The Influence of Apolipoproteins on the Hepatic Lipase-mediated Hydrolysis of High Density Lipoprotein Phospholipid and Triacylglycerol

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This study describes the influence of apolipoproteins on the hepatic lipase (HL)-mediated hydrolysis of phospholipids and triacylglycerol in high density lipoproteins (HDL). HL-mediated hydrolysis was assessed in well characterized, homogeneous preparations of spherical reconstituted high density lipoproteins (rHDL). The rHDL were comparable in size and lipid composition and contained either apoA-I ((A-I)rHDL) or apoA-II ((A-II)rHDL) as their sole apolipoprotein constituent. Preparations of rHDL containing only cholesteryl esters (CE) in their core, (A-I/CE)rHDL and (A-II/CE)rHDL, were used to assess phospholipid hydrolysis. Preparations of rHDL that contained triacylglycerol as their predominant core lipid, (A-I/TG)rHDL and (A-II/TG)rHDL, were used to assess both triacylglycerol and phospholipid hydrolysis. The rHDL contained trace amounts of either radiolabeled phospholipid or radiolabeled triacylglycerol. Hydrolysis was measured as the release of radiolabeled nonesterified fatty acids (NEFA) from the rHDL. Kinetic analysis showed that HL had a greater affinity for the phospholipids in (A-II/CE)rHDL (K_m(app) = 0.2 mM) than in (A-I/CE)rHDL (K_m(app) = 3.1 mM). This was also evident when hydrolysis was measured directly by quantitating NEFA mass. HL also had a greater affinity for the phospholipids and triacylglycerol in (A-II/TG)rHDL than in (A-I/TG)rHDL. The V_max for phospholipid hydrolysis was, by contrast, greater for (A-I/CE)rHDL than for (A-II/CE)rHDL: 395.3 versus 49.1 nmol of NEFA formed/ml of HL/h. Comparable V_max values were obtained for the hydrolysis of the phospholipids in (A-II/TG)rHDL and (A-I/TG)rHDL. In the case of triacylglycerol hydrolysis, the respective V_max values for (A-I/TG)rHDL and (A-II/TG)rHDL were 1154.8 and 240.2 nmol of NEFA formed/ml of HL/h. These results show that apolipoproteins have a major influence on the kinetics of HL-mediated phospholipid and triacylglycerol hydrolysis in rHDL.

Hepatic lipase (HL) is a 476-amino acid glycoprotein of molecular weight 64,000–69,000 (1) that is bound to liver sinusoidal endothelial cells (2). HL hydrolyzes acyl ester bonds of triacylglycerol and the sn-1 acyl ester bond of phospholipids. The main plasma substrates for HL are very low density lipoproteins and high density lipoproteins (HDL). The role of HL in HDL metabolism is of considerable importance, as shown by strong negative associations between HL activity and plasma HDL levels (3–5) and the dramatic reduction in the HDL levels of rabbits that have been made transgenic for human HDL (6).

Unlike lipoprotein lipase (LPL), which requires apolipoprotein C-II (apoC-II) for maximal activity, there is no known protein cofactor for HL. However, there is some conflicting evidence to suggest that the apoA-II in HDL may influence the HL-mediated hydrolysis of triacylglycerol in HDL (7–10). Some investigators have reported that apoA-II enhances (7, 8), while others have concluded that it inhibits, the HL-mediated hydrolysis of triacylglycerol in HDL (9, 10).

The present study was carried out in order to determine whether there are significant differences in the HL-mediated hydrolysis of phospholipids and triacylglycerol in HDL that differ in their apolipoprotein composition. This has been achieved by using well defined, homogeneous preparations of spherical reconstituted HDL (rHDL) as substrates for HL. The rHDL were comparable in size and lipid composition and contained either apoA-I or apoA-II as their sole apolipoprotein constituent. The results show that apolipoproteins not only have a major influence on the HL-mediated hydrolysis of the triacylglycerol and phospholipids in rHDL but also regulate the affinity of HL for the rHDL surface.

EXPERIMENTAL PROCEDURES

Purification of ApoA-I and ApoA-II—ApoA-I and apoA-II were prepared from pooled human plasma donated by the Transfusion Service, Royal Adelaide Hospital. HDL were isolated from the plasma by sequential ultracentrifugation in the 1.07 < d < 1.21 g/ml density range (11). The isolated HDL were delipidated (12), and the resulting apoHDL was subjected to anion exchange chromatography on Q Sepharose Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) (13). The purified apoA-I and apoA-II appeared as single bands following electrophoresis on a homogeneous 20% SDS-polyacylamide PhastGel (Amersham Pharmacia Biotech) and Coomassie staining.

Purification of Lecithin-Cholesterol Acyltransferase (LCAT)—LCAT was purified from pooled human plasma (Transfusion Service, Royal Adelaide Hospital) as described previously (14). The purified LCAT lipoprotein lipase; apo, apolipoprotein; rHDL, reconstituted high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; UC, unesterified cholesterol; CE, cholesteryl ester(s); CETP, cholesteryl ester transfer protein; LDL, low density lipoprotein(s); PLTP, phospholipid transfer protein; DPPIV, 1,2-dipalmitoylphosphatidylcholine; TBS, Tris-buffered saline; NEFA, nonesterified fatty acid(s); BSA, bovine serum albumin.
HL-mediated Hydrolysis of HDL Phospholipid and Triacylglycerol

Three pairs of radiolabeled rHDL containing either apoA-I or apoA-II as their sole apolipoprotein constituent were used to study phospholipid and triacylglycerol hydrolysis. The first pair of substrates, a and b, designated (A-I/CE)rHDL and (A-II/CE)rHDL, contained CE as their sole core lipid and either apoA-I (open ovals) or apoA-II (shaded ovals) as their sole apolipoprotein and were labeled with \([^{14}C]DPPC\) (black circles). This pair of substrates was used to study HL-mediated phospholipid hydrolysis in the absence of triacylglycerol. The second pair of radiolabeled substrates, c and d, contained triacylglycerol as the predominant core lipid and either apoA-I or apoA-II as the sole apolipoprotein, and these substrates were labeled with \([^{14}C]DPPC\). They were used to study phospholipid hydrolysis in the presence of triacylglycerol. The final two radiolabeled substrates, e and f, were identical to c and d, except they contained \([^{3}H]\)triolein (\(\ast TG\)) in their core. They were used to study triacylglycerol hydrolysis.

Fig. 1. Radiolabeled substrates for studying the kinetics of HL-mediated phospholipid and triacylglycerol hydrolysis. Three pairs of radiolabeled rHDL containing either apoA-I or apoA-II as their sole apolipoprotein and were labeled with \([^{14}C]DPPC\) (a, b), \([^{3}H]\)triolein (\(\ast TG\)) (c, d), or \([^{14}C]DPPC\) and \([^{3}H]\)triolein (\(\ast TG\)) (e, f). They were used to study phospholipid hydrolysis against a background of triacylglycerol hydrolysis. The final two substrates, e and f, contained triacylglycerol in their core and were labeled with \([^{3}H]\)triolein. They were used to monitor triacylglycerol hydrolysis. Since the rate of triacylglycerol hydrolysis was much greater than that of phospholipid hydrolysis for both (A-I/CE)rHDL and (A-II/CE)rHDL, we were not able to study the kinetics of phospholipid and triacylglycerol hydrolysis in rHDL, which were doubly labeled with \([^{14}C]DPPC\) and \([^{3}H]\)triolein.

Preparation of (A-I/CE)/rHDL and (A-II/CE)rHDL Labeled with \([^{14}C]DPPC\). Substrates a and b—Discoidal rHDL containing POPC, UC, and apo-A-I were prepared by the cholate dialysis method (20). The discs were incubated with LDL and LCAT as described previously (23) to generate spherical HDL with CE in their core and apo-A-I as the sole apolipoprotein constituent, (A-I/CE)rHDL. The (A-I/CE)rHDL were dialyzed extensively against 0.1 M Tris-buffered saline (TBS) (pH 7.4) containing 0.15 M NaCl, 0.005% (w/v) EDTA-\(N_a\), and 0.006% (w/v) Na\(_2\)EDTA before use. The (A-I/CE)rHDL were labeled with \([^{14}C]DPPC\) as follows. \([^{14}C]DPPC\)-labeled phospholipid vesicles were prepared by adding to a clean, dry test tube 0.35 mg POPC in chloroform/methanol (200 \(\mu l, 2.1\) \(\mu l/v)\), 1.25 \(\mu l\) of \([^{14}C]DPPC\), and 5 \(\mu l\) of 0.5 mM butyalted hydroxytoluene in ethanol. The lipids were dispersed as a thin film on the walls of the tube and dried under \(N_a\) for 2 h at 40 °C. The phospholipids were resuspended in 0.5 ml of TBS and sonicated for 3 × 5 min, using a Sonifier B-12 (Branson Sonic Power Company, Danbury, CT) equipped with a microtip. The mixture was then centrifuged at 15,000 rpm for 10 min, and the supernatant, which contained the \([^{14}C]DPPC\)-labeled phospholipid vesicles, was collected.

Spherical (A-I/CE)rHDL (6.6 \(\mu \text{mol phospholipid}\)) were added to the \([^{14}C]DPPC\)-labeled phospholipid vesicles (0.66 \(\mu \text{mol of phospholipid}\)) and incubated for 3 h at 37 °C in the presence of purified PLTP (final transfer activity = 247 nmol of PL transferred/ml of PLTP/h) and bovine serum albumin (BSA) (final concentration = 20 mg/ml). The final volume of the incubation mixture was 5.5 ml. When the incubation was complete, the radiolabeled (A-I/CE)rHDL were isolated by ultracentrifugation at 100,000 rpm in the density range 1.063 < \(d < 1.21\) g/ml using a TLA-100.4 rotor (Beckman Instruments, Fullarton, CA) with one 6-h spin at the lower density and another 16-h spin at the higher density. These procedures were carried out at 4 °C in a Beckman TL-100 tabletop ultracentrifuge (Beckman Instruments). The (A-I/CE)rHDL were isolated by ultracentrifugation at 100,000 rpm in the density range 1.063 < \(d < 1.21\) g/ml using a TLA-100.4 rotor (Beckman Instruments, Fullarton, CA) with one 6-h spin at the lower density and another 16-h spin at the higher density. These procedures were carried out at 4 °C in a Beckman TL-100 tabletop ultracentrifuge (Beckman Instruments).

Preparation of (A-I/CE)rHDL and (A-II/CE)rHDL Labeled with \([^{3}H]\)triolein—Spherical (A-I/CE)rHDL containing triacylglycerol (and a small amount of CE) in their core were prepared as described by Ryu et al. (17). Briefly, spherical (A-I/CE)rHDL (final CE concentration = 0.1 mmol/liter) were mixed with Intralipid (Kabi Pharmacia AB; final triacylglycerol concentration = 4 mmol/liter) and CETP (final concentration = 2.7 units/ml) and then incubated under \(N_a\) for 1.25 h at 37 °C. The final volume of the incubation mixture was 51.2 ml. The resulting (A-I/CE)rHDL were isolated by sequential ultracentrifugation in the density range 1.063 < \(d < 1.21\) g/ml using a TLA-100.4 rotor (Beckman Instruments) as described previously (17). \([^{3}H]\)triolein was incorporated into the (A-I/CE)rHDL as described for (A-I/CE)rHDL. Spherical (A-I/CE)rHDL labeled with \([^{14}C]DPPC\) were prepared by displacing all of the apo-A-I from spherical (A-I/CE)rHDL with lipid-free apo-A-II. These labeled rHDL preparations were used in experiments where phospholipid hydrolysis was determined directly by measuring nonesterified fatty acid (NEFA) mass.

Preparation of (A-I/CE)rHDL and (A-II/CE)rHDL Labeled with \([^{14}C]DPPC\). Substrates c and d—Spherical (A-I/CE)rHDL containing triacylglycerol (and a small amount of CE) in their core were prepared as described by Ryu et al. (17). Briefly, spherical (A-I/CE)rHDL (final CE concentration = 0.1 mmol/liter) were mixed with Intralipid (Kabi Pharmacia AB; final triacylglycerol concentration = 4 mmol/liter) and CETP (final concentration = 2.7 units/ml) and then incubated under \(N_a\) for 1.25 h at 37 °C. The final volume of the incubation mixture was 51.2 ml. The resulting (A-I/CE)rHDL were isolated by sequential ultracentrifugation in the density range 1.063 < \(d < 1.21\) g/ml using a TLA-100.4 rotor (Beckman Instruments) as described previously (17). \([^{3}H]\)triolein was incorporated into the (A-I/CE)rHDL as described for (A-I/CE)rHDL. Spherical (A-I/CE)rHDL labeled with \([^{14}C]DPPC\) were prepared by displacing all of the apo-A-I from (A-I/CE)rHDL. Spherical (A-I/CE)rHDL labeled with \([^{14}C]DPPC\) and \([^{3}H]\)triolein (A-I/CE)rHDL with lipid-free apo-A-II. These labeled rHDL preparations were used in experiments where phospholipid hydrolysis was determined directly by measuring nonesterified fatty acid (NEFA) mass.

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tion was carried out at 4 °C using a Ti-50.0 rotor in a Beckman L8–70M ultracentrifuge (Beckman Instruments). The 50,000 rpm spins were carried out at 4 °C using a Ti-50 rotor in a Beckman L5–70M ultracentrifuge (Beckman Instruments). The 100,000 rpm spins were carried out at 4 °C using a TLA-100.4 rotor in a Beckman TL-100 tabletop ultracentrifuge. The isolated HDL, which contained apoA-I as the predominant apolipoprotein, is designated (A-I)HDL2, as described for (A-I/CE)rHDL (A-II/CE)HDL2 labeled with radiolabeled DPPC were prepared by displacing all of the apoA-I from (A-I)DPPC-labeled (A-I/CE)HDL (23). The specific activities of the (A-I)DPPC-labeled (A-I/CE)HDL and (A-II/CE)HDL were 3.85 × 105 and 3.53 × 105 dpm/mg of phospholipid, respectively.

**Purification of Native (A-I)HDL2 and (A-II)HDL2**—The specific activities of the radiolabeled substrates added to the eluted fractions (8 ml) before dialysis against TBS containing 0.15 M NaCl (pH 7.4). HL was eluted from the column at a flow rate of 0.15 M NaCl (pH 7.4). HL was eluted from the column at a flow rate of 0.15 M NaCl (pH 7.4).

**Physical Properties of rHDL and Native HDL2 (Table I)**—The respective molar ratios of phospholipid/UC/E/A-I/II in the (A-I/CE)rHDL and (A-II/CE)rHDL labeled with [3H]triolein-labeled (A-I/TG)rHDL were prepared by displacing apoA-I from (A-I)HDL2 as described for (A-I/CE)rHDL. (A-II/CE)HDL2 labeled with [3H]triolein were prepared by displacing apoA-I from (A-II/CE)rHDL with lipid-free apoA-II (23).

**Calculations**—HL-mediated hydrolysis in the radiolabeled substates was determined as the amount of radiolabel in the NEFA relative to the total radiolabel in the substrate. HL-mediated hydrolysis in the unlabelled (A-I/CE)rHDL and (A-II/CE)rHDL was determined by direct mass assay of the NEFA formed. 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 >0.98. $V_{max}$ was determined as the reciprocal of the intercept on the $y$ axis. The $K_{m(app)}$ (app) was calculated as the product of the slope and $V_{max}$.

**Other Techniques**—All chemical analyses were carried out on a Beckman Model 23 refragual analyzer (Beckman Instruments, Fullerton, CA). Heparin was added to the pooled fractions to give a final concentration of 100 U/ml. Other techniques used in the present studies were described in a previous communication (25). NEFA were separated from the other rHDL (or native HDL2) lipids by thin layer chromatography. Triacylglycerol mass was measured by enzymatic assay (24) using Boehringer Mannheim kits were used for phospholipid, UC, and total cholesterol assays. CE concentrations were calculated as the difference between the total and UC concentrations. The concentrations of apoA-I and apoA-II were determined by an immunoturbidometric assay (26). The size of the rHDL and native HDL2 was determined by electrophoresis on 3–5% non-denaturning polyacrylamide gradient gels (Gradiopore, Sydney, Australia) (27).

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

**RESULTS**

**Physical Properties of rHDL and Native HDL2 (Table I)**—The respective molar ratios of phospholipid/UC/E/A-I/II in the (A-I/CE)rHDL and (A-II/CE)rHDL labeled with [3H]triolein-labeled (A-I/TG)rHDL were prepared by displacing apoA-I from (A-I)HDL2 as described for (A-I/CE)rHDL. (A-II/CE)HDL2 labeled with [3H]triolein were prepared by displacing apoA-I from (A-II/CE)rHDL with lipid-free apoA-II (23). As such, the (A-I/CE)rHDL and (A-II/CE)rHDL differed only in their apolipoprotein composition. This was also the case for the (A-I/CE)rHDL and (A-II/CE)rHDL and the (A-I/CE)rHDL and (A-II/CE)rHDL. The slight increase in the diameter of the (A-II/CE)rHDL and (A-II/CE)rHDL relative to their apoA-I-containing precursors is consistent with what has been reported earlier by this laboratory (23). The diameter of (A-II/CE)rHDL was also slightly larger than that of the (A-I/CE)rHDL.

**Kinetcs of the HL-mediated Hydrolysis of Phospholipids and Triacylglycerol in rHDL**—The aim of these studies was to determine how apolipoproteins influence the HL-mediated hydrolysis of phospholipids and triacylglycerol in rHDL. Preliminary experiments established that the hydrolysis of phospholipids and triacylglycerol in both (A-I)rHDL and (A-II)rHDL was linear up to 30% (results not shown). Consequently, the kinetic studies described below were all conducted under conditions that gave less than 30% phospholipid or triacylglycerol hydrolysis.

**Kinetics of HL-mediated Phospholipid Hydrolysis in (A-I/CE)rHDL and (A-II/CE)rHDL (Figs. 2 and 3 and Table II)**—(A-I/CE)rHDL and (A-II/CE)rHDL labeled with [3H]triolein were used to monitor the kinetics of the HL-mediated hydrolysis of phospholipids in the absence of triacylglycerol (Fig. 2).
In this experiment, the substrate concentration was increased progressively in incubations that contained a constant amount of HL. The duration of the incubation was 3 h. Fig. 2A shows that the rate of phospholipid hydrolysis increased as the concentration of the (A-I/CE)rHDL and (A-II/CE)rHDL increased from 0.05 to 0.4 mM phospholipid. For phospholipid concentrations from 0.05 to 0.2 mM, the rate of hydrolysis was greater in (A-II/CE)rHDL (open symbols) than in (A-I/CE)rHDL (closed symbols). The rate of hydrolysis as a function of substrate concentration is shown in A. The values are the means of triplicate determinations (*, p < 0.01). A double reciprocal plot of the kinetic data in A is shown in B.

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A range of concentrations of spherical rHDL were incubated with a constant amount of HL as described in the legends to Figs. 2–6. For the radiolabeled substrates, the resulting NEFA were separated from the other rHDL lipids by thin layer chromatography as described under "Experimental Procedures." Kinetic parameters were estimated from double-reciprocal plots of the rate of hydrolysis versus the concentration of rHDL phospholipid or triacylglycerol. All values are means of triplicate determinations. Since different preparations of HL were used for each study, the $V_{\text{max}}$ values cannot be compared for the different experiments.

**Table II**

Kinetic parameters of HL-mediated phospholipid and triacylglycerol hydrolysis in rHDL

| Spherical rHDL | Radiolabeled constituent | $K_m$(app) [mM] | $V_{\text{max}}$ [nmol NEFA/ml HL/h] | Catalytic efficiency | $V_{\text{max}}/K_m$(app) |
|---------------|--------------------------|-----------------|-------------------------------------|-------------------|-----------------------|
| (A-I/CE)rHDL  | PL $^a$                   | 3.1             | 309.3                               | 6.3               | 99.8                  |
| (A-I/CE)rHDL  | TG $^a$                   | 1.4             | 430.2                               | 3.1               | 307.3                 |
| (A-II/CE)rHDL | PL $^a$                   | 0.9             | 138.1                               | 2.5               | 460.3                 |
| (A-II/CE)rHDL | TG $^a$                   | 0.4             | 625.3                               | 2.5               | 694.8                 |
| (A-I/TG)rHDL  | PL $^a$                   | 1.0             | 1154.8                              | 4.8               | 1154.8                |
| (A-II/TG)rHDL | TG $^a$                   | 0.1             | 240.2                               |                   | 2402.0                |

$^a$ [14C]DPPC.

$^b$ A mass assay of NEFA was used to determine the extent of HL-mediated phospholipid hydrolysis in these spherical rHDL.

$^c$ [3H]Triolein.

**Symbols** ($p < 0.01$). At concentrations of 0.3 and 0.4 mM, the (A-I/CE)rHDL and (A-II/CE)rHDL exhibited comparable rates of phospholipid hydrolysis. A Lineweaver-Burk double-reciprocal plot of the data in Fig. 2A is shown in Fig. 2B. The kinetic parameters derived from the double reciprocal plot are shown in Table II. The $V_{\text{max}}$ was greater for (A-I/CE)rHDL than for (A-II/CE)rHDL (309.3 versus 49.1 nmol of NEFA formed/ml of HL/h). However, HL had a greater affinity for the phospholipids in (A-II/CE)rHDL ($K_m$(app) = 0.2 mM) than in (A-I/CE)rHDL ($K_m$(app) = 3.1 mM). The catalytic efficiency of the HL-mediated phospholipid hydrolysis ($V_{\text{max}}/K_m$(app)) was greater for (A-I/CE)rHDL than for (A-II/CE)rHDL.

To ensure that the hydrolysis of [14C]DPPC in the radiolabeled rHDL reflected the hydrolysis of the rHDL bulk phospholipid, NEFA formation was measured directly by mass assay in unlabeled (A-I/CE)rHDL and (A-II/CE)rHDL (Fig. 3A). As the NEFA mass assay is much less sensitive than the measurement of radiolabel, the lowest concentration of substrate used in these experiments was 0.08 mM phospholipid (compared with 0.05 mM phospholipid for the radiolabeled substrate). These results were comparable with what was obtained using [14C]DPPC-labeled (A-I/CE)rHDL and (A-II/CE)rHDL. When these results were transformed into a Lineweaver-Burk double reciprocal plot (Fig. 3B), it was apparent that, as with the radiolabeled substrate, HL has a greater affinity for the phospholipid in (A-II/CE)rHDL than in (A-I/CE)rHDL, with the $V_{\text{max}}$ being greater for (A-I/CE)rHDL than for (A-II/CE)rHDL. These results show that the hydrolysis of [14C]DPPC, when present in trace amounts in rHDL, reflects the hydrolysis of the bulk phospholipid.

**Kinetics of HL-mediated Phospholipid Hydrolysis in Native (A-I)HDL2 and (A-II)HDL2 (Fig. 4)** —To ensure that the above results are an accurate reflection of phospholipid hydrolysis in native HDL, an additional experiment was carried out with HDL2 that had been isolated from human plasma and radiolabeled with [14C]DPPC. ApoA-I constituted more than 90% of the apolipoproteins in this preparation (Table I). The HDL2 also contained a minimal amount of triacylglycerol. Native HDL2, in which apoA-II comprised more than 90% of the apolipoproteins, was prepared as described under "Experimental Procedures." The relationship between HL-mediated phospholipid hydrolysis in native (A-I)HDL2 and (A-II)HDL2 (Fig. 4) was comparable with what was observed in (A-I/CE)rHDL and (A-II/CE)rHDL. At HDL2 phospholipid concentrations of 0.05 and 1.0 mM, the rate of phospholipid hydrolysis was greater in (A-I)HDL2 (open symbols) than in (A-II)HDL2 (closed symbols) ($p < 0.01$). At HDL2 phospholipid concentrations of 0.7 and 1.0 mM, the rate of phospholipid hydrolysis was greater in (A-I)HDL2 than in (A-II)HDL2 ($p < 0.01$). Since transformation of this data into a Lineweaver-Burk double reciprocal plot did not yield a straight line, the kinetic parameters were not determined. The nonlinearity of the transformed data highlights the problems associated with using substrates that are heterogeneous in size and composition.

**Kinetics of HL-mediated Phospholipid Hydrolysis in (A-I/TG)rHDL and (A-II/TG)rHDL (Fig. 5, Table II)—(A-I)TG/rHDL and (A-II)TG/rHDL labeled with [14C]DPPC were used to assess the HL-mediated hydrolysis of phospholipids in the presence of triacylglycerol. The design of this study was identical to that described above for (A-I/CE)rHDL and (A-II/CE)rHDL. The rate of phospholipid hydrolysis as a function of rHDL phospholipid concentration is shown in Fig. 5A. At all concentrations of phospholipid, the rate of hydrolysis was greater in (A-I/TG)rHDL than in (A-II/TG)rHDL ($p < 0.01$). Since transformation of this data into a Lineweaver-Burk double reciprocal plot did not yield a straight line, the kinetic parameters were not determined. The nonlinearity of the transformed data highlights the problems associated with using substrates that are heterogeneous in size and composition.

**Kinetics of HL-mediated Triacylglycerol Hydrolysis in (A-I/TG)rHDL and (A-II/TG)rHDL (Fig. 6, Table II)—The kinetics...**
of triacylglycerol hydrolysis in (A-I/TG)rHDL and (A-II/TG)rHDL radiolabeled with [3H]triolein is shown in Fig. 6A. In this study, increasing concentrations of substrate were incubated for 1 h with a constant amount of HL. At rHDL triacylglycerol concentrations of 0.02 and 0.03 mM, the rate of hydrolysis was greater in (A-II/TG)rHDL (open symbols) than in (A-I/TG)rHDL (closed symbols) (see inset). The (A-I/TG)rHDL and (A-II/TG)rHDL exhibited comparable rates of hydrolysis at a triacylglycerol concentration of 0.1 mM. At rHDL triacylglycerol concentrations of 0.24 and 0.33 mM, the rate of triacylglycerol hydrolysis was greater in (A-I/TG)rHDL than in (A-II/TG)rHDL ($p$, 0.01). The $V_{\text{max}}$ for (A-I/TG)rHDL was greater than for (A-II/TG)rHDL (1154.8 versus 240.2 nmol of NEFA formed/ml of HL/h) (Table II). As with the phospholipid hydrolysis, HL had a greater affinity for the triacylglycerol in (A-II/TG)rHDL ($K_{\text{m(app)}}$) compared with (A-I/TG)rHDL ($K_{\text{m(app)}}$ = 1.0 mM). The $V_{\text{max}}/K_{\text{m(app)}}$ for triacylglycerol hydrolysis in (A-II/TG)rHDL was approximately double that for (A-I/TG)rHDL.

**DISCUSSION**

In the present study, we have used well characterized, homogeneous, apolipoprotein-specific preparations of spherical rHDL and preparations of native HDL2 to investigate the influence of apoA-I and apoA-II on the HL-mediated hydrolysis of HDL phospholipids and triacylglycerol. The (A-I)rHDL and (A-II)rHDL used in this study were comparable in size and lipid composition and differed only in their apolipoprotein content. The results show unequivocally 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 for both constituents is greater in (A-I)rHDL than in (A-II)rHDL. The HDL in human plasma consist of two major apolipoprotein-specific subpopulations of particles: those containing apoA-I without apoA-II, (A-I)HDL, and those with both apoA-I and apoA-II, (A-I/A-II)HDL (28). There is also a minor HDL subpopulation that contains apoA-II without apoA-I, (A-II)HDL (29). The present studies conducted in vitro with rHDL and native HDL2 raise the possibility that there may be major differences in the interaction of HL with (A-I)HDL and (A-II)HDL in vivo. Although it is not known whether (A-I/A-II)HDL resemble (A-I)HDL or (A-II)HDL in terms of their interactions with HL in plasma, it would be surprising if the presence of apoA-II in HDL did not have an impact on the HL-mediated hydrolysis of phospholipids and triacylglycerol in vivo.

There are several reasons why apolipoproteins may influence the interaction of HL with HDL. For instance, the different affinity of HL for (A-I)rHDL relative to (A-II)rHDL may reflect differences in particle charge. Rye and Barter (23) have reported that the surface of (A-II)rHDL is less negatively charged than that of (A-I)rHDL. Since HL has a net negative charge (30), it is likely to have a greater affinity for the surface of (A-II)rHDL compared with (A-I)rHDL. Consistent with this, Laboda et al. (30) showed that the HL-mediated hydrolysis of

**FIG. 5.** Kinetics of the hydrolysis of phospholipids in (A-I/TG)rHDL and (A-II/TG)rHDL. (A-I/TG)rHDL (●) and (A-II/TG)rHDL (○) were radiolabeled with [14C]DPPC, and aliquots in which the phospholipid concentration varied from 0.05 to 0.4 mM were incubated at 37 °C for 3 h with HL (10 μl of a preparation that hydrolyzed 102 nmol of triacylglycerol/ml of HL/h). The incubation mixtures also contained BSA and heparin at the same concentrations as in Fig. 2. The rate of phospholipid hydrolysis as a function of substrate concentration is shown in A. The values are the means of triplicate determinations (*, $p < 0.01$). A double reciprocal plot of the kinetic data in A is shown in B.

**FIG. 6.** Kinetics of the hydrolysis of triacylglycerol in (A-I/TG)rHDL and (A-II/TG)rHDL. (A-I/TG)rHDL (●) and (A-II/TG)rHDL (○) were radiolabeled with [3H]triolein, and aliquots in which the triacylglycerol concentration varied from 0.02 to 0.33 mM were incubated at 37 °C for 1 h with HL (9 μl of a preparation that hydrolyzed 47 nmol of triacylglycerol/ml of HL/h). The incubation mixtures also contained BSA and heparin at the same concentrations as in Fig. 2. The rate of triacylglycerol hydrolysis as a function of substrate concentration is shown in A. The inset shows an expanded view of the area of the graph below 0.075 mM triacylglycerol. Each value is the mean of triplicate determinations (*, $p < 0.01$; #, $p < 0.05$). A double reciprocal plot of the kinetic data in A is shown in B.
triolein is reduced when a negative charge is incorporated into a lipid monolayer. Another explanation for the different affinities of HL for (A-I)rHDL and (A-II)rHDL relates to lipid-water interfacial hydration. It has been shown in earlier work from this laboratory that the lipid-water interface of (A-I)rHDL is more hydrated than that of (A-II)rHDL (23). Since the catalytic sites of most lipases are hydrophobic (31, 32), it is conceivable that such enzymes would associate preferentially with the less hydrated lipid-water interface of (A-II)rHDL.

The present data show that when the substrate concentration is not rate-limiting, HL hydrolyzes both phospholipids and triacylglycerol more rapidly in (A-I)rHDL than in (A-II)rHDL. This is consistent with the phospholipid and triacylglycerol acyl chains in (A-I)rHDL being more accessible to the active site of HL than those in (A-II)rHDL. One explanation for this observation relates to the less ordered phospholipid head group packing in (A-I)rHDL compared with (A-II)rHDL (16). Such a difference may lead to significant structural changes in the microenvironment of the rHDL interface, which could influence access of the enzyme to its substrates in such a way as to enhance the hydrolysis of both phospholipids and triacylglycerol in (A-I)rHDL (33).

Tansey et al. reported recently on the HL-mediated hydrolysis of phospholipids in discoidal and spherical rHDL that had been prepared with a range of phospholipids, including DPPC (34). In that study, minimal phospholipid hydrolysis was observed in either the discoidal or spheroidal rHDL that contained DPPC. This is an interesting finding given the results of the present study, where [14C]DPPC was used as a tracer to monitor POPC hydrolysis. It should be noted that other investigators have also used [14C]DPPC successfully as a tracer to monitor phospholipid hydrolysis (35, 36). These discrepancies suggest that HL is sensitive to the phase state of substrate lipids. Indeed, Thuren et al. have found this to be the case in their monolayer studies (37). Thus, although DPPC is a poor substrate for HL when it is present as the bulk lipid in an interface, it is hydrolyzed readily by HL when it is present in trace amounts in a more physiological substrate. The current results show clearly that this is the case, since comparable results were obtained when phospholipid hydrolysis was determined using rHDL containing a trace amount of [14C]DPPC (Fig. 2) as well as in experiments where NEFA formation was measured directly with a mass assay (Fig. 3).

There are several conflicting reports as to the effects of apolipoproteins on the HL-mediated hydrolysis of phospholipids and triacylglycerol in HDL. On the one hand, it has been suggested that apoA-II inhibits (9, 10), while others have found that it enhances, HL-mediated hydrolysis of HDL triacylglycerol (7, 8, 38, 39). Morrow et al. (38) reported that hydrolysis of phospholipids and triacylglycerol in HDL is greater in (A-I/A-II)HDL compared with (A-I)HDL. However, these observations cannot be compared directly with what was observed in the present study, since the HDL2 used by Morrow et al. (38) were heterogeneous in size and composition. They also contained apolipoproteins other than apoA-I and apoA-II that may have influenced the hydrolysis (40). Finally, Morrow et al. (38) measured total fatty acid liberation (i.e. phospholipid and triacylglycerol hydrolysis combined) rather than the hydrolysis of individual lipids.

The results of Zhong et al. (9) are, to a large extent, consistent with the present findings. Those investigators found that the HL-mediated hydrolysis of triacylglycerol in a lipid emulsion was inhibited by the addition of HDL from mice that had been made transgenic for either human apoA-I or human apoA-II or for both human apoA-I and apoA-II. Since the HDL from the apoA-II and apoA-I/apoA-II transgenic mice mediated a greater reduction in the rate of HL-mediated triacylglycerol hydrolysis than the HDL from the apoA-I transgenic mice, it was concluded that apoA-II inhibits HL activity more than apoA-I. Given the findings in the present study, which show that HL has a greater affinity for the less reactive apoA-II-containing rHDL, it would be predicted that (A-II)rHDL would be more effective than (A-I)rHDL as inhibitors of triacylglycerol hydrolysis in lipid emulsions.

In another study, Jahn et al. (39) found that reconstituted particles containing apoA-II and HDL lipids enhanced the HL-mediated hydrolysis of triacylglycerol in microemulsions. These investigators obtained similar results when the reconstituted particles were substituted with ultracentrifugally isolated HDL. Jahn et al. (39) explained their observations in terms of apoA-II partitioning from the reconstituted particles (or isolated HDL) to the triacylglycerol emulsion and thus increasing the affinity of HL for the triacylglycerol emulsion. However, these investigators also found that the triacylglycerol hydrolysis in the microemulsions decreased at high concentrations of reconstituted particles (or HDL). This may have been caused by the high concentrations of reconstituted particles or HDL competing with the triacylglycerol emulsion for HL.

The results of the present study may explain some of the conflicting conclusions that have been drawn about the relative effects of apoA-I and apoA-II on HL-mediated phospholipid and triacylglycerol hydrolysis (9, 38). The lower Km values for both the phospholipids and triacylglycerol in (A-II)rHDL relative to (A-I)rHDL suggest that HL has a greater affinity for HDL that contain apoA-II than for HDL that contain apoA-I. In other words, at a low concentration of substrate, the amount of HL interacting with HDL (and hydrolyzing HDL lipids) may be much greater in HDL that contain apoA-II than in (A-I)rHDL. Therefore, when a study is conducted at a low substrate concentration, it may be concluded that apoA-II-containing HDL are superior to (A-I)rHDL as substrates for HL. By contrast, if experiments are conducted at high substrate concentrations, under conditions where substrate availability is no longer a limiting factor, and the Vmax for phospholipid and triacylglycerol hydrolysis is greater in (A-I)rHDL than in (A-II)rHDL, it may be concluded that (A-I)rHDL are superior substrates compared with HDL that contain apoA-II.

In conclusion, these studies provide the first description of the kinetics of the HL-mediated hydrolysis of phospholipids and triacylglycerol in spherical, apolipoprotein-specific rHDL. In addition, they show that apolipoproteins have a major impact on these processes. It remains to be determined whether the interaction of (A-II)HDL with HL resembles that of either the (A-I)HDL or (A-II)rHDL or whether it is distinct from each.

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