Different reactivities of high density lipoprotein$_2$ subfractions with hepatic lipase

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Abstract Human high density lipoproteins$_2$ (HDL_2) consist of particles that contain both apolipoprotein (apo) A-I and apoA-II (A-I/A-II-HDL_2) and others that contain apoA-I but are devoid of apoA-II (A-I-HDL_2). When postprandial lipemia is pronounced, a fraction of HDL_2 is converted into HDL_3-like particles. These HDL_3 exhibit lower apoA-I/apoA-II ratios than the parent HDL_2, suggesting preferential conversion of A-I/A-II-HDL_2 into HDL_3 (*J. Clin. Invest.* 1984. **74:** 2017–2023). Triglyceride transfer from triglyceride-rich lipoproteins to HDL_2 and subsequent lipolysis by hepatic lipase are thought to mediate the conversion of HDL_2 into HDL_3. To understand why A-I/A-II-HDL_2 are preferentially converted into HDL_3, we separated postprandial HDL_2 into A-I-HDL_2 and A-I/A-II-HDL_2 species by immunoaffinity chromatography using a monoclonal antibody for apoA-II, and determined the ability of HDL_2 species *i)* to participate in protein-mediated lipid transfer; and *ii)* to interact with hepatic lipase in vitro. Triglyceride transfer from/to triglyceride-rich lipoproteins was similar for the two HDL_2 species. In contrast, A-I/A-II-HDL_2 were twice as effective as A-I-HDL_2 in liberating hepatic lipase immobilized on HDL_3-Sepharose. Lipolysis of triglycerides by hepatic lipase was 60% higher in postprandial A-I/A-II-HDL_2 than in postprandial A-I-HDL_2. Hydrolysis of phosphatidylcholine by hepatic lipase was threefold higher in A-II-containing HDL_2 when compared with HDL_2 devoid of apoA-II. The different lipolytic rates in HDL_2 subspecies correlated with the size reduction of substrate lipoproteins. Reconstitution of postprandial A-I-HDL_2 with apoA-II enhanced the rate of lipolysis by hepatic lipase to that observed in A-I/A-II-HDL_2. We conclude that it is the interaction with hepatic lipase rather than the rate of triglyceride transfer that results in the preferred conversion of postprandial A-II-containing HDL_2 into HDL_3, and that apoA-II exerts a crucial role in this process.—**Mowri, H-O., W. Patsch, L. C. Smith, A. M. Gotto, Jr., and J. R. Patsch.** Different reactivities of high density lipoprotein$_2$ subfractions with hepatic lipase. *J. Lipid Res.* 1992. **33:** 1269–1279.

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In virtually every major epidemiologic study, a powerful inverse association has been found between coronary artery disease (CAD) and high density lipoprotein (HDL)-cholesterol (1–4). HDL are traditionally divided into two major subclasses, HDL_2 and HDL_3. As a strong correlation exists between HDL-cholesterol and plasma levels of HDL_2, but not of HDL_3, plasma concentrations of HDL_2 may better reflect the "protective" function of HDL-cholesterol. Even though epidemiologic studies do not always support this view (5), the importance of HDL_2 is suggested by clinicopathological studies (6) and the relationship between prevalence or incidence of CAD and HDL_2 plasma levels as observed in males and females (7). Furthermore, the beneficial effects of high HDL_2 levels are also apparent in an autosomal dominant inherited condition, familial hyperalphacholesterolemia, which reportedly is associated with longevity (8). In these subjects, most of the HDL-cholesterol is found in the HDL_2 fraction, while concentrations of HDL_3 are similar to those in controls (9). Therefore, the mechanisms controlling the plasma levels of HDL_2 are central to our understanding of the inverse relationship between CAD and HDL-cholesterol.

The metabolism of triglyceride-rich lipoproteins (TGRL) is a major determinant of HDL_2 concentrations in blood. The assimilation of TGRL-derived surface material, predominantly phospholipids, by HDL promotes the formation of HDL_2-like particles from HDL_3 (10). Conversely, impaired metabolism of TGRL, as ascertained in the postprandial state, reduces the concentration of HDL_2. When the concentration of TGRL is increased postprandially, cholesteryl esters are translocated from HDL_2 to TGRL in exchange for roughly equimolar amounts of triglycerides (11–14). Triglycerides transferred to HDL are hydrolyzed by hepatic lipase. Dependent on the enrich-

Abbreviations: HDL, high density lipoprotein; VLDL, very low density lipoprotein; TGRL, triglyceride-rich lipoproteins; CAD, coronary artery disease; TG, triglyceride; PL, phospholipid.

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ment of postprandial HDL_2, this process may lead to conversion of the larger HDL_2 into smaller HDL_3 (15). In vitro incubation of postprandial triglyceride-enriched HDL with hepatic lipase showed that HDL_3-like particles generated had a two- to threefold lower molar ratio of apoA-I to apoA-II when compared to particles remaining within the HDL_2 flotation range. Furthermore, particles remaining in the HDL_2 flotation range after incubation showed an increased molar ratio of apoA-I to apoA-II when compared with parent HDL_2 (16). This suggested preferential conversion of apoA-II-containing HDL_2 into HDL_3 which could have resulted from enhanced translocation of triglycerides from TGRL to apoA-II-containing HDL_2 or enhanced hydrolysis of core lipids in apoA-II-containing HDL_2 by hepatic lipase. However, shedding of apolipoproteins during lipolysis could have caused changes in the apolipoprotein composition of HDL subclasses as well. To distinguish among these possibilities, we have isolated postprandial HDL_2 containing apoA-II (A-I/A-II-HDL_2) and devoid of apoA-II (A-I-HDL_2) by immunoaffinity chromatography. We have determined the ability of these two HDL_2 species to participate in protein-mediated lipid transfer and to interact with hepatic lipase. We report here that it is not the selective participation in the neutral lipid exchange process of apoA-II-containing HDL_2 but rather their interaction with hepatic lipase that confers specificity to the conversion of apoA-II-containing HDL_2 into HDL_3 (part of this research was presented in abstract form, ref. 17).

METHODS

Blood from fasting subjects was obtained after an overnight fast. Postprandial blood was obtained 6 h after a standardized fatty meal (18). Blood was collected into tubes containing 1.5 mg EDTA/ml of blood, and plasma was separated by centrifugation (1500 *g*, 20 min, 4°C). For isolation of HDL_2 and HDL_3 zonal ultracentrifugation was used (19). Volume fractions containing HDL_2 were subjected to gel permeation chromatography using Bio-gel A-5M in 2.5 cm × 95 cm columns. Purified lipoprotein fractions were dialyzed against standard buffer containing 0.9% NaCl, 0.3 mM EDTA, 0.01 M Tris-HCl, pH 7.8.

For separation of HDL_2 subfractions containing apoA-I/apoA-II or apoA-I without apoA-II, immunoaffinity chromatography was used (20). Mab 32, a monoclonal anti-apoA-II antibody, was purified from murine ascites fluid (21). After treatment of ascites fluid with Lipoclean (Calbiochem, San Diego, CA) and 50% ammonium sulfate precipitation, anti-apoA-II antibody was solubilized in 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 1 mM EDTA, and 0.02% NaN_3. After centrifugation at 1000 *g* for 10 min, the supernate was recycled twice through an HDL_3-Sepharose 4B column, 1.5 × 20 cm, at a flow rate of 40 ml/h. The HDL_3-Sepharose 4B was prepared from HDL_3 containing 104 mg protein and 10 g of CNBr-activated Sepharose 4B according to the instructions of the manufacturer (Pharmacia, Piscataway, NJ). Nonspecifically bound proteins were removed by washing the column with standard buffer until the A_{280} was less than 0.02 units. Mab directed against apoA-II was eluted from the column with 0.2 M glycine buffer, pH 2.8, containing 1 mM EDTA and 0.02% NaN_3. Four-ml fractions were collected into tubes containing 1.4 ml of 1 M Tris-HCl, pH 8.0. Reactivity of affinity-purified antibodies against HDL_3 was established by ELISA using alkaline phosphatase-conjugated anti-mouse IgG as second antibody.

Affinity-purified Mab 32 was covalently coupled to CNBr-activated Sepharose 4B at a ratio of 9–11 mg per 1 g of gel. The Mab 32-Sepharose 4B was extensively washed with several cycles of 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0, and 0.1 M Na acetate, 0.5 M NaCl, pH 4.0, followed by 0.2 M glycine, pH 2.8. The gel was then equilibrated in Tris-HCl, pH 7.4, containing 0.02% NaN_3 and stored at 4°C.

For separation of A-I/A-II-HDL_2 and A-I-HDL_2, HDL_2 containing up to 5 mg protein was diluted to a volume of 10 ml with Tris-buffered saline and applied to Mab 32-Sepharose 4B columns (1 × 7 cm) at a flow rate of 8 ml/h. The eluate was recycled twice through the column prior to collecting unbound HDL in 1-ml fractions. After collection of 30 fractions, the elution buffer was changed to 0.2 M glycine buffer, pH 2.8, and 1-ml fractions were collected into tubes containing 0.35 ml of 1 M Tris-HCl, pH 8.0. In larger scale experiments, 10 mg HDL_2-protein was applied to 1.5 × 19 cm columns.

Protein was measured by the method of Lowry et al. (22) using bovine serum albumin as standard. Cholesterol, esterified cholesterol, and triglycerides were determined by enzymatic procedures (23–25). Lipid phosphorus was determined by the procedure of Bartlett (26). For analysis of particle size, HDL fractions were subjected to electrophoresis in 4–30% polyacrylamide gels (27). Apolipoproteins were separated by polyacrylamide gel electrophoresis in 0.1% SDS (28) or by isoelectric focusing (29). Relative abundance of apoA-I and apoA-II was determined by scanning of IEF-gels using a laser densitometer (LKB Ultrascan XL, Pharmacia LKB Biotechnology Inc., Piscataway, NJ) as described (30).

To determine transfer of triglycerides between TGRL and HDL fractions, d < 1.006 g/ml lipoproteins, isolated by ultracentrifugation, were labeled with [^{14}C]triolein according to the method of Simard et al. (31). Ten μCi (89 nmol) of tri[^{14}C]oleoylglycerol (DuPont, Wilmington, DE) dispersed in 50 μl ethanol, were added to 10 ml plasma, and incubated for 3.5 h at 37°C in the presence of 0.25 mM EDTA and 0.01% NaN_3. TG-rich lipoproteins were then isolated at a density of 1.006 g/ml by ultracentrifugation and further purified by gel permeation chromatography on a Sephadex G-50 column (1 × 15

cm). ^{14}C-labeled VLDL (100 μg TG) was incubated together with HDL fractions and 5,5-dithio-2-nitrobenzoic acid (2 mM) at 37°C in the presence or absence of lipoprotein-deficient plasma (5 mg protein) in 0.05 M phosphate buffer, pH 7.4. The total assay volume was 700 μl. After stopping the reaction by placing the tubes on crushed ice for 15 min, 250 μl saline, 50 μl LDL (6.4 mg protein/ml), 100 μl HDL_2 (5.8 mg protein/ml), and 120 μl of a mixture containing 0.1% dextran sulfate and 1 M $MgCl_2$ were added. After mixing, tubes were allowed to stand on ice for 15 min prior to centrifugation at 3,500 *g* for 10 min. Radioactivity in supernates was determined by liquid scintillation counting. Triglyceride exchange activity was calculated by the formula (32):

$$F = \frac{-\ln\left[1 - \frac{C_H}{M_V(S_{V(0)} - S_{Eq})}\right]}{\frac{M_V + M_H}{M_V \cdot M_H}\, t}$$

where F is the rate of exchange in nmol/h per incubation between VLDL and HDL. M_V is the pool size of triglycerides in VLDL in nmol/incubation, M_H the pool size of cholesteryl ester and triglycerides in HDL in nmol/incubation, C_H is the radioactivity in HDL in counts/min at time t, where t is expressed in hours. $S_{V(0)}$ denotes counts/M_V (cpm/nmol) at time 0 and S_{Eq} counts/(M_V + M_H) in cpm/nmol at equilibrium.

Hepatic lipase was isolated and purified from healthy volunteers. They were injected with 2,280 U/m² heparin and blood was obtained 10–15 min after heparin administration. Purification steps included adsorption of the enzyme to Intralipid (Vitrum, Stockholm, Sweden) and affinity chromatography with heparin-Sepharose (Pharmacia, Piscataway, NJ) (33). A stepwise elution with 0.9 M NaCl was used, and enzyme activities were measured in column fractions. Activities of lipoprotein lipase and hepatic lipase were determined according to Huttunen et al. (34), using [^{14}C]triolein-labeled substrate stabilized with gum arabic and an antiserum directed against hepatic lipase as described (15). The specific activity of hepatic lipase preparations averaged 5.7 μmol fatty acids released/min per mg protein. Enzyme preparations contained less than 3% lipoprotein lipase activity as judged by lipolytic activity recovered after precipitation with an antiserum directed against hepatic lipase that has been used previously (15).

For displacement studies with HDL_2 subspecies, hepatic lipase was immobilized to HDL_3-Sepharose by incubating 50 μg of the enzyme preparation with 1 ml packed gel and 0.8 ml of equilibration buffer containing 20 mM Tris-HCl, 0.1 M NaCl, and 1% (g/v) bovine serum albumin at 4°C for 60 min (35). After two washes with buffer, 100-μl aliquots of the gel were incubated with 200 μl equilibration buffer containing various concentrations of A-I- or A-I/A-II-HDL_2 at 4°C for 30 min. Incubation mixtures were centrifuged at 3,500 *g* for 1 min, and supernates were collected. Pellets were washed with 200 μl of equilibration buffer, and twice with 200 μl of 5 mM deoxycholic acid in 20 mM Tris-HCl, pH 8.5. All supernates were assayed for hepatic lipase activity. For these experiments, a batch of hepatic lipase complexed to HDL_3-Sepharose was divided into aliquots that were used only once to avoid potential effects of deoxycholic acid on the integrity of the matrix.

To determine substrate properties of HDL_2 subspecies, A-I- and A-I/A-II-HDL_2 were incubated with hepatic lipase in 250 μl of 100 mM Tris-HCl, pH 8.0, containing 4% (w/v) bovine serum albumin. After various incubation periods, lipids were extracted (36). [^{14}C]triolein was added to monitor recovery of triglycerides. Lipids were separated by thin-layer chromatography using a modification of the two-step procedure of Macala, Yu, and Ando (37). Plates were developed to half their height with chloroform–methanol–acetic acid–formic acid–water 70:30:12:4:2 (v/v) and then with hexane–diethyl ether–acetic acid 80:20:1 (v/v). After visualization with iodine vapor, lipids were scraped from the plate and extracted three times with chloroform–methanol 2:1 (v/v). Extracts were analyzed for triglycerides, cholesteryl ester, and phosphorus in phospholipids as described above. Fatty acids were measured according to Stajner and Suva (38). To determine the initial rates of fatty acid release, fatty acid concentrations were measured in such incubation mixtures using an enzymatic procedure (Free Fatty Acids Kit, Boehringer Mannheim, Mannheim, Germany).

To increase the triglyceride content of HDL_2 subfractions, HDL_2 (10 mg protein) was incubated with VLDL (0.5 or 2.0 mg protein) and lipoprotein-deficient plasma (20 mg protein/ml) in a volume of 40 ml of 10 mM Tris-HCl, 140 mM NaCl, 1 mM EDTA, 0.02% NaN_3 (w/v), 2 mM dithionitrobenzoic acid, pH 7.4, at 37°C. After an incubation period of 16 h, HDL_2 were isolated by zonal ultracentrifugation, and A-I- and A-I/A-II-HDL_2 were separated by immunoaffinity chromatography and used as substrates for hepatic lipase.

To estimate effects of lipolysis on particle size, postprandial A-I-HDL_2 and A-I/A-II-HDL_2 were labeled by the ICl procedure (39) to a specific activity of 10^7 cpm/mg protein. Labeled HDL_2 subspecies were incubated with hepatic lipase in incubation mixtures described. After various incubation periods, aliquots of mixtures were analyzed by electrophoresis in 5–20% of polyacrylamide gradients. Gels were dried prior to exposure to X-ray film.

To reconstitute A-I-HDL_2 with apoA-II, A-I-HDL_2 containing 1.5 mg protein was incubated with 0.75 mg apoA-II in 1.5 ml of 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 0.02% NaN_3, and 1 mM EDTA at 25°C for 2 h (40). Incubation mixtures were brought to a density

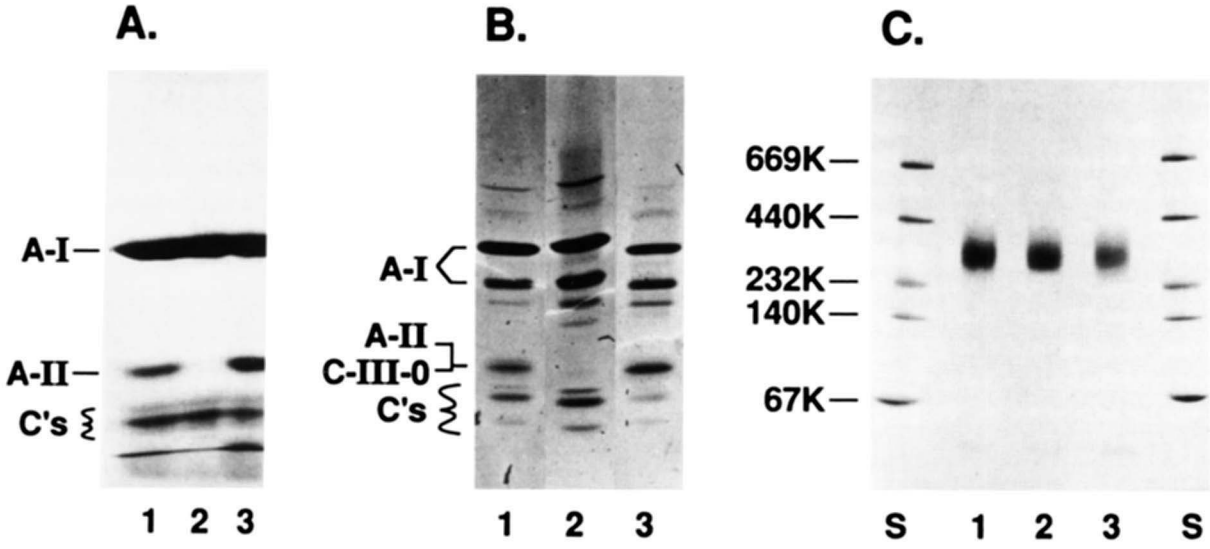

Fig. 1. Analysis of postprandial HDL_2 species by SDS-polyacrylamide gel electrophoresis (A), isoelectric focusing (B), and 4–30% polyacrylamide gel electrophoresis under nondenaturing conditions (C); lanes 1, 2, and 3 are total HDL_2, A-I-HDL_2, and A-I/A-II-HDL_2, respectively. Molecular weight standards are in lanes S.

of 1.21 g/ml by using KBr and ultracentrifuged in an SW 40 rotor (Beckman, Palo Alto, CA) at 40,000 rpm, 10°C for 24 h. The top fraction containing the reconstituted particles was dialyzed and applied to affinity chromatography on Mab 32-Sepharose 4B. The retained fraction was eluted as described before, dialyzed, characterized, and used for incubation studies.

RESULTS

Affinity chromatography using Mab 32-Sepharose 4B was effective in separating postprandial HDL_2 subspecies based on their apoA-II content. As judged by polyacrylamide gel electrophoresis in 0.1% SDS and isoelectric focusing, the unbound fraction was devoid of apoA-II, while the apoA-II content of the retained fraction increased as the dye uptake ratio of A-I/A-II decreased from 11.6 in parent HDL_2 to 5.2 in A-I/A-II-HDL_2 (**Fig. 1A and B**). A-I- and A-I/A-II-HDL_2 exhibited similar size as estimated by nondenaturing gradient gel electrophoresis (Fig. 1C). Postprandial A-I/A-II-HDL_2 exhibited a lower content of triglycerides and unesterified cholesterol when compared with postprandial A-I-HDL_2 (**Table 1**). The phospholipid composition of HDL_2 subspecies was similar. As determined in two preparations, mean percentage contribution of lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine to phospholipids was 0.9, 9.1, 86.8, and 3.2 in A-I-HDL_2 and 1.0, 9.4, 86.7, and 2.9 in A-I/A-II-HDL_2, respectively.

To determine the ability of HDL-subspecies to participate in protein-mediated lipid transfer, translocation of ^{14}C-labeled triglyceride between labeled VLDL and HDL-subspecies was measured using lipoprotein-deficient plasma as source of transfer activity. In three different HDL preparations, initial rates of triglyceride transfer were virtually the same for the two HDL_2 subspecies (**Fig. 2**). Using two concentrations of HDL_2 subfractions and incubation periods of 16 h, no significant difference in triglyceride transfer was found in HDL_2 preparations

TABLE 1. Weight percentage chemical composition of postprandial A-I-HDL_2 and A-I/A-II-HDL_2

	Protein	PL	UC	CE	TG
A-I-HDL_2	38.7 (2.0)	34.0 (1.8)	3.7 (1.3)	17.4 (2.1)	6.6 (2.5)
A-I/A-II-HDL_2	41.5 (2.9)	32.3 (2.9)	3.0 (0.9)*	17.8 (1.4)	5.7 (2.1)*

Results are means (SD) of four preparations. PL, phospholipids; UC, unesterified cholesterol; CE, cholesteryl ester; TG, triglycerides.
*$P < 0.05$.

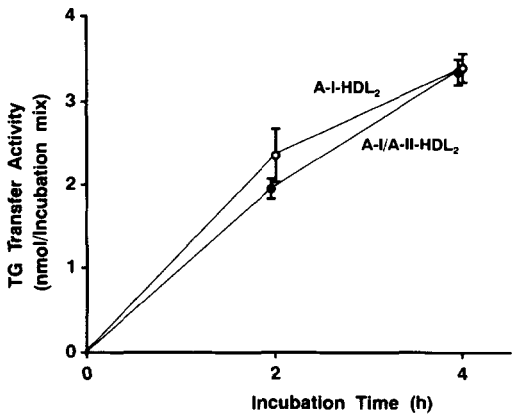

Fig. 2. Initial rates of triglyceride transfer between triglyceride-rich lipoproteins and A-I- and A-I/A-II-HDL_2. Incubation mixtures contained 100 μg triglycerides of [^{14}C]triolein-labeled triglyceride-rich lipoproteins, 25 μg protein of A-I- (○) or A-I/A-II-HDL_2 (●), and 5 mg protein of lipoprotein-deficient plasma in a total volume of 0.7 ml. The incubation temperature was 37°C. Values are means ± SD of three different HDL preparations.

obtained from three different subjects (data not shown). Thus, in none of our experiments was the transfer of triglycerides to and from A-I/A-II-HDL_2 greater when compared with A-I-HDL_2.

Bengtsson and Olivecrona (35) previously demonstrated that hepatic lipase binds to HDL that is covalently attached to Sepharose. We reasoned that a matrix containing immobilized enzyme would be suitable for displacement studies of enzyme by various HDL_2 fractions. In control experiments, HDL_2 subspecies labeled in their protein moiety were incubated with hepatic lipase immobilized to the HDL_3 matrix using the conditions applied for subsequent displacement studies. There was no difference in the proportions of HDL-associated radioactivity between A-I-HDL_2 and A-I/A-II-HDL_2 which was recovered in the various elution steps (**Table 2**). To determine the interaction of postprandial HDL subspecies with hepatic lipase, the ability of these fractions to displace hepatic lipase immobilized to HDL_3-Sepharose was measured at increasing concentrations (**Table 3**). On the average, A-I/A-II-HDL_2 were about twice as effective as A-I-HDL_2 to liberate immobilized enzyme ($P < 0.05$, two-way analysis of variance). In these experiments, effects of HDL_2 species and deoxycholic acid on apparent activity of hepatic lipase in the respective supernates were taken into account. In other control experiments, enzyme activity was measured in the presence of HDL_2 species present at concentrations representative for the various supernates (Tables 2 and 3). At the highest HDL concentration used in Table 3, apparent lipolytic activity was reduced to 91% and 84% of control by A-I- and A-I/A-II-HDL_2, respectively. Deoxycholic acid, present in supernate III, did not alter lipolytic activity under our assay conditions. Thus, consistent with our earlier studies (15), HDL_2 had only minor inhibitory effects on apparent lipolytic activity which were used to correct the data shown in Table 3.

To directly compare substrate properties of A-I- and A-I/A-II-HDL_2, lipolysis was measured after incubation of the two HDL_2 subspecies with hepatic lipase. Initially, postabsorptive HDL_2 subfractions were incubated for 20 h. Lipolysis of triglycerides and phosphatidylcholine in A-I/A-II-HDL_2 was 68 and 43%, respectively, higher than in A-I-HDL_2 as determined by mass measurements before and after incubation. A 4-h incubation experiment with postprandial HDL_2 subfractions clearly demonstrated the superior substrate properties of A-I/A-II-HDL_2 (**Table 4**). In our separate preparations, lipolysis of triglycerides in postprandial A-I/A-II-HDL_2 by hepatic lipase was 159 ± 5.25% (mean ± SD, $P < 0.02$) of that in postprandial A-I-HDL_2. Hydrolysis of phosphatidylcholine measured in three different HDL preparations was about threefold higher in A-I/A-II-HDL_2 than in A-I-HDL_2 (Table 4). To compare substrate properties of HDL_2 subspecies more rigorously, initial rates of fatty acid liberation were measured over a 4-h incubation period. At all time points, mean values of fatty acids

TABLE 2. Distribution of ^{125}I-labeled A-I-HDL_2 and ^{125}I-labeled A-I/A-II-HDL_2 in supernates after incubation with hepatic lipase immobilized to HDL_3-Sepharose

HDL_2-Species	HDL_2-Protein	Supernate I	Supernate II	Supernate III	Total Recovery
	mg/incubation		*% of radioactivity applied*		%
A-I-HDL_2	0.16	64.3	19.9	13.7	97.9
A-I-HDL_2	0.32	65.4	19.4	13.0	97.8
A-I/A-II-HDL_2	0.16	64.5	21.8	14.6	100.9
A-I/A-II-HDL_2	0.32	67.3	22.1	11.7	101.1

Aliquots of 0.1 ml of HDL_3-Sepharose containing immobilized hepatic lipase were incubated with ^{125}I-labeled HDL_2 species at two concentrations each in a total volume of 0.3 ml at 4°C for 30 min. Radioactivity was determined in supernates. Supernate I was obtained by centrifugation of the incubation mixture; supernate II was obtained after washing the pellet with equilibration buffer; and supernate III was obtained after washing the pellet of the first wash with 5 mM deoxycholic acid (see Methods).

TABLE 3. Liberation of hepatic lipase activity immobilized to HDL_3-Sepharose by A-I-HDL_2 and A-I/A-II-HDL_2

HDL_2 Subfraction (protein/incubation)	Lipolytic Activity Liberated		
	Supernate I	Supernate II	Supernate III
	% of activity recoverd		
A-I-HDL_2			
0.16 mg	1.1	0.1	98.8
0.32 mg	3.0	2.1	95.0
0.64 mg	8.8	4.0	87.2
A-I/A-II-HDL_2			
0.16 mg	2.8	1.9	95.3
0.32 mg	6.4	4.1	89.5
0.64 mg	16.5	5.8	77.7

Hepatic lipase was bound to HDL_3-Sepharose; 0.1-ml aliquots of the matrix were incubated with increasing doses of postabsorptive HDL_2 species in a total volume of 0.3 ml at 4°C for 30 min. Enzyme activity was then determined in supernates obtained by centrifugation. Supernate I was obtained by centrifugation of the incubation mixture; supernate II was obtained after washing the pellet with incubation buffer; and supernate III was obtained by a second wash of the pellet with 5 mM deoxycholic acid. Lipolytic activities in the respective supernates were corrected for effects of the HDL_2 species on apparent lipolytic activity under our assay conditions. Results are expressed as percentage of enzyme activity released from the matrix and are the means of duplicate determinations which differed by less than 15%. Lipolytic activity of the various incubation mixtures which was recovered in the three supernates was 444.1 ± 43.5 nmol/h (mean ± SD).

released were higher in incubation mixtures containing A-I/A-II-HDL_2 (**Fig. 3**, $P < 0.001$, analysis of covariance, equality of slopes test). Furthermore, the velocity of fatty acid release was greater for A-I/A-II-HDL_2 at all substrate concentrations studied (**Fig. 4**, $P < 0.001$, analysis of covariance, equality of slopes test).

Lipolysis of surface phospholipids and core triglycerides by hepatic lipase results in reduction of particle mass and size. To relate differences in substrate properties of A-I- and A-I/A-II-HDL_2 to size changes, labeled A-I- and A-I/A-II-HDL_2 were incubated with hepatic lipase and the sizes of parent and product lipoproteins were estimated from autoradiograms of nondenaturing polyacrylamide gels. As shown in **Fig. 5**, migration distances of parent and control incubated HDL_2 subspecies were identical, but greater increases in mobility were noted in A-I/A-II-HDL_2 after incubation with hepatic lipase for 4 or 16 h. Stokes radii of A-I-HDL_2 estimated from standards were 11.1, 11.0, and 10.8 nm at the 0, 4 and 16 h time points. The respective values for A-I/A-II-HDL_2 were 11.2, 10.9, and 10.6 nm. In addition to the major band representing the respective HDL_2 subspecies, faint bands with an apparent molecular mass of less than 67 kDa are visible in this figure. These bands, which could have represented free apolipoproteins, did not dramatically increase during lipolysis of either HDL subspecies, suggesting that only minor, if any, apolipoprotein shedding occurred as a result of lipolysis.

TABLE 4. Hydrolysis of triglycerides and phosphatidylcholine in A-I-HDL_2 and A-I/A-II-HDL_2 by hepatic lipase

	Triglyceride Hydrolyzed[a]	Phosphatidylcholine Hydrolyzed[b]
	nmol/incubation	
A-I-HDL_2	4.65 ± 0.95	1.33 ± 0.34
A-I/A-II-HDL_2	7.38 ± 1.69[c]	3.92 ± 0.31[d]

[a]Results are means ± SD of four HDL preparations and show the decrease of triglycerides in nmol/incubation. Incubation mixtures contained 19 μg HDL-triglyceride in 250 μl. Incubation was for 4 h at 28°C.
[b]Results are means ± SD of three different preparations and show the decrease of phosphatidylcholine in nmol/incubation mixture. Incubation mixtures contained 100 μg HDL-phospholipid and 7 μg hepatic lipase in 250 μl. Incubation was for 4 h at 28°C.
[c]$P < 0.02$, Mann-Whitney test.
[d]$P < 0.05$, Mann-Whitney test.

To determine whether the distinctiveness in substrate properties of HDL_2 subspecies is modulated by their triglyceride content, the cores of postabsorptive HDL_2 subfractions were enriched with triglycerides in vitro through the neutral lipid transfer reaction. The changes in chemical composition induced are shown in **Table 5**. At the same substrate concentration of HDL-triglycerides, initial rates of fatty acid release were higher in incubation mixtures containing A-I/A-II-HDL_2, irrespective of the enrichment with triglycerides over the range studied (**Table 6**, $P < 0.05$, two-way analysis of variance). Thus, the difference in substrate properties was apparent over a threefold range in the TG/PL ratio. In contrast, TG transfer rates were not different for A-I- and A-I/A-II-HDL_2 subspecies with similar core composition.

As the major qualitative difference between postprandial HDL_2 subspecies was their apoA-II content (Table 1 and Fig. 1A and B), a role of apoA-II in enhancing sub-

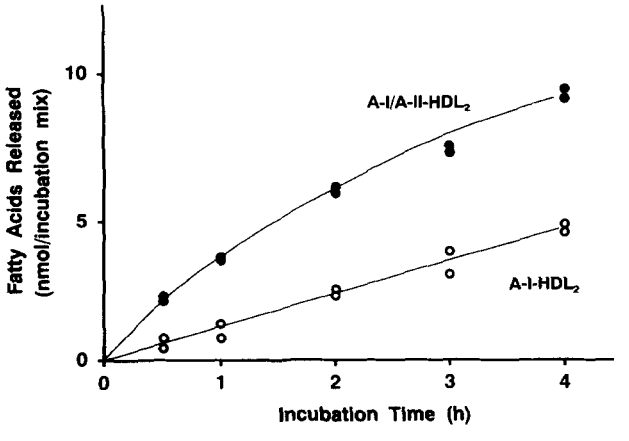

Fig. 3. Time course of fatty acid liberation from A-I- and A-I/A-II-HDL_2 by hepatic lipase. A-I- (○) and A-I/A-II-HDL_2 (●) (4 μg triglyceride) were incubated with 1.4 μg of hepatic lipase in 50 μl of 100 mM Tris-HCl, pH 8.0, containing 4% bovine serum albumin (w/v).

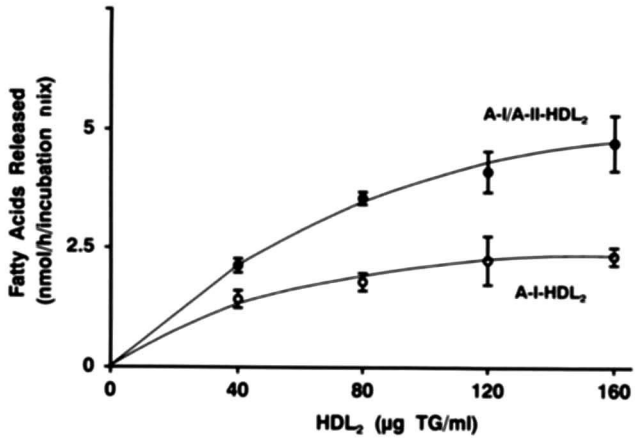

Fig. 4. Effect of substrate concentration on rates of fatty acid liberation from A-I- and A-I/A-II-HDL_2 by hepatic lipase. A-I- (○) and A-I/A-II-HDL_2 (●) were incubated at various concentrations for 1 h with 1.4 μg of hepatic lipase in 50 μl of 100 mM Tris-HCl, pH 8.0, containing 4% bovine serum albumin (w/v). Means ± SD of triplicate determinations are shown.

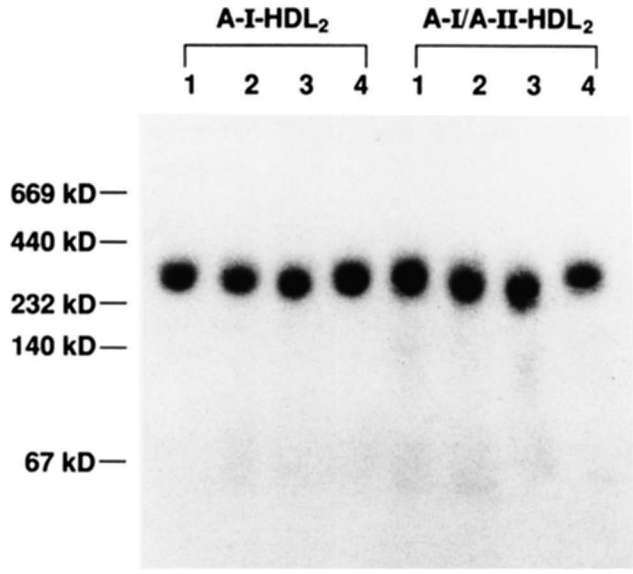

Fig. 5. Effect of lipolysis by hepatic lipase on size of substrate particle as determined by autoradiography of 5–20% polyacrylamide gels. ^{125}I-labeled A-I- and ^{125}I-labeled A-I/A-II-HDL_2 (4 μg triglyceride) were incubated with or without hepatic lipase. Lane 1, no incubation; lane 2, 4 h incubation with 3 μg of enzyme; lane 3, 16 h incubation with 3 μg of enzyme each added at 0 and 8 h of incubation; lane 4, 16 h incubation without enzyme.

strate properties was suggested. We therefore reconstituted A-I/A-II-HDL_2 from postprandial A-I-HDL_2 and apoA-II. The apoA-I/apoA-II dye uptake ratio was 4.8 in reconstituted particles as compared to 5.8 in postprandial A-II-containing HDL_2, and no major changes in size were observed upon reconstitution as judged by gradient gel electrophoresis (**Fig. 6**). In reconstituted particles, the protein content was increased to 45.6% compared to 39.3% and 40.1% in A-I- and A-I/A-II-HDL_2 isolated from this subject. Composition of lipids and main phospholipids was similar to that of the A-I-HDL_2 used for reconstitution (data not shown). Association of A-I-HDL_2 with apoA-II clearly enhanced its substrate properties as determined by mass measurements of triglycerides and fatty acids (Table 7). Similar results were obtained with a different preparation of postprandial A-I-HDL_2 reconstituted with apoA-II.

DISCUSSION

Our main conclusion is that the preferential conversion of apoA-II-containing HDL_2 into HDL_3 results from enhanced interaction of hepatic lipase with A-I/A-II HDL_2 and not from enhanced triglyceride transfer from TGRL

TABLE 5. Chemical composition of postabsorptive HDL_2 subspecies before and after enrichment with triglycerides in vitro

	Protein	PL	UC	CE	TG	CE + TG	TG/CE + TG	TG/PL
Postabsorptive								
A-I-HDL_2	41.4	29.5	3.4	21.7	4.1	25.8	0.159	0.139
A-I/A-II-HDL_2	46.6	26.5	2.6	19.6	4.7	24.3	0.193	0.177
Triglyceride-enriched (a)								
A-I-HDL_2	40.1	30.8	3.5	16.3	9.3	25.6	0.363	0.302
A-I/A-II-HDL_2	43.1	29.0	2.7	16.1	9.2	25.3	0.364	0.317
Triglyceride-enriched (b)								
A-I-HDL_2	38.3	30.0	1.2	16.9	13.6	30.5	0.446	0.453
A-I/A-II-HDL_2	41.3	30.2	1.0	16.7	10.8	27.5	0.393	0.358

Results are weight percentages and represent means of duplicate measurements. PL, phospholipids; UC, unesterified cholesterol; CE, cholesteryl ester; TG, triglycerides. Postabsorptive HDL_2 were enriched with triglycerides by incubating lipoprotein-deficient plasma with the HDL_2 and VLDL at a protein weight ratio of 20 and 5 in (a) and (b), respectively. The resulting triglyceride-enriched HDL_2 was isolated by zonal ultracentrifugation, and A-I-HDL_2 and A-I/A-II-HDL_2 were separated by immunoaffinity chromatography (see Methods).

TABLE 6. Release of fatty acids by hepatic lipase in postabsorptive triglyceride-enriched HDL_2 subspecies and triglyceride transfer between these subspecies and triglyceride-rich lipoproteins

HDL_2 Fraction	Fatty Acids Released	Triglyceride Transferred
	nmol/h/incubation	
Postabsorptive		
A-I-HDL_2	4.2	0.69
A-I/A-II-HDL_2	7.1	0.68
Triglyceride-enriched (a)		
A-I-HDL_2	2.8	0.74
A-I/A-II-HDL_2	6.0	0.78
Triglyceride-enriched (b)		
A-I-HDL_2	3.9	0.87
A-I/A-II-HDL_2	5.8	0.82

Postabsorptive HDL_2 was enriched with triglycerides by incubating lipoprotein-deficient plasma with HDL_2 and VLDL at a protein weight ratio of 20 or 5 in (a) and (b), respectively. Hepatic lipase activity was measured by incubating 4 μg of HDL_2 triglyceride and 3 μg of enzyme in 50 μl at 28°C for 60 min. For TG-transfer analysis, each HDL_2 subfraction (25 μg protein) was incubated at 37°C for 4 h with 100 μg triglyceride of [^{14}C]triolein-labeled triglyceride-rich lipoproteins and 5 mg protein of lipoprotein-deficient plasma in a volume of 700 μl. Results are means of duplicate determinations which differed by less than 15%.

to A-I/A-II-HDL_2 during the postalimentary phase. This conclusion is based on the following findings: *i*) Postprandial apoA-II-containing HDL_2 contained less triglycerides in their core when compared with postprandial HDL_2 devoid of apoA-II (Table 1). *ii*) The rate of triglyceride transfer from TGRL to A-I/A-II-HDL_2 never exceeded the rate of transfer to A-I-HDL_2 in in vitro experiments (Fig. 2). *iii*) A-I/A-II-HDL_2 were more effective in liberating hepatic lipase immobilized to HDL_3-Sepharose (Table 3). *iv*) Lipolysis of triglycerides and phospholipids in postprandial A-I/A-II-HDL_2 was significantly higher than in postprandial A-I-HDL_2 (Table 4); this difference was also observed in initial rates of fatty acid release (Fig. 3) and was apparent at all substrate concentrations tested (Fig. 4). Furthermore, differences in substrate properties were reflected in commensurate reductions of particle size (Fig. 5). *v*) Reconstitution of postprandial A-I-HDL_2 with apoA-II enhanced its substrate properties for hepatic lipase (Table 7).

Consistent with our data for postprandial HDL, A-I/A-II-HDL_2 contained less triglycerides than A-I-HDL_2 in the postabsorptive state (41). In vitro studies with reconstituted HDL suggest that an increased content of triglycerides and free cholesterol decreases the ability of HDL to accept cholesteryl esters transferred by cholesteryl ester transfer protein (CETP) (42). However, greater than twofold changes in the relative abundance of triglycerides and free cholesterol were required in these model lipoproteins to demonstrate the inhibition of transfer. Using a solid phase radioimmunoassay for measurement of CETP mass, Marcel et al. (43) found a significant correlation of CETP with apoA-I, apoE, HDL-cholesterol, and HDL-triglyceride while no correlation existed with apoA-II. Furthermore, CETP was present mostly in small HDL_3 particles, known to contain primarily apoA-I (20). These studies argue as well against a specific interaction between apoA-II and CETP.

While our results suggest a role of apoA-II in the interaction of HDL_2 species with hepatic lipase, apoA-II is not obligatory for hepatic lipase action as significant lipolysis of triglycerides was observed in A-I-HDL_2 albeit at a lesser rate. The enhanced liberation of hepatic lipase from HDL_3-Sepharose by A-I/A-II-HDL_2 suggests that apoA-II facilitates the association of the enzyme with its substrate. A role of apoA-II in the activation of hepatic lipase is also supported by in vivo data, as the concentration of A-I/A-II-HDL_2 was found to be increased in hepatic lipase deficiency, while the concentration of A-I/A-II-HDL_3 was

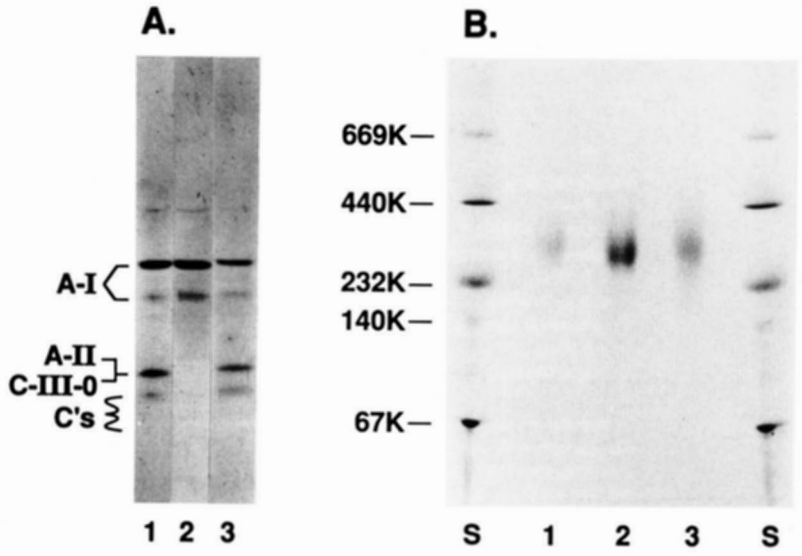

Fig. 6. Isoelectric focusing gels (A) and 4–30% polyacrylamide gradient gels (B) of postprandial A-I-HDL_2 reconstituted with apoA-II (lane 1), A-I-HDL_2 used for reconstitution (lane 2), and A-I/A-II-HDL_2 obtained from the same subject (lane 3). Molecular weight markers are shown in lanes S.

TABLE 7. Hepatic lipase-catalyzed hydrolysis of triglycerides in native postprandial HDL_2 subspecies and in A-I-HDL_2 reconstituted with apoA-II

HDL_2 Fraction	Decrease of Triglycerides	Fatty Acid Increase
	nmol/incubation	
A-I/A-II-HDL_2	5.5 ± 1.1^{a}	19.9 ± 4.1
A-I-HDL_2	$3.4 \pm 0.3^{a,b}$	15.0 ± 3.6^{c}
A-I-HDL_2 reconstituted with apoA-II	6.2 ± 1.2^{b}	22.3 ± 1.2^{c}

Average triglyceride content of incubation mixtures was 17, 16, and 15 nmol triglycerides for assays containing A-I/A-II-, A-I-, and A-I-HDL_2 reconstituted with apoA-II, respectively. Incubation was at 28°C for 4 h. Results are means ± SD of triplicate determinations.

[a,b,c]Pairs differing from each other ($P < 0.05$, analysis of variance).

decreased (44). Previous in vitro studies on the effect of apoA-II on hepatic lipase activity are contradictory in that both specific stimulation (45) and nonspecific inhibition (46–48) of enzyme activity by apoA-II have been reported. Some studies used artificial substrates to determine effects of apoA-II on hepatic lipase activity (45, 48). Shinomiya et al. (46) used tri[^{3}H]oleoylglycerol-labeled HDL_2 that was reconstituted with various amounts of apoA-II, to show an inhibitory effect of apoA-II on lipolysis of triglycerides by hepatic lipase. It is possible that lack of discrimination in apoA-II enrichment between A-I- and A-I/A-II-HDL has confounded these results. Inhibition of hepatic lipase activity may become evident when the substrate surface is saturated with apolipoproteins (46, 48). Such an inhibition is nonspecific and can also be demonstrated with synthetic lipid binding model peptides (33). Saturation of the substrate surface with apolipoproteins changes the interfacial properties by reducing the availability of substrate. Clearly, additional experiments in model lipoproteins are necessary to define the stoichiometry of apo-A-II to lipids and other apoproteins required for binding and/or activation of hepatic lipase.

A number of studies indicate that A-I- and A-I/A-II-HDL are metabolically distinct and fulfill different functions. Clinical studies showed that drug treatment alters the proportion of A-I-HDL and A-I/A-II-HDL (47). The well-established male–female difference in plasma levels of HDL_2 reflects increased concentrations of apoA-I-HDL in HDL_2 of females (49). A cholesterol-rich diet also alters the partition of A-I and A-I/A-II particles between HDL_2 and HDL_3 (H. Mowri and W. Patsch, unpublished observation). Particles containing A-I may be the physiologic acceptor of cellular cholesterol (50). Our studies further specify functional differences between A-I- and A-I/A-II-HDL. The preferential conversion of apoA-II-containing HDL_2 to HDL_3 during postprandial lipemia may provide the volume necessary to accommodate cholesteryl esters synthesized de novo by LCAT. Conversely, A-I/A-II-HDL_3 may be converted into larger HDL_2-like particles through assimilation of surface components liberated during lipolysis of TGRL. ApoA-II-containing HDL could thus represent a shuttle in going from HDL_2 to HDL_3 via lipid exchange with TGRL and lipolysis by hepatic lipase and from HDL_3 to HDL_2 through acquisition of surface components.

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