nmol of NEFA (mL of HL)$^{-1}$ h^{-1}]. Incubations containing mixtures of (A-I)rHDL (final concentration 0.1−0.9 mM phospholipid) plus (A-II)rHDL (final concentration either 0.2 or 0.4 mM phospholipid) were also carried out. All the incubations contained fatty acid free BSA (final concentration 20 mg/mL). The final incubation volume was 50 μL. At the end of the incubations the NEFA were separated from the other rHDL lipids by thin-layer chromatography. Phospholipid hydrolysis was quantitated by liquid scintillation counting of the liberated NEFA and the phospholipid substrate. All values are the means of triplicate determinations ± SD. [b] The rate of phospholipid (PL) hydrolysis in incubations containing only (A-I)rHDL. [c] The rate of phospholipid hydrolysis in incubations containing only (A-II)rHDL. [d] The rate of phospholipid hydrolysis in incubations containing mixtures of (A-I)rHDL and (A-II)rHDL.

Table 3: Rate of HL-Mediated Phospholipid Hydrolysis in Incubation Mixtures Containing both [^{14}C]DPPC-Labeled (A-I)rHDL and [^{14}C]DPPC-Labeled (A-II)rHDL: Predicted versus Observed Values[a]

(A-I)rHDL + (A-II)rHDL (0.2 mM PL)[b]						(A-I)rHDL + (A-II)rHDL (0.4 mM PL)					
total rHDL (mM PL)	(A-I) rHDL (mM PL)	(A-II) rHDL (mM PL)	PL hydrolysis [nmol of NEFA (mL of HL)$^{-1}$ h^{-1}]		ratio obsd/ pred	total rHDL (mM PL)	(A-I) rHDL (mM PL)	(A-II) rHDL (mM PL)	PL hydrolysis [nmol of NEFA (mL of HL)$^{-1}$ h^{-1}]		ratio obsd/ pred
			obsd[b]	pred[b]					obsd	pred	
0.3	0.1	0.2	90.6 ± 3.5	37.0	2.5						
0.5	0.3	0.2	105.4 ± 4.9	56.2	1.9	0.5	0.1	0.4	155.7 ± 2.5	43.2	3.6
0.7	0.5	0.2	126.1 ± 5.6	71.2	1.8	0.7	0.3	0.4	173.2 ± 13.6	57.0	3.0
0.9	0.7	0.2	151.1 ± 10.7	83.3	1.8	0.9	0.5	0.4	162.8 ± 19.9	68.4	2.4
1.1	0.9	0.2	164.7 ± 10.6	93.2	1.8	1.1	0.7	0.4	190.7 ± 28.3	78.0	2.4

[a] (A-I)rHDL and (A-II)rHDL were labeled with [^{14}C]DPPC as described under Experimental Procedures. The incubations contained mixtures of (AI)rHDL (final concentration 0.1−0.9 mM phospholipid) and (A-II)rHDL (final concentration either 0.2 or 0.4 mM phospholipid) as described in Table 2. The values for the observed rates of phospholipid hydrolysis are the means of triplicate determinations ± SD. The predicted rates of phospholipid hydrolysis were determined from eq 1. [b] PL, phospholipid; obsd, observed; pred, predicted.

Table 4: Rate of HL-Mediated Phospholipid Hydrolysis in Incubation Mixtures Containing both (A-I)rHDL and (A-II)rHDL As Determined by Mass Assay: Predicted versus Observed Values[a]

total rHDL (mM PL)	(A-I)rHDL (mM PL)	(A-II)rHDL (mM PL)	PL[b] hydrolysis [nmol of NEFA (mL of HL)$^{-1}$ h^{-1}]		ratio obsd/ pred
			obsd[b]	pred[b]	
0.5	0.1	0.4	281.2 ± 30.5	187.4	1.5
0.7	0.3	0.4	336.6 ± 24.3	224.7	1.5
0.9	0.5	0.4	356.9 ± 30.2	257.4	1.4
1.1	0.7	0.4	357.6 ± 19.3	286.1	1.3

[a] Mixtures of unlabeled (A-I)rHDL (final concentration 0.1−0.7 mM phospholipid) plus unlabeled (A-II)rHDL (final concentration 0.4 mM phospholipid) were incubated at 37 °C for 3 h with HL [30 μL of a preparation which generated 682 nmol of NEFA (mL of HL)$^{-1}$ h^{-1}]. The incubations contained fatty acid free BSA (final concentration 20 mg/mL) in a final incubation volume of 120 μL. The rate of phospholipid hydrolysis was determined by mass assay of the generated NEFA. All values are the means of triplicate determinations ± SD. The predicted rates of phospholipid hydrolysis were determined from eq 1. [b] PL, phospholipid; obsd, observed; pred, predicted.

the predicted rates of phospholipid hydrolysis. As was the case for the radiolabeled substrates, the observed rate of phospholipid hydrolysis in incubations containing mixtures of (A-I)rHDL and (A-II)rHDL was greater than the rate predicted by the mathematical model.

HL-Mediated Phospholipid Hydrolysis in Incubations Containing Mixtures of [^{14}C]DPPC-Labeled (A-I)rHDL and Unlabeled (A-II)rHDL (Figure 1; Table 1B). Additional experiments were carried out to determine whether the greater than expected rate of phospholipid hydrolysis in mixtures of (A-I)rHDL and (A-II)rHDL was due to an enhanced rate of hydrolysis in the (A-I)rHDL or enhanced hydrolysis in the (A-II)rHDL. In the first experiment [^{14}C]-DPPC-labeled (A-I)rHDL (final concentration 0.1−0.7 mM phospholipid) was incubated with a constant amount of HL and unlabeled (A-II)rHDL (final concentration either 0.2 or 0.4 mM phospholipid). The physical properties of these preparations of rHDL are shown in Table 1B. As the formation of [^{14}C]-labeled NEFA was measured in this incubation, the results reflect phospholipid hydrolysis in the [^{14}C]DPPC-labeled (A-I)rHDL and not the hydrolysis in the unlabeled (A-II)rHDL. Figure 1 shows the rate of phospholipid hydrolysis in [^{14}C]DPPC-labeled (A-I)rHDL (final concentration 0.1, 0.3, 0.5, and 0.7 mM phospholipid) in the absence of unlabeled (A-II)rHDL (open bars) or in the presence of a final concentration of either 0.2 (hatched bars)

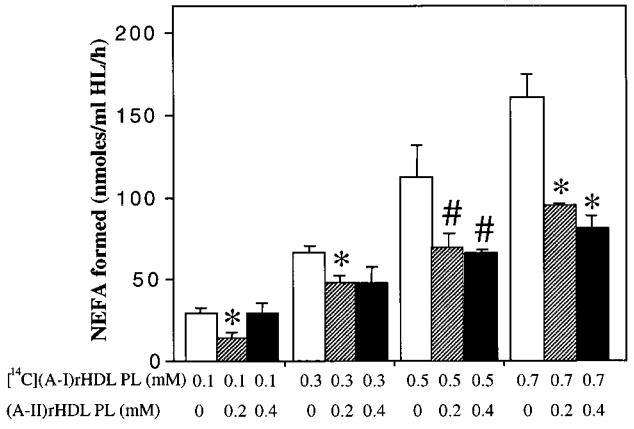

FIGURE 1: Effect of unlabeled (A-II)rHDL on HL-mediated phospholipid hydrolysis in [^{14}C]DPPC-labeled (A-I)rHDL. (A-I)-rHDL (final concentration 0.1−0.7 mM phospholipid) were radiolabeled with [^{14}C]DPPC and incubated at 37 °C for 3 h with HL [20 μL of a preparation which generated 745 nmol of NEFA (mL of HL)$^{-1}$ h^{-1}]. The incubations were carried out in the absence (□) or presence of unlabeled (A-II)rHDL at a final phospholipid concentration of either 0.2 mM (▨) or 0.4 mM (■). All incubations contained fatty acid free BSA (final concentration 20 mg/mL). The final incubation volume was 50 μL. The figure shows the rate of hydrolysis of [^{14}C]DPPC in (A-I)rHDL. The results are the means of triplicate determinations ± SD [*, $p < 0.01$; #, $p < 0.05$, difference from (A-I)rHDL only incubations].

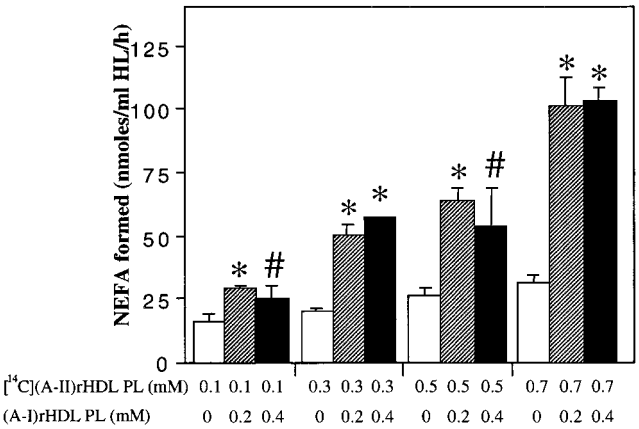

FIGURE 2: Effect of unlabeled (A-I)rHDL on HL-mediated phospholipid hydrolysis in [^{14}C]DPPC-labeled (A-II)rHDL. (A-II)rHDL (final concentration 0.1−0.7 mM phospholipid) were radiolabeled with [^{14}C]DPPC and incubated at 37 °C for 3 h with HL [20 μL of a preparation which generated 745 nmol of NEFA (mL of HL)$^{-1}$ h^{-1}]. The incubations were carried out in the absence (□) or presence of unlabeled (A-I)rHDL at a final phospholipid concentration of either 0.2 mM (▨) or 0.4 mM (■). All incubations contained fatty acid free BSA (final concentration 20 mg/mL). The final incubation volume was 50 μL. The figure shows the rate of hydrolysis of [^{14}C]DPPC in (A-II)rHDL. The results are the means of triplicate determinations ± SD [*, $p < 0.01$; #, $p < 0.05$, difference from (A-II)rHDL only incubations].

or 0.4 mM (closed bars) unlabeled (A-II)rHDL phospholipid. With the exception of the incubation containing 0.1 mM (A-I)rHDL phospholipid plus 0.4 mM (A-II)rHDL phospholipid, the rate of phospholipid hydrolysis in (A-I)rHDL decreased when (A-II)rHDL were present. This indicates that (A-II)-rHDL are, as expected, competitive inhibitors of HL-mediated phospholipid hydrolysis in (A-I)rHDL.

HL-Mediated Phospholipid Hydrolysis in Incubations Containing Mixtures of [^{14}C]DPPC-Labeled (A-II)rHDL and Unlabeled (A-I)rHDL (Figure 2; Table 1C). To determine if the greater than predicted rate of phospholipid hydrolysis in incubations containing mixtures of (A-I)rHDL and (A-II)rHDL was a result of the (A-I)rHDL enhancing the rate of hydrolysis in the (A-II)rHDL, unlabeled (A-I)rHDL (final concentration either 0.2 or 0.4 mM phospholipid) were added to incubations containing [^{14}C]DPPC-labeled (A-II)rHDL (final concentration 0.1−0.7 mM phospholipid) and a constant amount of HL. The physical properties of these rHDL are shown in Table 1C. The hydrolysis observed in this experiment reflects that of the phospholipids in [^{14}C]-DPPC-labeled (A-II)rHDL. Figure 2 shows the rate of phospholipid hydrolysis in [^{14}C]DPPC-labeled (A-II)rHDL (final concentration 0.1, 0.3, 0.5, and 0.7 mM phospholipid) in the absence of unlabeled (A-I)rHDL (open bars) or in the presence of a final concentration of either 0.2 (hatched bars) or 0.4 mM (closed bars) unlabeled (A-I)rHDL phospholipid. In all cases the hydrolysis of (A-II)rHDL phospholipids was enhanced significantly when unlabeled (A-I)rHDL were present in the incubations. When taken together with the results of the preceding experiment, this result indicates that the greater than predicted rate of phospholipid hydrolysis in incubations containing mixtures of (A-I)rHDL and (A-II)-rHDL is due to enhanced phospholipid hydrolysis in (A-II)-rHDL and not in (A-I)rHDL.

HL-Mediated Phospholipid Hydrolysis in (A-I/A-II)rHDL (Figure 3; Tables 5 and 6). Further experiments were conducted to exclude the possibility that the enhanced rate of phospholipid hydrolysis in mixtures of (A-I)rHDL and (A-II)rHDL was due to the formation of rHDL that contained both apoA-I and apoA-II on the same particle, (A-I/A-II)-rHDL. (A-I)rHDL (final concentration 0.1 mM phospholipid) and (A-II)rHDL (final concentration 0.4 mM phospholipid) were incubated with HL for 3 h. The incubation mixture was then applied to an anti-human apoA-I immunoaffinity column. Only 3% of the apoA-II bound to the column (results not shown), indicating that there was minimal formation of (A-I/A-II)rHDL during the incubation.

To further confirm that the enhanced phospholipid hydrolysis in incubations containing mixtures of (A-I)rHDL and (A-II)rHDL could not be explained by the formation of (A-I/A-II)rHDL, the phospholipid hydrolysis in (A-I/A-II)-rHDL was compared directly with that in (A-I)rHDL alone and in (A-II)rHDL alone. The (A-I/A-II)rHDL were prepared as described under Experimental Procedures. The composition of the (A-I)rHDL, (A-II)rHDL, and (A-I/A-II)rHDL are shown in Table 5. As none of the rHDL were radiolabeled, NEFA formation as a result of phospholipid hydrolysis was determined directly by mass assay. The rate of HL-mediated phospholipid hydrolysis in (A-I)rHDL (closed circles), (A-II)rHDL (open circles), and (A-I/A-II)rHDL (closed squares) is shown in Figure 3. In all cases the rate of hydrolysis increased as the final rHDL phospholipid concentration increased from 0.05 to 0.8 mM. From 0.2 to 0.8 mM rHDL phospholipid, the rate of phospholipid hydrolysis in the (A-I/A-II)rHDL was between that of the (A-I)rHDL and the (A-II)rHDL. This is reflected in the kinetic parameters for phospholipid hydrolysis in the three types of rHDL (Table 6). The V_{max} for (A-I/A-II)rHDL [778.3 nmol of NEFA formed (mL of HL)$^{-1}$ h^{-1}] was between that for (A-I)rHDL and (A-II)rHDL [1159.4 and 395.1 nmol of NEFA formed (mL of HL)$^{-1}$ h^{-1}, respectively]. Likewise, the affinity of HL for the phospholipids in (A-I/A-II)rHDL (apparent K_m

Table 5: Physical Properties of (A-I)rHDL, (A-I/A-II)rHDL, and (A-II)rHDL[a]

spherical rHDL	stoichiometry[b] (% mass) PL	UC	CE	A-I	A-II	Stokes diameter[c] (nm)
(A-I)rHDL	35.4	2.1	17.9	44.6	0.0	9.1
(A-I/A-II)rHDL	31.9	0.4	20.2	23.0	24.5	9.9
(A-II)rHDL	32.8	1.8	15.9	2.1	47.4	9.9

[a] (A-I/A-II)rHDL were prepared by incubating discoidal (A−I)rHDL and (A-II)rHDL with low-denstiy lipoproteins and LCAT as described (*31*). [b] Stoichiometries were calculated from the means of triplicate determinations which varied by less than 8%. Abbreviations: PL, phospholipid; UC, unesterified cholesterol; CE, cholesteryl ester; A-I, apoA-I; A-II, apoA-II. [c] All of the rHDL preparations contained a single, homogeneous population of particles as judged by nondenaturing gradient gel electrophoresis.

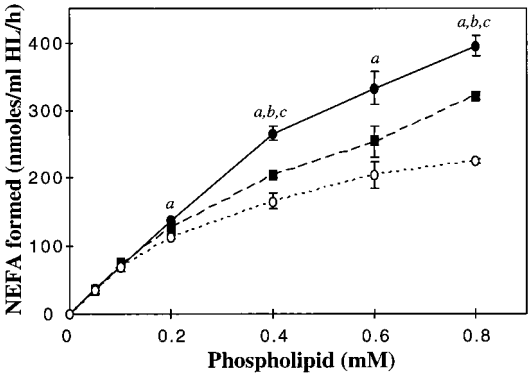

FIGURE 3: Kinetics of HL-mediated phospholipid hydrolysis in (A-I)rHDL, (A-I/A-II)rHDL, and (A-II)rHDL. Unlabeled (A-I)rHDL (●), (A-II)rHDL (○), and (A-I/A-II)rHDL (■) were incubated at 37 °C for 3 h with HL [20 μL of a preparation which generated 540 nmol of NEFA (mL of HL)$^{-1}$ h^{-1}]. The final rHDL phospholipid concentration varied from 0.05 to 0.8 mM, and the incubation mixtures contained fatty acid free BSA (final concentration 20 mg/mL). The final incubation volume was 120 μL. The rate of phospholipid hydrolysis was determined by mass assay of the generated NEFA. The values are the means of triplicate determinations ± SD [*a*, $p < 0.01$ (A-I)rHDL versus (A-II)rHDL; *b*, $p < 0.01$ (A-I)rHDL versus (A-I/A-II)rHDL; *c*, $p < 0.01$ (A-II)rHDL versus (A-I/A-II)rHDL].

Table 6: Kinetic Parameters of HL-Mediated Phospholipid Hydrolysis in (A-I)rHDL, (A-I/A-II)rHDL, and (A-II)rHDL[a]

spherical rHDL	K_m(app) (mM PL[b])	V_{max} [nmol of NEFA (mL of HL)$^{-1}$ h^{-1}]
(A-I)rHDL	1.52	1159.4
(A-I/A-II)rHDL	1.05	778.3
(A-II)rHDL	0.51	395.1

[a] Spherical rHDL (final concentration 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 mM phospholipid) were incubated with a constant amount of HL as described in the legend to Figure 3. The resulting NEFA were measured by mass assay as described under Experimental Procedures. Kinetic parameters were estimated from double-reciprocal plots of the data shown in Figure 3. [b] PL, phospholipid.

= 1.05 mM) was between that for (A-I)rHDL and (A-II)rHDL (apparent K_m = 1.52 and 0.51 mM, respectively).

HL-Mediated Phospholipid Hydrolysis in Incubations Containing Mixtures of [^{14}C]DPPC-Labeled (A-I/A-II)rHDL and Unlabeled (A-I)rHDL (Tables 7 and 8). The effect of (A-I)rHDL on phospholipid hydrolysis in (A-I/A-II)rHDL was also investigated. The physical properties of the (A-I)rHDL and the (A-I/A-II)rHDL used in these experiments are shown in Table 7. On the basis of the results for the kinetics of phospholipid hydrolysis in (A-I)rHDL and (A-I/A-II)rHDL (Table 6), eq 2 (see Experimental Procedures) was used to predict that the rate of phospholipid hydrolysis in (A-I/A-II)rHDL should decrease when (A-I)rHDL are also present in the incubation (Table 8). These predictions were tested experimentally in incubations containing unlabeled (A-I)rHDL (final concentration 0.2 mM phospholipid) and [^{14}C]DPPC-labeled (A-I/A-II)rHDL (final concentration 0.1−0.5 mM phospholipid) in the presence of a constant amount of HL. The hydrolysis in this experiment reflects that of the phospholipids in [^{14}C]DPPC-labeled (A-I/A-II)rHDL. The observed rate of phospholipid hydrolysis in the (A-I/A-II)rHDL decreased, as predicted, when (A-I)rHDL were present in the incubations. This result was in contrast to the observed increase in the rate of (A-II)rHDL phospholipid hydrolysis in incubation mixtures that also contained (A-I)rHDL (Figure 2).

Table 7: Physical Properties of Unlabeled (A-I)rHDL and [^{14}C]DPPC-Labeled (A-I/A-II)rHDL[a]

spherical rHDL	stoichiometry[b] (% mass) PL	UC	CE	A-I	A-II	Stokes diameter[c] (nm)
unlabeled (A-I)rHDL	54.3	5.6	17.4	22.7	0.0	9.2
[^{14}C]DPPC−(A-I/AII)rHDL	41.5	1.7	22.9	19.7	14.1	9.1

[a] (A-I/A-II)rHDL were prepared by incubating discoidal (A-I)rHDL and (A-II)rHDL with low-density lipoproteins and LCAT as described (*31*). The (A-I/A-II)rHDL were labeled with [^{14}C]DPPC as described under Experimental Procedures. [b] Stoichiometries were calculated from the means of triplicate determinations. Abbreviations: PL, phospholipid; UC, unesterified cholesterol; CE, cholesteryl ester; A-I, apoA-I; A-II, apoA-II. [c] The rHDL preparations contained a single, homogeneous population of particles as judged by nondenaturing gradient gel electrophoresis.

Table 8: Rate of HL-Mediated Phospholipid Hydrolysis in [^{14}C]DPPC-Labeled (A-I/A-II)rHDL in the Absence and Presence of Unlabeled (A-I)rHDL: Observed and Predicted Values[a]

[^{14}C]DPPC-labeled (A-I/A-II)rHDL (mM PL)	unlabeled (A-I)rHDL (mM PL)	phospholipid hydrolysis in (A-I/A-II)rHDL [nmol of NEFA (mL of HL)$^{-1}$ h^{-1}] observed	predicted
0.1	0	71.5 ± 4.9	67.7
0.1	0.2	35.4 ± 6.1*	60.4
0.3	0	176.4 ± 37.5	173.0
0.3	0.2	112.4 ± 24.7	156.9
0.5	0	243.2 ± 58.8	251.1
0.5	0.2	181.8 ± 64.8	230.5

[a] (A-I/A-II)rHDL were labeled with [^{14}C]DPPC as described under Experimental Procedures. Aliquots of [^{14}C]DPPC-labeled (A-I/A-II)rHDL (final concentration 0.1, 0.3, and 0.5 mM phospholipid) were incubated at 37 °C for 3 h with HL [10 μL of a preparation which generated 959 nmol of NEFA (mL of HL)$^{-1}$ h^{-1}]. The incubations were carried out in the absence or presence of unlabeled (A-I)rHDL (final concentration 0.2 mM phospholipid). All incubations contained fatty acid free BSA (final concentration 20 mg/mL). The final incubation volume was 50 μL. The values for the observed rates of hydrolysis of [^{14}C]DPPC in (A-I/A-II)rHDL are the means of triplicate determinations ± SD [*, $p < 0.01$, difference from incubation in the absence of (A-I)rHDL]. The predicted values for the rate of phospholipid hydrolysis in (A-I/A-II)rHDL were determined from eq 2 using the kinetic parameters shown in Table 5.

DISCUSSION

We have previously used well-characterized, homogeneous preparations of spherical rHDL containing either apoA-I or

apoA-II as the sole apolipoprotein constituent to show that apolipoproteins have a major influence on HL-mediated phospholipid and triacylglycerol hydrolysis in HDL (*21*). The results of those studies demonstrated that, although HL has a higher affinity for the phospholipids and triacylglycerol in (A-II)rHDL than in (A-I)rHDL, the maximal rate of hydrolysis of both constituents is greater in (A-I)rHDL than in (A-II)rHDL. One aim of the present study was to investigate whether this was also the case for HL-mediated phospholipid hydrolysis in mixtures of (A-I)rHDL and (A-II)rHDL.

Given that HL has a higher affinity for (A-II)rHDL phospholipids than for (A-I)rHDL phospholipids but hydrolyzes phospholipids more slowly in (A-II)rHDL than in (A-I)rHDL (*21*), it follows that (A-II)rHDL may function as a competitive inhibitor of HL-mediated phospholipid hydrolysis in (A-I)rHDL. Thus, the presence of (A-II)rHDL in an incubation mixture containing HL and (A-I)rHDL should decrease the rate of rHDL phospholipid hydrolysis. It was surprising, therefore, to find that the rate of hydrolysis in mixtures of (A-I)rHDL and (A-II)rHDL was substantially greater than that observed in incubations containing equivalent concentrations of (A-I)rHDL only. In addition, the rate of phospholipid hydrolysis was much greater than what was expected from the kinetic parameters for the (A-I)rHDL and (A-II)rHDL alone. This was the case when phospholipid hydrolysis was determined either by measuring formation of radiolabeled NEFA (Table 3) or by measuring NEFA formation using a mass assay (Table 4).

There are five possible explanations for this observation: (i) the hydrolysis of (A-I)rHDL phospholipids increases when (A-II)rHDL are present, (ii) the hydrolysis of (A-II)rHDL phospholipids increases when (A-I)rHDL are present, (iii) phospholipid hydrolysis in both (A-I)rHDL and (A-II)rHDL is increased, (iv) (A-I/A-II)rHDL are formed during the incubation and HL hydrolyzes the phospholipids in these particles more rapidly than the phospholipids in either (A-I)rHDL or (A-II)rHDL, and (v) a previously unidentified substrate is formed during the incubation, and the phospholipids in this substrate are hydrolyzed very rapidly.

The first three possibilities were investigated by incubating mixtures of (A-I)rHDL and (A-II)rHDL with HL and measuring phospholipid hydrolysis in one substrate at a time. This was achieved by incorporating [^{14}C]DPPC into only one of the substrates and measuring the release of radiolabeled NEFA. In this way the rate of phospholipid hydrolysis was measured in the radiolabeled substrate, not in the unlabeled substrate. The dramatic differences in the results shown in Figures 1 and 2 exclude the possibility that the transfer of [^{14}C]DPPC between the rHDL preparations could significantly influence our results.

When unlabeled (A-II)rHDL were added to incubations containing [^{14}C]DPPC-labeled (A-I)rHDL and HL, phospholipid hydrolysis in (A-I)rHDL decreased. This was predicted from the kinetics of the phospholipid hydrolysis in the individual substrates and was consistent with (A-II)rHDL acting as a competitive inhibitor of HL-mediated hydrolysis of (A-I)rHDL phospholipids. However, when unlabeled (A-I)rHDL were present in incubations containing [^{14}C]DPPC-labeled (A-II)rHDL and HL, there was a large and unexpected enhancement of the rate of (A-II)rHDL phospholipid hydrolysis. These results indicate that the greater than predicted rate of phospholipid hydrolysis in incubations containing mixtures of (A-I)rHDL and (A-II)rHDL was due to enhanced phospholipid hydrolysis in (A-II)rHDL. It was of interest to note that this enhancement did not increase when the concentration of (A-I)rHDL was doubled from 0.2 to 0.4 mM phospholipid (Figure 2).

Two approaches were used to exclude the possibility that the enhanced phospholipid hydrolysis in incubation mixtures containing (A-I)rHDL and (A-II)rHDL was due to the formation of (A-I/A-II)rHDL. First, anti-apoA-I immunoaffinity chromatography was used to demonstrate that the formation of (A-I/A-II)rHDL during the incubations was minimal. Second, we showed that the rate of hydrolysis of (A-I/A-II)rHDL phospholipids is intermediate between that of (A-I)rHDL and (A-II)rHDL. Therefore, even if significant quantities of (A-I/A-II)rHDL were formed, their presence could not explain the higher than expected rate of phospholipid hydrolysis that was observed in mixtures of (A-I)rHDL and (A-II)rHDL.

The possibility that a substrate other than (A-I/A-II)rHDL, which is preferentially hydrolyzed by HL, is generated during the incubation of (A-I)rHDL and (A-II)rHDL cannot be discounted. However, it is difficult to envisage precisely what such a substrate could be. While it is possible that surface constituents may dissociate from the (A-II)rHDL and act as substrates for HL, this is unlikely because of the very high affinity of apoA-II for lipid (*39*).

To understand why (A-I)rHDL enhance phospholipid hydrolysis in (A-II)rHDL, it is necessary to consider why the rate of phospholipid hydrolysis in (A-I)rHDL alone is faster than in (A-II)rHDL alone. Studies of human pancreatic lipase, an enzyme that is structurally similar to HL, support the view that HL contains a catalytic domain and a COOH-terminal domain (*40*). It has been proposed that the initial interaction of HL with its substrate occurs in the COOH-terminal domain (*41*). This interaction changes the conformation of the catalytic domain and enables the substrate access to the active site (*42*, *43*). The change in conformation most likely involves the opening of a "lid" which is normally closed and covers the catalytic site of HL (*42*, *44*). It is also possible that this lid may be partially, rather than fully, opened when substrate is bound to the COOH-terminal domain of HL (*45*). The higher rate of phospholipid hydrolysis in (A-I)rHDL, compared to (A-II)rHDL, in the present study suggests that the lid is more open when (A-I)rHDL bind to HL. This enhances access of the substrate to the catalytic site. When (A-II)rHDL bind to HL, the lid opens less than for (A-I)rHDL. This decreases access of the substrate to the active site, and phospholipid hydrolysis is reduced.

Structural and functional evidence supports the model of active, dimeric HL proposed by Hill et al. (*43*). In this model, two HL monomers, each comprising a COOH-terminal domain and a catalytic domain, are arranged in a "head-to-tail" fashion so that the COOH-terminal domain of one subunit is in close proximity to the catalytic domain of the other subunit. When a substrate binds to the COOH-terminal domain of one momomer, the catalytic site of that monomer becomes accessible to substrate that is bound to the other monomer. Our results showing that (A-I)rHDL enhance phospholipid hydrolysis in (A-II)rHDL can be explained by this model if we assume that the binding of these substrates to HL differentially exposes the active site as explained in

the preceding paragraph. Thus, when (A-II)rHDL bind to HL, the active site is only partly exposed. On the other hand, binding of (A-I)rHDL to HL enhances exposure of the active site to substrate. Thus, binding of (A-I)rHDL to an HL subunit enables (A-II)rHDL associated with the opposing subunit enhanced access to the active site. This explains why (A-II)rHDL phospholipid hydrolysis is enhanced in mixtures of (A-I)rHDL and (A-II)rHDL.

There was no difference in the enhancement of phospholipid hydrolysis in (A-II)rHDL when either 0.2 or 0.4 mM (A-I)rHDL phospholipids were present (Figure 2). This suggests that once (A-I)rHDL binds to the COOH-terminal domain of one HL subunit, multiple (A-II)rHDL particles can bind to the other subunit and access the more exposed active site. This supports the notion that (A-I)rHDL changes the conformation of the catalytic site of HL in a way that enhances hydrolysis of (A-II)rHDL phospholipids and that further increases in the concentration of (A-I)rHDL have no additional effect because the active site of HL is already more exposed.

The result showing that phospholipid hydrolysis in (A-I/A-II)rHDL is greater than in (A-II)rHDL indicates that apoA-I enhances the rate of HL-mediated phospholipid hydrolysis even when it is present in the same particle as the apoA-II. This suggests that apoA-I, whether in (A-I)rHDL or (A-I/A-II)rHDL, exposes the active site of HL more than occurs with (A-II)rHDL. Thus, while (A-II)rHDL require the presence of (A-I)rHDL in order to have enhanced access to the active site of HL, (A-I/A-II)rHDL may already have enhanced access to the active site and do not require additional (A-I)rHDL. This view is consistent with the absence of enhanced phospholipid hydrolysis in (A-I/A-II)rHDL in incubation mixtures that were supplemented with (A-I)rHDL (Table 8). However, the fact that the rate of phospholipid hydrolysis in (A-I/A-II)rHDL is less than in (A-I)rHDL (Figure 3) indicates that the apoA-II in (A-I/A-II)rHDL must have some effect on the conformation of the lid such that access of phospholipids to the active site of HL is somewhat decreased.

(A-I/A-II)HDL and (A-I)HDL are the major subpopulations of HDL in human plasma (*46*). Our observation that the rate of HL-mediated phospholipid hydrolysis in (A-I/A-II)rHDL is decreased relative to that of (A-I)rHDL but greater than that of (A-II)rHDL (Table 6, Figure 3) is in agreement with what has been shown for mice transgenic for human apoA-II and CETP (*19*). In those studies the apoA-II-containing HDL were enriched with triacylglycerol relative to the (A-I)HDL. This indicates that the HL-mediated hydrolysis of phospholipid and triacylglycerol in the (A-I/A-II)HDL in these animals is reduced relative to the hydrolysis in (A-I)HDL.

In conclusion, this study shows that the rate of HL-mediated phospholipid hydrolysis in mixtures of (A-I)rHDL and (A-II)rHDL is much greater than expected from the kinetic parameters of the individual types of rHDL. We have shown that this is due to the (A-I)rHDL increasing the phospholipid hydrolysis in the (A-II)rHDL. In addition, we found that the rate of phospholipid hydrolysis in (A-I/A-II)rHDL is greater than in (A-II)rHDL. When these results are considered together, it is apparent that apoA-I enhances HL-mediated phospholipid hydrolysis in apoA-II-containing HDL. Furthermore, this enhancement occurs irrespective of whether the apoA-I is present in the same particle as the apoA-II, as in (A-I/A-II)rHDL, or whether it is present as a component of particles that contain apoA-I only.

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