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Hepatic lipase treatment of chylomicron remnants increases exposure of apolipoprotein E

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Abstract The consequences of hepatic lipase treatment of chylomicron remnants were studied. Rats were fed corn oil to induce production and secretion of chylomicrons and were then injected with polyclonal antiserum raised against hepatic lipase to specifically and quantitatively inhibit hepatic lipase activity in vivo. A fraction enriched in chylomicron remnants was isolated from rat plasma by a brief centrifugation step that preferentially isolates triglyceride-rich apolipoprotein (apo) B-48-containing lipoproteins. The chylomicron remnants were then treated with hepatic lipase in vitro, or incubated under identical conditions in the absence of enzyme (control incubations). Hepatic lipase-treated and control chylomicron remnants were isolated by a second brief centrifugation step using discontinuous salt gradients. Control lipoproteins were collected from one discrete band at $d < 1.02$ g/ml. Hepatic lipase-treated chylomicron remnants formed two discrete bands and were collected at two densities: $d < 1.02$ g/ml and $1.02 < d < 1.04$ g/ml. The buoyant ($d < 1.02$ g/ml) subfraction of hepatic lipase-treated chylomicron remnants was depleted of 62% of the total phospholipid when compared to control $d < 1.02$ g/ml lipoproteins. The dense ($1.02 < d < 1.04$ g/ml) subfraction of hepatic lipase-treated chylomicron remnants was depleted of 65% of particle phospholipid content and 90% of particle triglyceride content when compared to control $d < 1.02$ g/ml lipoproteins. The dense ($1.02 < d < 1.04$ g/ml) subfraction of hepatic lipase-treated chylomicron remnants showed 5- to 7-fold greater immunoreactivity of apoE when compared to control lipoproteins in competitive displacement immunoassays. These data suggest that extensive hydrolysis of chylomicron remnant phospholipid and triglyceride leads to the formation of a dense remnant particle that contains highly exposed apoE. This increased exposure of apoE may be the key to the previously observed increased degradation of chylomicron remnants treated with hepatic lipase because more exposed apoE may bind better to cell surface lipoprotein receptors. Furthermore, the data imply that hepatic lipase cleaves chylomicron remnant phospholipid and triglyceride in a sequential fashion; hydrolytic intermediates depleted only of phospholipid precede the formation of a smaller dense remnant particle depleted of phospholipid and triglyceride.—**Brasaemle, D. L., K. Cornely-Moss, and A. Bensadoun.** Hepatic lipase treatment of chylomicron remnants increases exposure of apolipoprotein E. *J. Lipid Res.* 1993. **34:** 455–465.

Supplementary key words lipolysis • triglyceride hydrolysis • phospholipid hydrolysis • chylomicron remnant subfractions • immunoreactivity • competitive displacement immunoassay • ELISA

Dietary lipids are packaged into chylomicrons in intestinal epithelial cells. Nascent chylomicrons are large (> 1000 Å) triglyceride-rich lipoproteins containing apolipoprotein A-I (apoA-I), apoA-IV, and apoB-48 (reviewed in refs. 1 and 2). Chylomicrons are secreted into the lymph and enter the general circulation after passage through the thoracic duct. Circulating chylomicrons acquire apoC-II which acts as a co-factor for lipoprotein lipase. Lipoprotein lipase associated with endothelial cell surfaces of capillary beds hydrolyzes a large portion of triglyceride in the lipoprotein core. This lipolysis leads to remodeling of the lipoprotein characterized by loss of both core and excess surface components. The resulting chylomicron remnant acquires apoE from circulating lipoproteins.

Chylomicron remnants are removed from the circulation primarily by the liver, and by bone marrow and spleen in some species (3, 4). Clearance of chylomicron remnants is mediated by interaction of apoE on the lipoprotein surface with specific cell surface receptors (5–9).

Hepatic lipase appears to play a role in the metabolism of triglyceride-rich lipoproteins. Several examples of heritable hepatic lipase deficiency in humans have been described (10–17). Common features among these subjects include elevated fasting serum triglyceride levels and the accumulation of triglyceride-enriched remnant particles isolated by centrifugation from the density ranges of intermediate density lipoproteins and low density lipoproteins.

Abbreviations: VLDL, very low density lipoprotein.

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Additionally, hepatic lipase catalytic activity has been blocked by injection of specific polyclonal antisera into both fasted and lipid-fed experimental animals to yield similar accumulations of remnant particles in plasma (18–25). These observations suggest that hepatic lipase is necessary to clear triglyceride-rich lipoprotein remnants from the circulation.

The probable mechanism of hepatic lipase-assisted metabolism of triglyceride-rich lipoproteins involves hydrolysis of lipids in the circulating lipoproteins by the enzyme. In vitro, hepatic lipase acts as a phospholipase and a hydrolase of neutral glycerides in a number of model systems including isolated lipoproteins (reviewed in refs. 26 and 27). Hepatic lipase is synthesized in hepatocytes and, after secretion, binds to endothelial cell surfaces in liver, adrenal gland, and ovary (reviewed in ref. 28). From these locations, it comes into contact with circulating lipoproteins; ensuing hydrolysis of phospholipids and triglycerides may increase the exposure of apolipoproteins essential for receptor-mediated uptake of the lipoproteins.

The purpose of this study was to examine the consequences of hepatic lipase-catalyzed hydrolysis of chylomicron remnant lipids and to test the hypothesis that this activity of hepatic lipase increases the immunoreactivity of apoE on chylomicron remnants. We collected triglyceride-rich lipoproteins from corn oil-fed rats injected with polyclonal antibodies raised against hepatic lipase to block enzyme activity. The collected lipoproteins were thus subjected to lipolysis by lipoprotein lipase in vivo, but not to hydrolysis by hepatic lipase. A portion of the collected lipoproteins was treated with hepatic lipase in vitro; the remaining lipoproteins were incubated under identical conditions in the absence of enzyme. The lipid and apolipoprotein contents of hepatic lipase-treated and untreated (control) lipoproteins were compared. Hepatic lipase-treated chylomicron remnants were found to contain more immunoreactive apoE relative to total apoE than untreated lipoproteins; hence, hepatic lipase treatment increased the exposure of apoE on chylomicron remnants.

MATERIALS AND METHODS

Preparation of antiserum against rat apoE and anti-rat apoE immunoglobulin preparation

ApoE was purified from rat d<1.04 g/ml lipoproteins following methods adapted from previously published procedures (29, 30). Antiserum to purified rat apoE was raised in a goat. Goat anti-rat apoE immunoglobulins were purified from antisera by affinity chromatography using an apoE-Sepharose® 4B column prepared by coupling apoE to CNBr-activated Sepharose® 4B (Pharmacia) following the manufacturer's procedures.

ELISA conditions for the determination of total apoE concentration of lipoproteins

ApoE concentrations of lipoproteins were determined by a sandwich ELISA using affinity-purified polyclonal antibodies raised against rat apoE for both the capturing antibody and the horseradish peroxidase-tagged (31) antibody. A sample buffer of 0.5 M NaCl, 1% bovine serum albumin (BSA), 0.05% Tween 20, 10 mM sodium phosphate, pH 7.4, was used for sample and standard dilutions, and horseradish peroxidase–antibody conjugate addition. This assay gave a linear increase in absorbance at 490 nm with increasing concentrations of highly purified apoE to 1 ng. The affinity-purified polyclonal antibody used in this assay showed no cross reactivity to apoB; 100 ng of apoB-48 gave no detectable absorbance at 490 nm.

To maximally expose epitopes of apoE on the surface of triglyceride-rich (d<1.02 g/ml) lipoproteins, lipoprotein samples (and purified apoE standards) were incubated for 1 h at 37°C in 10 mM sodium dodecyl sulfate (SDS) before further dilution and addition to the assay plates. As SDS interferes with the detection of apoE, the final concentrations of SDS in all diluted samples and standards were adjusted to be equal and less than or equal to 0.5 mM.

ELISA conditions for the determination of exposed apoE on lipoprotein fractions

Exposure of apoE on the surfaces of lipoproteins was assayed using a competitive displacement immunoassay. Alternate rows of 96-well ELISA plates were coated with purified rat apoE; alternate rows were coated with buffer containing no apoE, to serve as controls for nonspecific binding. Serial dilutions of lipoproteins were made in 3% BSA, phosphate-buffered saline (PBS), pH 7.4. The goat anti-rat apoE immunoglobulin–horseradish peroxidase conjugate was diluted in 3% BSA, PBS, pH 7.4, and was added to the serial dilutions of lipoproteins. The lipoprotein solutions were incubated briefly on ice and then added to both apoE-coated and control (uncoated) wells of the ELISA plates. Soluble lipoprotein/antibody complexes were removed by washing, and an *o*-phenylenediamine substrate solution was used to quantitate immunoglobulin–horseradish peroxidase complexed with immobilized apoE.

Data were expressed as the ratio of the absorbances at 490 nm of sample wells (B) to the average absorbance at 490 nm of wells incubated with dilute goat anti-rat apoE immunoglobulin–horseradish peroxidase conjugate without competing lipoprotein (B_O). The B/Bo ratio was then plotted as a function of the total apoE content of lipoprotein samples determined by ELISA of SDS-treated lipoproteins. Binding curves were analyzed simultaneously using the ALLFIT program (1992 version developed by DeLean, A., P. J. Munson, V. Guardabasso, and D. Rodbard, National Institutes of Health, Bethesda,

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MD) on an IBM-PC; this program fits families of sigmoidal curves using the four-parameter logistic equation (32). Slopes of fitted curves and midpoint values of binding isotherms (ED_{50}) were determined.

Preparation of antiserum against rat apoB-100 and anti-rat apoB-48 immunoglobulin preparation

ApoB-100 and apoB-48 were isolated from rat very low density lipoproteins (VLDL) using the procedures of Zilversmit and Shea (33). Purified rat apoB-100 was used to immunize a goat. Goat anti-rat apoB-48 immunoglobulin was purified from antisera raised against apoB-100 using an apoB-48–Affi-gel 10 column prepared by coupling apoB-48 to Affi-gel 10 (Bio-Rad) following the manufacturer's instructions.

ELISA conditions for the determination of total apoB-48 concentration of lipoproteins

ApoB concentrations of lipoproteins were determined by a sandwich ELISA using affinity-purified apoB-48-specific polyclonal antibodies for both the capturing antibody and the horseradish peroxidase-tagged (31) antibody. A sample buffer of 1% BSA, 0.025% Tween 20, PBS, pH 7.4, was used for sample and standard dilutions, and horseradish peroxidase–antibody conjugate addition. This assay gave a linear increase in absorbance at 490 nm with increasing concentrations of highly purified apoB-48 or apoB-100 to 20 ng; the slope of the standard curve for apoB-100 was approximately 50% of the slope of the standard curve for apoB-48. The affinity-purified polyclonal antibody used in this assay showed no cross-reactivity to apoE; 100 ng of apoE gave no detectable absorbance at 490 nm.

To maximally expose epitopes of apoB on the surface of triglyceride-rich ($d < 1.02$ g/ml) lipoproteins, lipoprotein samples (and purified apoB-48 standards) were incubated for 1 h at 37°C in 5% Triton X-100 before further dilution and addition to the assay plates. Incubation of lipoproteins with 5% Triton X-100 was more effective at increasing exposure of epitopes on apoB than incubation of lipoproteins with 10 mM SDS, 1% Triton X-100, rat hepatic lipase, or bee venom phospholipase A_2. Concentrations of Triton X-100 ranging from 0.1% to 5% interfered minimally with the ability to quantitate apoB-48 by ELISA; nevertheless, the Triton X-100 concentrations of all samples and standards were adjusted to be equal and less than or equal to 0.1%.

Preparation of antiserum against rat hepatic lipase

Hepatic lipase was purified from rat liver perfusate and used to immunize a goat as described previously (34). The titers of two antisera used in these studies were determined by the method described previously by Kompiang, Bensadoun, and Yang (35). One ml of antiserum inhibited hepatic lipase activity at a level equivalent to 16,900 or 29,400 μmol fatty acid released per h, as determined using a tri[^{3}H]oleoylglycerol substrate.

Purification of rat hepatic lipase for lipolysis of lipoproteins in vitro

Hepatic lipase was purified from rat liver perfusate by new methods that eliminate low levels of contaminant apoE and apoB (Bensadoun, A., D. L. Brasaemle, J. Hsu, and L. B. Hughes, unpublished results). Briefly, perfusate was collected from 60 adult male rat livers and concentrated using PM30 Diaflo Ultrafiltration Membranes (Amicon Corporation, Danvers, MA) in an Amicon concentration cell at 4°C. All subsequent steps were carried out at 4°C.

The concentrated liver perfusate was dialyzed against 20 mM Tris, pH 7.2, and loaded onto a 1.5 cm × 11 cm Q-Sepharose® (Pharmacia) column equilibrated with 2.5 mM n-decylsucrose (CALBIOCHEM), 20 mM Tris, pH 7.2. The column was washed extensively with 20 mM Tris, pH 7.2, and 0.2% Triton X-100, 20 mM Tris, pH 7.2. Bound hepatic lipase was eluted with a 400-ml gradient of 0–1.0 M NaCl in 2.5 mM n-decylsucrose, 20 mM Tris, pH 7.2. Column fractions containing enzyme activity were pooled, the buffer was adjusted to contain 20% glycerol, and the pooled fractions were loaded onto a 2.6 cm × 31 cm heparin Sepharose® (Pharmacia) column equilibrated with 20 mM Tris, pH 7.2. The column was washed extensively with 20% glycerol, 20 mM Tris, pH 7.2, and 0.1% Triton N101, 20% glycerol, 20 mM Tris, pH 7.2. Bound hepatic lipase was eluted with a 400-ml gradient of 0–1.2 M NaCl in 2.5 mM n-decylsucrose, 20 mM Tris, pH 7.2. Column fractions containing hepatic lipase catalytic activity were pooled and dialyzed against 2.5 mM n-decylsucrose, 20% glycerol, 10 mM sodium phosphate, pH 7.2

Pooled and dialyzed fractions containing hepatic lipase activity were loaded onto a 1.6 cm × 7 cm S-Sepharose® (Pharmacia) column equilibrated with 20% glycerol, 10 mM sodium phosphate, pH 7.2. The column was washed extensively with the column equilibration buffer, and then eluted with a 300-ml gradient of 0–1.0 M NaCl in 2.5 mM n-decylsucrose, 20% glycerol, 10 mM sodium phosphate, pH 7.2. Hepatic lipase purified by these methods showed a major band at 55,000 daltons and a faint band at 59,000 daltons in Coomassie blue R-250-stained SDS-polyacrylamide gels, and showed no contamination by apoE or apoB as determined by ELISA. The specific activity of hepatic lipase was 6500 μmol fatty acid released per h per mg protein. Fractions from S-Sepharose® containing hepatic lipase catalytic activity were pooled and concentrated for use in experiments by elution over a small (1 g) heparin Sepharose® column equilibrated with 0.1% BSA, 30% glycerol, 10 mM sodium phosphate, pH 7.4. The column was washed with the column equilibration buffer, and then eluted with 1% BSA, 1.0 M NaCl,

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30% glycerol, 10 mM sodium phosphate, pH 7.4. Concentrated hepatic lipase was stored at −80°C before use.

Collection of chylomicron remnants

Male Long-Evans rats (Harlan Sprague-Dawley), 270–370 g, were fed commercial rat chow ad libitum throughout the study. To increase absorbed lipid, rats were fed 2 ml of corn oil emulsified by sonication with 5% sodium taurodeoxycholate in a 1:1 (v/v) ratio by gastric intubation. Immediately after corn oil gavage, each rat was anesthetized with ether, and injected via the jugular vein with 1 ml goat antiserum raised against rat hepatic lipase. The antiserum used in this study had a titer 7- to 13-fold higher than that previously demonstrated to specifically and completely inhibit the activity of heparin-releasable hepatic lipase when injected into rats (22). After 3 h, the rats were injected with an additional 1 ml goat anti-rat hepatic lipase serum via the jugular vein. Six h after the corn oil gavage and initial injection of antiserum, the animals were exsanguinated by cardiac puncture.

Triglyceride-rich, apoB-48-containing lipoproteins were isolated from plasma by centrifugation. The density of the collected plasma was adjusted to d = 1.04 g/ml with a salt solution containing NaCl and NaBr; centrifuge tubes containing this plasma were overlaid with d 1.02 g/ml salt solution and centrifuged for 25 min at 202,000 *g* (average) in a Beckman SW 40 rotor at 14°C. Lipoproteins of d<1.02 g/ml were collected by slicing the tubes and were stored at 4°C overnight.

Treatment of triglyceride-rich lipoproteins with purified rat hepatic lipase in vitro and further isolation of remnant lipoproteins

Triglyceride-rich d<1.02 g/ml lipoproteins were treated with purified rat hepatic lipase in vitro for 1 h at 35°C. Hepatic lipase was added to the lipoprotein solutions at a level of 19–39 units of enzyme activity (μequivalents fatty acid released from triglyceride per h) per mg lipoprotein triglyceride. The incubation mixture contained lipoprotein and hepatic lipase in 10 mg/ml BSA, 0.5 M NaCl, 10 mM $CaCl_2$, 0.2 M Tris, pH 8.6. A control incubation contained all components except hepatic lipase.

Control and hepatic lipase-treated remnants were isolated by ultracentrifugation using discontinuous salt gradients. The density of each incubation mixture was adjusted to 1.08 g/ml with a salt solution containing NaCl and NaBr, and the lipoprotein solutions were overlaid with d 1.04 g/ml, and then d 1.02 g/ml salt solutions. The gradients were centrifuged for 75 min at 202,000 *g* in a Beckman SW40 rotor at 14°C. Lipoproteins were harvested by slicing the tubes.

Analysis of chylomicron remnant composition

The compositions of control and hepatic lipase-treated chylomicron remnants were determined. Apolipoprotein concentrations were determined by ELISAs within 8 h of lipoprotein isolation. Total phospholipid content was calculated from the total phosphorus content (36) of solvent lipid extracts (37). Total and partial glyceride analysis was conducted by thin-layer chromatography of lipid extracts (37) followed by quantitation of glycerol content (38) of mono-, di-, and triglyceride extracts from thin-layer chromatography silica gel scrapings. Total cholesterol content (cholesterol and cholesteryl esters) was determined using an enzymatic cholesterol assay kit from Boehringer-Mannheim (Kit #1236691). Total protein content was determined by the method of Lowry et al. (39), using a BSA standard. SDS-PAGE of lipoprotein fractions and purified apolipoproteins was conducted using the methods of Laemmli (40). Statistical significance of composition differences between control and hepatic lipase-treated chylomicron remnants were evaluated by Student's paired *t*-test.

RESULTS

Isolation of apoB-48-containing lipoproteins from corn oil-fed rats

Triglyceride-rich lipoproteins were collected from corn oil-fed rats with functionally inactive hepatic lipase. These lipoproteins were, however, exposed to lipoprotein lipase in vivo. The conditions used to collect triglyceride-rich lipoproteins from corn oil-fed rats were designed to maximize the recovery of apoB-48-containing lipoproteins with minimal contamination of the preparation with apoB-100-containing lipoproteins and minimal loss of apoE. When very brief centrifugations of 25 min with a SW40 rotor were used, d<1.02 g/ml lipoprotein fractions were highly enriched in apoB-48 (see **Fig. 1,** lane 2). Levels of apoB-100 determined by densitometric scanning of Coomassie Blue R-250-stained SDS-PAGE gels (33) typically totaled less than 5% of the levels of apoB-48 (data not shown). Previous studies have documented the secretion of apoB-48-containing lipoproteins by rat liver as well as intestine (41–45). Although we cannot rule out the presence of lipoproteins originating from liver in the collected d<1.02 g/ml fraction, the feeding of a bolus of corn oil to the rats should ensure an abundant supply of intestinally derived chylomicron remnants in circulation.

Changes in chylomicron remnant density profiles after hepatic lipase treatment

ApoB-48-containing d<1.02 g/ml lipoproteins were treated with hepatic lipase in vitro. The lipolysis reaction was followed by isolation of the remnant products by ultracentrifugation of salt gradients. Lipoproteins from control incubations (no hepatic lipase) were collected in the same density range (d<1.02 g/ml) as the original triglyceride-rich lipoprotein. In four experiments,

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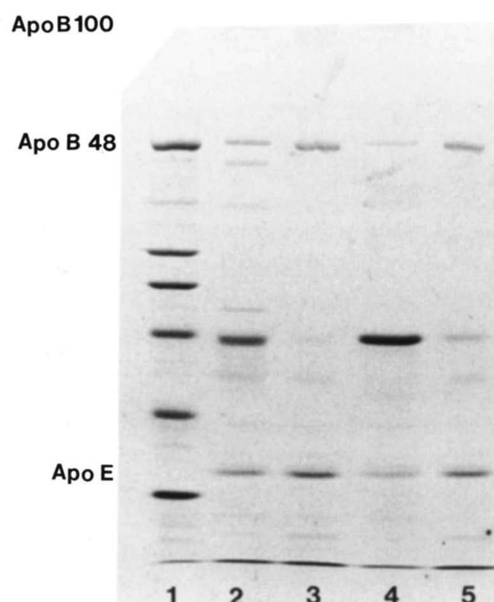

Fig. 1. Coomassie Blue R250-stained SDS-PAGE gel of lipoprotein fractions from density gradients. Lane 1 contains molecular mass standards myosin (205 kD), β-galactosidase (116 kD), phosphorylase B (97.4 kD), bovine serum albumin (66 kD), egg albumin (45 kD), and carbonic anhydrase (29 kD). Lane 2 contains d<1.02 g/ml lipoproteins from the initial isolation of chylomicron remnants from plasma. Lanes 3–5 contain lipoproteins from various density fractions obtained from the centrifugal isolation of chylomicron remnants after hepatic lipase treatment. Lane 3 contains d<1.02 g/ml control lipoproteins from incubations in the absence of hepatic lipase. Lane 4 contains 1.04<d<1.02 g/ml dense remnants from hepatic lipase incubations. Lane 5 contains d<1.02 g/ml lipoproteins from hepatic lipase incubations. Lanes 2–5 contain equal amounts of total protein.

92–98% of the apoB-48 recovered from control incubation gradients was collected in this density range (**Table 1**). The 1.02<d<1.04 g/ml fraction of the control lipoprotein gradients showed a very low concentration of apoB-48. Comparison of the protein composition of d<1.02 g/ml lipoproteins from the control incubation (Fig. 1, lane 3) to that of the original d<1.02 g/ml lipoproteins (Fig. 1, lane 2) indicated a decrease in albumin content and an enrichment of the relative apolipoprotein content of the lipoprotein fraction following the second centrifugation.

Dense chylomicron remnants were formed during the treatment of triglyceride-rich lipoproteins with hepatic lipase. The majority of chylomicron remnants from hepatic lipase incubations were collected from two density ranges of the salt gradients: d<1.02 g/ml and 1.02<d<1.04 g/ml. In four experiments, 56–73% of the apoB-48 recovered from the hepatic lipase treatment gradients was collected from the d<1.02 g/ml density range, 21–32% from the 1.02<d<1.04 g/ml range, and 4–12% from the d>1.04 g/ml range (Table 1). Comparison of the protein composition of the hepatic lipase treatment d<1.02 g/ml fraction (Fig. 1, lane 5) to the control d<1.02 g/ml fraction (Fig. 1, lane 3) showed similar apolipoprotein content. The protein composition of the 1.02<d<1.04 g/ml fraction, "dense remnants," (Fig. 1, lane 4) indicates an enrichment of albumin relative to apolipoproteins. The increased albumin in the dense remnant fraction originated from BSA added to the lipolysis mixture. The addition of albumin during lipolysis is necessary to remove monoacylglycerides, lysophosphatidylcholine, and free fatty acid from the hydrolyzed lipoproteins (46, 47). Enrichment of albumin in the dense remnant fraction may result from increased affinity of the dense chylomicron remnants for albumin or from decreased washing effects for lipoprotein collected lower in the gradient. As the various lipoprotein fractions contained a variable content of contaminating albumin, comparisons of lipid content of these fractions were made on a per particle basis using apoB-48 content as an indicator of particle number.

TABLE 1. Recovery of apolipoproteins E and B-48 in salt density gradient fractions

	Lipoprotein Density Fractions		
Sample	d<1.02 g/ml	1.02<d<1.04 g/ml	d≥1.04 g/ml
		% of apoE recovered	
Control	66 ± 11	2[a]	32 ± 10
Hepatic lipase-treated	56 ± 3[b]	31 ± 5	14 ± 5[c]
		% of apoB-48 recovered	
Control	95 ± 3	1[a]	4 ± 4
Hepatic lipase-treated	66 ± 7[d]	26 ± 5	8 ± 4[e]

Data are means ± standard deviations for the average of duplicate ELISA analyses from four experiments.
[a]Average of determinations from two experiments.
[b]Differences between apoE recovery data of control d<1.02 g/ml fractions and hepatic lipase-treated d<1.02 g/ml fractions are significant at a level of $P < 0.10$ by Student's paired *t*-test.
[c]ApoE recovery data: control d≥1.04 g/ml fractions vs. hepatic lipase-treated d≥1.04 g/ml fractions; $P < 0.05$.
[d]ApoB-48 recovery data: control d<1.02 g/ml fractions vs. hepatic lipase-treated d<1.02 g/ml fractions; $P < 0.005$.
[e]ApoB-48 recovery data; control d≥1.04 g/ml fractions vs. hepatic lipase-treated d≥1.04 g/ml fractions; $P < 0.005$.

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Changes in chylomicron remnant lipid composition after hepatic lipase treatment

Hepatic lipase hydrolyzes both surface phospholipid and core triglyceride of chylomicron remnants. Hepatic lipase-treated chylomicron remnants isolated at d<1.02 g/ml showed depletion of 62% of total phospholipid relative to apoB-48 content when compared to control (d<1.02 g/ml) lipoproteins; triglyceride content of this fraction was similar to control lipoproteins (**Table 2**). Dense hepatic lipase-treated remnants (1.02<d<1.04 g/ml) showed depletion of 65% of total phospholipid and 90% of triglyceride when compared to control (d<1.02 g/ml) lipoproteins. Therefore, the shift of a portion of hepatic lipase-treated chylomicron remnants to the higher density range between 1.02 g/ml and 1.04 g/ml was due to the large decrease in particle triglyceride content in addition to depletion of phospholipid. Additionally, dense hepatic lipase-treated chylomicron remnants showed an increase in monoglyceride content relative to control lipoproteins. No significant differences in total cholesterol content per particle were observed.

Measurements of lipid composition relative to apoB showed large variability between experiments. A potential source of variability was a wide range of particle size of the initial d<1.02 g/ml lipoprotein. Triglyceride content of control lipoproteins covered a range of 80 to 180 μg triglyceride/μg apoB-48; thus, the starting chylomicron remnant core size varied considerably. The degree of hepatic lipase-catalyzed triglyceride hydrolysis of the starting d<1.02 g/ml lipoprotein fraction also varied between experiments. For four experiments, 21–32% of total apoB-48 (hence, 21–32% of the particles) was recovered in the dense remnant (1.02<d<1.04 g/ml) subfraction of hepatic lipase-treated lipoprotein; the triglyceride content of this dense subfraction varied from 6 to 23 μg triglyceride/μg apoB-48. Despite experimental variability in lipid composition measurements, the pattern for these parameters was the same and significant differences were observed for all four experiments.

Changes in apoE exposure and content with hepatic lipase treatment of chylomicron remnants

The exposure of apoE on chylomicron remnants was studied using polyclonal antibodies raised against rat apoE in competitive displacement immunoassays. Immunoreactivity of apoE on the hepatic lipase-treated dense subfraction of chylomicron remnants (1.02<d<1.04 g/ml) was greater than that of control (d<1.02 g/ml) lipoproteins (**Fig. 2** and **Table 3**). The midpoints of the binding isotherms (ED_{50}s) were used to compare the relative exposure of apoE in the two chylomicron remnant subfractions (Table 3); apoE on hepatic lipase-treated dense remnants (1.02<d<1.04 g/ml) was 5- to 7-fold more immunoreactive than apoE on control lipoproteins. Additionally, the slopes of binding isotherms from hepatic lipase-treated dense remnants (1.02<d<1.04 g/ml) were steeper than the slopes of the control (d<1.02 g/ml) lipoprotein binding curves (Table 3), implying increased binding affinity of polyclonal antibodies for apoE on hepatic lipase-treated chylomicron remnants. These data suggest that hepatic lipase treatment increases the number of exposed epitopes on apoE for polyclonal antibody binding and that the consequent increased binding occurs with higher affinity.

Similar competitive displacement immunoassays were used to compare the relative exposure of apoE on d<1.02 g/ml hepatic lipase-treated chylomicron remnants to control (d<1.02 g/ml) lipoproteins. In three experiments, the ratios of ED_{50} values of binding isotherms of control over hepatic lipase-treated d<1.02 g/ml lipoproteins were 0.9, 3.6, and 3.0. Student's paired *t*-test analysis of ED_{50} values and slopes indicated that differences between the two

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TABLE 2. Lipoprotein lipid composition

Lipid Component	Control d<1.02 g/ml Fraction	Hepatic Lipase Treatment d<1.02 g/ml Fraction	Hepatic Lipase Treatment Dense Remnants 1.02<d<1.04 g/ml
		μg lipid/μg apoB-48	
Triglyceride	129 ± 40	115 ± 26	13.1 ± 7.2[a,b]
Diglyceride	13.0 ± 6.3	9.72 ± 5.12	5.09 ± 1.84
Monoglyceride	4.28 ± 2.94	8.65 ± 5.26	7.12 ± 4.81[c]
Total cholesterol	5.81 ± 1.85	5.99 ± 1.61	5.90 ± 2.14
Phospholipid	18.78 ± 4.73	7.10 ± 2.40[d]	6.56 ± 3.64[c]

Data are the means ± standard deviation for duplicate lipid analyses of four separate experiments.
[a] Differences between control d<1.02 g/ml fractions and hepatic lipase treatment dense remnants (1.02<d<1.04 g/ml) are significant at a level of $P < 0.01$ by Student's paired *t*-test.
[b] Hepatic lipase treatment d<1.02 g/ml fractions vs. hepatic lipase treatment dense remnants; $P < 0.01$.
[c] Control d<1.02 g/ml fractions vs. hepatic lipase treatment dense remnants; $P < 0.05$.
[d] Control d<1.02 g/ml fractions vs. hepatic lipase treatment d<1.02 g/ml fractions; $P < 0.05$.

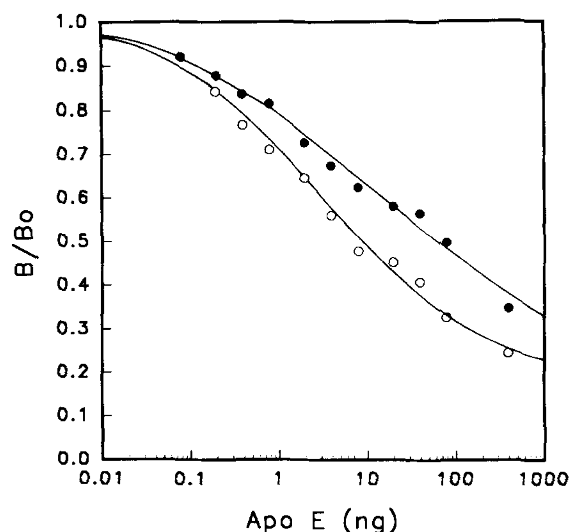

Fig. 2. Competition by control (closed symbols) and hepatic lipase-treated (open symbols) chylomicron remnants with immobilized apoE for binding to soluble polyclonal goat anti-rat hepatic lipase IgG conjugated to horseradish peroxidase. Lipoprotein concentration is expressed as total apoE mass determined by ELISA of SDS-treated lipoproteins. B/Bo values represent absorbance of ELISA sample wells containing lipoprotein/absorbance of wells containing only immobilized apoE. Data shown are averages of duplicate values from one representative experiment out of four.

lipoprotein fractions were not statistically significant (P = 0.19); hence, it is unclear whether the hydrolysis of phospholipids observed in hepatic lipase-treated d < 1.02 g/ml lipoproteins leads to increased exposure of apoE.

ApoE recovery data from lipoprotein fractions isolated by density gradients suggest that a portion of apoE is loosely associated with chylomicron remnants. Approximately 30% of apoE was isolated in d ≥ 1.04 g/ml fractions of control lipoproteins (Table 1); apoB content of the same fractions was very low (4% of recovered apoB). These data imply that apoE recovered from d ≥ 1.04 g/ml fractions of control samples is not associated with lipoproteins. In agreement with our findings, other researchers have observed the dissociation of apoE from lipoproteins during centrifugation (48, 49). Less apoE was recovered in d ≥ 1.04 g/ml fractions from density gradients of hepatic lipase-treated lipoproteins than from the same fractions of control lipoproteins; greater apoB content was measured in these dense fractions from hepatic lipase-treated lipoproteins (compared to control lipoproteins). These data suggest that at least some of the apoE was associated with lipoproteins in the d ≥ 1.04 g/ml fractions of hepatic lipase-treated chylomicron remnants. Additional apoE and apoB were found at an intermediate density (1.02 < d < 1.04 g/ml) in the dense chylomicron remnants fraction of hepatic lipase-treated lipoproteins.

Competitive displacement immunoassays were used to determine whether apoE on the original d < 1.02 g/ml chylomicron remnants isolated after a single centrifugation step was more immunoreactive than apoE remaining on control d < 1.02 g/ml lipoproteins after the second centrifugation step. Immunoreactivity of apoE on control d < 1.02 g/ml chylomicron remnants after a second centrifugation was equal to that of the original d < 1.02 g/ml lipoproteins (**Fig. 3**). This observation demonstrates that loss of 30% of the total apoE from the initial chylomicron remnant does not change the immunoreactivity of the remaining apoE.

Hepatic lipase-treated chylomicron remnants collected at d < 1.02 g/ml and 1.02 < d < 1.04 g/ml showed an enrichment of apoE relative to apoB when compared to d < 1.02 g/ml lipoproteins from control (untreated) incubations (**Table 4**). This observation coupled with the lower content of free apoE in high density fractions (d ≥ 1.04 g/ml) of hepatic lipase-treated lipoproteins (Table 1), suggest that chylomicron remnants exposed to hepatic lipase contain more tightly associated apoE than the untreated control lipoproteins.

TABLE 3. Effect of hepatic lipase treatment on immunoreactivity of apoE on chylomicron remnants

Experiment #	Control d < 1.02 g/ml Fraction		Hepatic Lipase Treatment Dense Remnants (1.02 < d < 1.04 g/ml)		ED_{50} Control/ ED_{50} Hepatic Lipase Treatment Dense Remnants
	ED_{50}	Slope	ED_{50}	Slope	
1	5.27	0.282	1.10	0.565	4.79
2	16.84	0.366	2.42	0.612	6.96
3	21.83	0.316	3.08	0.541	7.09
4	15.23	0.366	3.04	0.473	5.01
Mean	14.79	0.332	2.41	0.548	5.96
± SD	± 6.94	± 0.041	± 0.92[a]	± 0.058[a]	± 1.23

Competitive binding isotherm midpoint (ED_{50}) values and slopes were determined using the ALLFIT program as described in Materials and Methods.
[a]Differences between control d < 1.02 g/ml fraction and hepatic lipase treatment dense remnants are significant at a level of $P < 0.05$ by Student's paired t-test.

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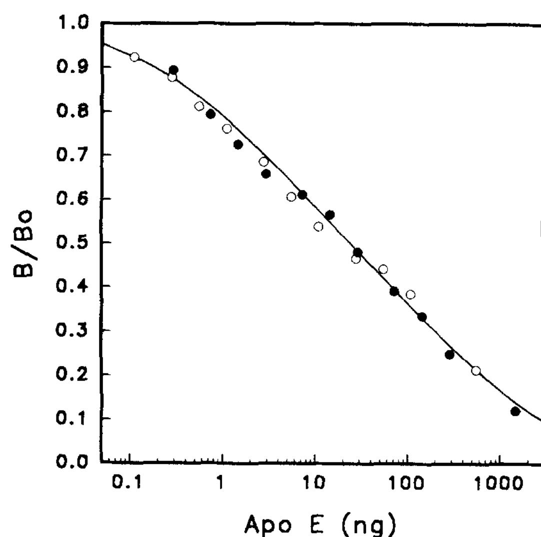

Fig. 3. Competition by d<1.02 g/ml lipoprotein isolated after one centrifugation step (closed symbols) and control d<1.02 g/ml lipoprotein isolated after mock hepatic lipase incubation and a second centrifugation step (open symbols) with immobilized apoE for binding to soluble polyclonal goat anti-rat hepatic lipase IgG conjugated to horseradish peroxidase. Lipoprotein concentration is expressed as total apoE mass determined by ELISA of SDS-treated lipoproteins. B/Bo values represent absorbance of ELISA sample wells containing lipoprotein/absorbance of wells containing only immobilized apoE. Data shown are averages of duplicate values.

DISCUSSION

The major findings of this study are that *1)* hydrolysis of lipids by hepatic lipase leads to increased immunoreactivity of apoE on chylomicron remnants, and *2)* hepatic lipase hydrolyzes chylomicron remnant phospholipid and triglyceride in a stepwise fashion in vitro. Evidence supporting sequential hydrolysis of phospholipid and triglyceride was obtained from the isolation of a hydrolytic intermediate depleted only of phospholipid, in addition to a small remnant particle extensively depleted of phospholipid and triglyceride. Significant increases in apoE immunoreactivity were observed in this dense subfraction of chylomicron remnants formed in vitro following extensive phospholipid and triglyceride hydrolysis.

The isolation of chylomicron remnants extensively hydrolyzed by hepatic lipase in vivo has proven difficult to accomplish. Chylomicrons are cleared very rapidly from circulation ($t_{1/2}$ = approximately 10 min) in rats (50). Furthermore, difficulty in obtaining chylomicron remnants exposed to hepatic lipase in vivo may be related to liver morphology and location of hepatic lipase in the liver. Triglyceride-rich lipoproteins that are small enough to pass through the fenestrae of liver endothelial cells become sequestered in the Space of Disse before interaction with cell surface receptors on hepatocytes. Recent studies have found a concentration of hepatic lipase in the Space of Disse (E. J. Blanchette-Mackie and R. O. Scow, personal communication) where it may be bound to heparan sulfate proteoglycans (28) and come into contact with sequestered lipoproteins. Within the Space of Disse, lipoproteins may be acted upon by hepatic lipase, bind cell surface receptors, and then be rapidly internalized.

Increases in chylomicron remnant apoE exposure after hydrolysis of remnant lipids by hepatic lipase may be the key to increased binding of chylomicron remnants to cell surface lipoprotein receptors. Previous studies have shown that rat lymph chylomicrons treated with hepatic lipase are taken up more readily by liver than native chylomicrons or chylomicron remnants treated solely with lipoprotein lipase (51). Uptake of chylomicron remnants by the liver is mediated by apoE binding to cell surface receptors (5–9). Our data demonstrate that hepatic lipase hydrolytic activity increases the immunoreactivity of apoE, and may thereby enhance chylomicron remnant clearance. The increased exposure of apoE may be essential for binding of the lipoprotein to cell surface receptors. Further experimentation is necessary to test this hypothesis.

The chylomicron remnants used in our studies were the product of lipolysis by lipoprotein lipase in vivo and hepatic lipase in vitro. Previous studies have shown that depletion of core and surface lipids by hydrolysis of VLDL with bovine milk lipase increased immunoreactivity of apoB (52, 53); however, no change in im-

TABLE 4. Apolipoprotein E/apolipoprotein B molar ratios for lipoprotein fractions

Experiment #	Control d<1.02 g/ml Fraction	Hepatic Lipase Treatment d<1.02 g/ml Fraction	Hepatic Lipase Treatment Dense Remnants (1.02<g<1.04 g/ml)
1	1.44	1.83	2.41
2	1.32	2.04	1.55
3	1.58	1.93	4.25
4	1.50	1.54	2.60
Mean ± SD	1.46 ± 0.11	1.84 ± 0.21[a]	2.70 ± 1.13[b]

[a]Differences between control d<1.02 g/ml fractions and hepatic lipase treatment d<1.02 g/ml fractions are significant at a level of P = 0.074 by Student's paired t-test.
[b]Control d<1.02 g/ml fractions vs. hepatic lipase treatment dense remnants; P = 0.094.

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munoreactivity was observed when two different polyclonal antibody preparations were used to probe relative apoE exposure (52). Although immunoreactivity of apoE varies among lipoprotein populations (54, 55), no clear correlations between apoE exposure and lipoprotein size or lipid composition have previously been made. Our studies begin to characterize the contribution that hepatic lipase makes to apoE exposure, but do not address the relative contribution of lipoprotein lipase. It is possible that lipoprotein lipase can create a similar dense remnant particle during extended circulation of chylomicron remnants. Alternatively, lipoprotein lipase may initiate the lipolysis of chylomicrons, thus facilitating entry of lipoproteins into the Space of Disse in the liver and allowing subsequent lipolysis by hepatic lipase.

Possible mechanisms for the increased exposure of apoE after hepatic lipase treatment of chylomicron remnants include hydrolysis of surface lipids covering regions of apoE, and conformational changes of apoE resulting from hydrolysis of the lipoprotein core lipid. Data obtained in this study indicate that large increases in exposure of apoE result only after considerable hydrolysis of both surface phospholipid and core triglyceride; hence, extensive remodelling of the lipoprotein structure is required for the observed increased immunoreactivity detected using polyclonal antibodies raised against apoE. Several studies have demonstrated that depletion of chylomicron remnant phospholipid content greatly increases the rate of chylomicron uptake by perfused liver (56, 57). Our studies suggest that triglyceride hydrolysis in addition to phospholipid hydrolysis is necessary for increased exposure of apoE. It is possible that hepatic lipase-catalyzed phospholipid hydrolysis increases exposure of essential receptor-binding regions of apoE to an extent that is below the sensitivity of our assay. Alternatively, surface phospholipid hydrolysis may expose underlying core triglycerides for further hydrolysis by hepatic lipase. Additional experimentation is necessary to resolve the relative contributions of hepatic lipase-catalyzed phospholipid and triglyceride hydrolysis to increased binding of chylomicron remnants to cell surface receptors.

Chylomicron remnants depleted of phospholipid and triglyceride content by hepatic lipase treatment had a higher content of apoE per particle than control (untreated) chylomicron remnants. The relative increase in apoE appeared to be due to decreased stripping of apoE from the lipid-depleted particles during centrifugation used to isolate the remnants. These observations suggest that chylomicron remnants depleted of phospholipid or phospholipid and triglyceride contain more tightly associated apoE than lipid-rich remnants. Current studies are probing the binding of exogenous free apoE to chylomicron remnants generated by hepatic lipase treatment in vitro to determine whether these lipoproteins have an enhanced affinity for apoE. ■

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