Particle size determines the specificity of apolipoprotein E-containing triglyceride-rich emulsions for the LDL receptor versus hepatic remnant receptor in vivo

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Abstract Apolipoprotein E (apoE) is an important determinant for the uptake of triglyceride-rich emulsions and lipoproteins by the liver, and exerts affinity for both the LDL receptor (LDLr) and a distinct liver-specific recognition site. Our current aim was to assess the mechanism underlying the receptor-specificity of apoE-carrying lipoproteins. Triglyceride-rich emulsions were synthesized, with mean sizes of 50, 80, and 150 nm. These fractions efficiently acquired apoE from rat serum, and were processed by LPL in vivo with a similar efficiency. Upon injection of the [^{3}H]cholesteryl oleate-labeled emulsions into rats, the liver association rate was positively correlated with particle size (24 ± 2%, 54 ± 1%, and 64 ± 3% of the injected dose at 20 min after injection, respectively) and the liver uptake was predominantly exerted by parenchymal cells. The role of the LDLr in emulsion clearance was established in wild-type versus LDLr knockout mice. In the absence of the LDLr, an 8-fold increased serum half-life was observed for the small emulsion, concomitant with a 6- and 15-fold decreased uptake by the liver and adrenals at 60 min after injection, respectively. In contrast, the in vivo behavior of the large emulsion was independent of the LDLr. Both the ratio of apoE:C on the emulsions upon serum incubation and the α-helical content of apoE were inversely correlated with particle size, indicating that these factors may be involved in the emulsion size-dependent receptor specificity in vivo. It is concluded that the contribution of the LDLr to the apoE-mediated clearance of emulsions by the liver and adrenals strongly increases with decreasing particle size, while large particles initially associate with a distinct liver-specific recognition site. As these emulsions mimic chylomicrons, we anticipate that the apoE-dependent metabolic behavior of chylomicrons (remnants) is largely dependent on their size.—**Rensen, P. C. N., N. Herijgers, M. H. Netscher, S. C. J. Meskers, M. van Eck, and T. J. C. Van Berkel.** Particle size determines the specificity of apolipoprotein E-containing triglyceride-rich emulsions for the LDL receptor versus hepatic remnant receptor in vivo. *J. Lipid Res.* 1997. **38:** 1070–1084.

Supplementary key words apoC-III • chylomicrons • conformation • hepatic lipase • lipoprotein lipase • lipoproteins • remnant lipoprotein • metabolism • transgenic mice

Apolipoprotein E (apoE), a 34-kDa glycoprotein, plays a key role in the hepatic metabolism of triglyceride-rich lipoproteins such as chylomicrons and very low density lipoproteins (VLDL) (1, 2), and emulsions (3, 4). Within the blood compartment, triglyceride-rich lipoproteins are converted into remnants through the hydrolysis of core triglycerides by lipoprotein lipase (LPL) (5) and the concomitant enrichment with apoE (6). These remnants are subsequently taken up by the liver, but the precise mechanism of recognition is still unclear. Various apoE-recognizing systems have been proposed to participate in remnant removal, including the LDL (apoB,E) receptor (LDLr) (4, 7–13), a distinct specific apoE receptor (1, 14), the low density receptor-related protein/α_2-macroglobulin receptor (LRP) (12, 15, 16), heparan sulfate proteoglycans (HSPG) (4, 17), as well as LPL (18) and hepatic lipase (HL) (19) in concert with proteoglycans and/or LRP. Whereas the lipases (LPL and HL) and proteoglycans may be involved in the initial low-affinity binding of remnants to the liver, the dominant role of both the LDLr and a distinct receptor, possibly LRP, in endocytosis of remnants has been clearly demonstrated (11, 12, 20).

The existence of an apoE-recognizing receptor in addition to the LDLr has been suggested by several independent lines of evidence (10, 11, 21–23). Chylomicron remnants appeared not to accumulate in Watanabe

Abbreviations: apo, apolipoprotein; BSA, bovine serum albumin; CD, circular dichroism; HL, hepatic lipase; HSPG, heparan sulfate proteoglycan(s); LDL(r), low density lipoprotein(receptor); LPL, lipoprotein lipase; LRP, low density lipoprotein receptor-related protein; PBS, phosphate-buffered saline; *rec-*, recombinant; β-VLDL, β-migrating very low density lipoprotein.

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heritable hyperlipidemic (WHHL) rabbits (21), LDLr knockout mice (10), or human familial hypercholesterolemia homozygotes (23). The essential role of apoE in lipid homeostasis is indicated by the massive accumulation of remnants in apoE-deficient mice (11) and in type III hyperlipoproteinemic humans, as caused by a genetic change in apoE (22). Whereas the sole absence of the LDLr does not result in a marked elevation of triglyceride-rich lipoprotein levels, a strong elevation of chylomicron remnants has been observed in LDLr knockout mice that also lack the apoE gene (11) and in LDLr knockout mice in which LRP has been inactivated by adenovirus-mediated transfer of the 39-kDa receptor associated protein (RAP) (12). Accordingly, a combination of an anti-LDLr antibody and RAP decreased the hepatic uptake of chylomicron remnants in mice by 80% (20).

The individual contribution of both receptor systems to the liver uptake of remnants is difficult to assess, and has been estimated as 50–76% for the LDLr (9, 20, 24) and 17–23% for LRP in mice (20). Although short-term kinetic studies in rats, mice, and rabbits indicate a prolonged plasma half-life of remnants in case the LDLr is blocked (9, 20, 24) or absent (10, 25), the apoE-specific non-LDLr recognition site may account for the clearance of virtually all of the remnants in the absence of the LDLr, albeit at prolonged circulation periods (11, 12).

The affinity of the LDLr for VLDL is clearly heterogeneous (26, 27). It has been suggested from in vitro studies that the capacity of LDL receptors on human fibroblasts to bind VLDL decreases with increasing particle size (28, 29). In vivo, large VLDL (>45 nm) showed a faster plasma decay in humans (30) and New Zealand White (NZW) rabbits (25) than total VLDL. The difference in plasma half-life was even more apparent in WHHL rabbits, indicative of participation of the non-LDLr recognition site in the binding of large VLDL (25). Recently, Windler et al. (31) reported that uptake of small chylomicron remnants by the isolated perfused rat liver paralleled the activity of the LDLr, as estimated from LDLr mRNA. In contrast, the liver uptake of remnants from large postprandial chylomicrons was not influenced by LDLr modulation (31). These studies thus suggest the involvement of (remnant) particle size in receptor recognition in vitro and ex vivo.

At present, it is still unclear which factor(s) decide about the specificity of remnants for the different receptor systems in vivo. Recently, we described a triglyceride-rich apolipoprotein-free emulsion model of native chylomicrons with well-defined physicochemical characteristics (3, 32). Upon intravenous injection into rats, the emulsion was extensively processed by LPL (32), acquired apolipoproteins, e.g., apoE and apoCs, and was subsequently taken up by liver parenchymal cells via lactoferrin-sensitive apoE-specific receptors just like endogenous chylomicrons (3). The rate of liver uptake was greatly increased by preloading the emulsion with recombinant (*rec*-) apoE. In the present study, we fractionated this emulsion into well-defined size-populations in order to evaluate the effects of emulsion size versus composition on the relative roles of the LDLr and the distinct apoE-specific receptor in the hepatic clearance. In addition to rats, we also used wild-type versus LDLr knockout mice, allowing unequivocal evaluation of the role of the LDLr. The contribution of the LDLr to the apoE-mediated uptake of emulsions by the liver (and adrenals) clearly increased with decreasing particle size. As these emulsions mimic chylomicrons, we anticipate that the apoE-dependent metabolic behavior of chylomicrons (remnants) is largely dependent on their size.

MATERIALS AND METHODS

Chemicals

Recombinant human apolipoprotein (*rec*-apo) E was a generous gift from Tikva Vogel, Bio-Technology General, Ltd., Israel, and was supplied as a lyophilized powder containing 76% *rec*-apoE, 11.7% L-cysteine, and 12.0% $NaHCO_3$ (33). ApoE was dissolved in phosphate-buffered saline (PBS) pH 7.4 (2.0 mg/ml) and stored under argon at −80°C. [1α,2α-^{3}H]cholesteryl oleate, glycerol tri[9, 10-^{3}H]oleate ([^{3}H]triolein), 1-[1-^{14}C]palmitoyl L-lyso-3-phosphatidylcholine, and ^{125}I (carrier-free) in NaOH were purchased from Amersham, Buckinghamshire, UK. Triolein (99% pure) and egg yolk phosphatidylcholine (98%) were from Fluka, Buchs, Switzerland. L-α-lysophosphatidylcholine (99%), cholesterol (>99%), bovine serum albumin (BSA, fraction V), 17α-ethinyl estradiol (EE), and purified bovine milk LPL (5800 units/mg) were obtained from Sigma, St. Louis, MO. Cholesteryl oleate (97%) was from Janssen, Beersse, Belgium. Cholesterol oxidase, cholesterol esterase, peroxidase type II (200 units/mg), Precipath® L, and EDTA were from Boehringer Mannheim, Germany. HEPES was from Merck, Darmstadt, Germany. Human lactoferrin (lyophilized, salt-free) was from Serva, Heidelberg, Germany, and heparin was from Leo Pharmaceutical Products B.V., Weesp, The Netherlands. All other chemicals were of analytical grade.

Preparation of emulsion fractions

Emulsions were prepared according to the sonication and ultracentrifugation procedure of Redgrave and Maranhao (34) from 100 mg of total lipid at a weight ratio triolein: egg yolk phosphatidylcholine: lysophosphatidylcholine: cholesteryl oleate: cholesterol of 70:

22.7: 2.3: 3.0: 2.0, using a Soniprep 150 (MSE Scientific Instruments, UK) at 18 μ output that is equipped with a water bath for temperature (54°C) maintenance (3). The emulsion was fractionated into three size populations by consecutive density gradient ultracentrifugation steps in a Beckman SW 40 Ti rotor. After centrifugation for 22 min at 20,000 rpm at 20°C, an emulsion fraction containing large emulsion particles (fraction 1) was removed from the top of the tube by aspiration and replaced by NaCl buffer of similar density (i.e., 1.006 g/ml) (34). Similarly, emulsion fractions 2 and 3 were isolated upon subsequent centrifugation at 40,000 rpm for 22 min and 4–5 h, respectively. Lipid composition of the emulsion fractions was determined with the Boehringer Mannheim (Mannheim, Germany) enzymatic kits for triacylglycerols, phospholipids, and cholesterol. The distribution of lysophosphatidylcholine over the emulsion fractions was determined by adding 1.25 μCi of [^{14}C]lysophosphatidylcholine before emulsion synthesis. Particle size and homogeneity of the fractions were assayed by photon correlation spectroscopy using a Malvern 4700 C system (Malvern Instruments, UK). Measurements were performed at 25°C and a 90° angle between laser and detector. The net negative surface charge of the emulsions was determined by subjecting the fractions to 0.75% (w/v) agarose gel electrophoresis at pH 8.8 using 0.075 M Tris-HCl, 0.080 M hippuric acid, 0.65 mM EDTA buffer. R_f values were determined relative to the front marker bromphenol blue. Emulsions were stored at 20°C under argon and used for characterization and metabolic studies within 5 days following preparation, in which period no physicochemical changes occurred. For synthesis of radiolabeled emulsions, 10 μCi of [^{14}C]cholesteryl oleate and 75 μCi of glycerol tri[^{3}H]oleate, or 25–150 μCi of [^{3}H]cholesteryl oleate were added to 100 mg of total lipid. Further preparation and isolation of the emulsions were similar as described above.

Incubation of emulsion fractions with rat serum

Serum, obtained from blood that was collected by puncture of the abdominal aorta from diethyl ether-anesthetized rats, was depleted of VLDL as previously described (34). Emulsion fractions (1.13 mg of triglycerides) were incubated with 3.0 ml of VLDL-free serum for 30 min at 37°C, and reisolated as described (34). Associated protein was quantified according to Lowry et al. (35), with BSA as an internal standard. Individual proteins (10–20 μg) were separated on 5–20% SDS-polyacrylamide gels. The relative amounts of (apolipo)proteins were calculated by densitometric scanning of Coomassie Brilliant Blue R-250-stained gels using a GS300 Scanning Densitometer from Hoefer Scientific Instruments (San Francisco, CA).

Association of apoE with the emulsion fractions

ApoE was radioiodinated at pH 10.0 with carrier-free ^{125}I according to a modification of the ICl method as described earlier (32). Free ^{125}I was removed by Sephadex G-25 gel filtration and extensive dialysis against 8 mM PBS containing 1 mM EDTA, pH 7.4, with repeated changes of buffer. More than 97% of the label in apoE was trichloroacetic acid-precipitable. The specific activity of ^{125}I-labeled apoE was 450–473 dpm/ng. ^{125}I-labeled apoE was then incubated with the emulsion fractions at various ratios for 30 min at 37°C, and emulsion-bound activity was separated from free activity using density gradient ultracentrifugation as described previously (32).

Isolation and radiolabeling of LDL

Human LDL was isolated from the blood of healthy volunteers by differential ultracentrifugation ($1.019 < d < 1.063$ g/ml) as described (36), dialyzed against 8 mM PBS containing 1 mM EDTA, pH 7.4, with repeated changes of buffer, and radiolabeled with ^{125}I-labeled tyramine cellobiose as described earlier (37).

Liver uptake and serum decay of emulsion fractions in rats

Fasted male Wistar rats weighing 210–270 g were anesthetized by intraperitoneal injection of sodium pentobarbital (15 mg/kg body weight) and the abdomens were opened. [^{3}H]cholesteryl oleate-labeled or tri[^{3}H]oleoyl glycerol [^{14}C]cholesteryl oleate double-labeled emulsion fractions (0.50 mg of emulsion triglyceride) were injected via the vena cava inferior. When indicated, rats (weighing 180–210 g) received a preinjection of lactoferrin (70 mg/kg) at 1 min before injection of the radiolabeled emulsions, or a preinjection of heparin (500 units/kg) via the left arteria carotis that had been cannulated, 10 min prior to injection of the emulsions. At the indicated times, blood samples of 300 μl were taken from the vena cava inferior and allowed to clot for 30 min. ^{3}H radioactivity in duplicate serum samples of 50 μl, obtained after centrifugation for 3 min at 2500 *g*, was counted in 3 ml of Emulsifier Safe (Packard, Downers Grove, IL). The total amount of radioactivity in the serum was calculated using the equation: serum volume (ml) = [0.0219 × body weight (g)] + 2.66 (38). In order to determine liver uptake, liver lobules were tied off, excised, and weighed at the indicated times. The amount of liver tissue tied off during the experiment did not exceed 15% of the total liver weight. At 30 min, rats were killed, and organs were excised and weighed. Radioactivity in liver and other tissue samples was counted after combustion (recovery >97%) in a Packard Tri-Carb 306 Sample Oxidizer and

corrected for the serum radioactivity in the tissues at the time of sampling (3).

Liver uptake and serum decay of lipoproteins and emulsion fractions in mice

LDLr knockout [LDLr(−/−)] mice, created from hybrids of C57Bl/6J and 129SvJ strains by targeted gene disruption as described by Ishibashi et al. (10) were obtained from the Jackson Laboratories (Bar Harbor, ME), and bred in our local facility under specific pathogen-free conditions, as well as wild-type C57Bl/6KH mice. The in vivo studies with β-VLDL and emulsions were performed essentially similarly as described for rats. Mice weighing 20–27 g, fasted overnight, were anesthetized by intraperitoneal injection of sodium pentobarbital (15 mg/kg body weight) or a mixture of diazepam (5 mg/kg) and Hypnorm (10 mg/kg fluanisone and 0.3 mg/kg fentanyl citrate), and the abdomens were opened. [^{3}H]cholesteryl oleate-labeled emulsions (150 μg of triglyceride) were injected, and blood (<50 μl) as well as liver samples were taken and processed as described for rats. The amount of liver tissue tied off did not exceed 17% of the total liver weight. In case of ^{125}I-labeled β-VLDL, apoB-associated radioactivity was determined after the selective precipitation of apoB with isopropanol (39). The total serum volumes of C57Bl/6KH and LDLr (−/−) mice were 1.068 ± 0.066 ml (mean ± SE; n = 5) and 1.177 ± 0.049 ml (mean ± SE; n = 3), respectively, as determined with ^{125}I-labeled BSA similarly as previously described for rats (38). These data correspond well with the plasma volume (4.5% of body weight), as estimated for C57Bl/6 mice and LDLr (−/−) mice by Herz et al. (13). Organ distributions were performed at 60 min after injection, similarly as described for rats. The radioactivity in liver and other tissue samples was corrected for the serum radioactivity in the tissues assumed to be present at the time of sampling, as determined by performing organ distributions of ^{125}I-labeled BSA at 10 min after injection. These values are for C57Bl/6KH and LDLr (−/−) mice, respectively: liver: 84.7 ± 8.9 and 68.6 ± 3.3; heart: 68.1 ± 2.9 and 57.8 ± 0.9; lungs: 125.9 ± 13.2 and 127.3 ± 7.9; stomach: 22.9 ± 3.2 and 24.1 ± 1.5; kidneys: 135.2 ± 7.2 and 142.8 ± 2.2; adrenals: 110.2 ± 9.5 and 99.9 ± 3.6; spleen: 64.6 ± 9.2 and 72.6 ± 3.4; small intestine: 46.3 ± 9.0 and 35.8 ± 2.8; large intestine: 35.2 ± 3.0 and 23.3 ± 1.0; muscles: 13.7 ± 2.5 and 20.2 ± 1.2; thymus: 46.7 ± 6.5 and 32.3 ± 2.6; skin: 13.1 ± 3.4 and 12.9 ± 1.3 μl serum/g wet weight (mean ± SE; n = 5 and 3, respectively). To determine the serum decay of ^{125}I-labeled LDL in fed conscious mice at prolonged circulation periods (>60 min), both injection (15 μg of protein) and blood sampling were done via the tall vein.

Intrahepatic hydrolysis of emulsion cholesteryl oleate in mice

To determine the rate of [^{3}H]cholesteryl oleate hydrolysis in the liver of mice, emulsion fraction 1 was injected into wild-type and LDLr (−/−) mice as described above. At the indicated times, liver lobules were taken and immediately frozen in liquid N_2. Upon homogenization in ice-cold PBS, pH 7.4, lipids were extracted according to Bligh and Dyer (40), and separated using thin-layer chromatography (heptane–diethyl ether–acetic acid 60:40:1 (v/v/v). Cholesteryl oleate (R_f 0.85) and cholesterol (R_f 0.23) were visualized with iodine vapor, scraped off, and counted in 15 ml of Hionic Fluor (Packard, Downers Grove, IL). Using this method, 99.5% of the emulsion-associated ^{3}H radioactivity appeared as [^{3}H]cholesteryl oleate.

Circular dichroism (CD) spectra and protein secondary structure analysis

To probe the conformation of apoE on the emulsion fractions, CD spectra were measured on a CD6 Dichrograph (Instruments S.A., Jobin-Yvon, Longjumeau, France) using a quartz cell of 1 mm path length at room temperature. Spectra were recorded of apoE (34 μg/ml), with or without previous incubation (30 min at 37 °C) with emulsions (1.7 mg/ml) that were previously dialyzed against PBS, pH 7.4. At these emulsion-triglyceride: apoE = 50:1 weight ratios, the presence of unbound apoE is prevented. Ten scans per sample were recorded in a continuous scanning mode and averaged in order to improve the signal to noise ratio. Spectra were also measured for the emulsions alone to allow correction for the contribution of lipids to the CD spectra (29). The mean residue ellipticity was calculated using the equation $[\theta]_m = (3300 \times \Delta A \times MRW) / (L \times C)$, where ΔA is the measured difference in absorption of left and right circularly polarized light, MRW the mean residue molecular weight (i.e., 115), L the cell path length in cm, and C the apoE concentration in g/L. The α-helical content (f_α) of apoE was calculated from the mean residue ellipticity at 222 nm using $f_\alpha = (-[\theta]_{222} + 2340)/30300)$ (41), in accordance with others (29, 42).

RESULTS

Characterization of emulsion fractions

The differential ultracentrifugation procedure from Redgrave and Maranhao (34) was used to divide the initial emulsion into three populations, based on their particle size-dependent flotation properties. The size

TABLE 1. Size, electrophoretic mobility, and lipid composition of emulsion fractions

	Emulsion Fraction		
	1	2	3
Mean diameter (nm)	152.3 ± 7.0	80.0 ± 2.4	47.8 ± 1.7
R_f on agarose	0.09 ± 0.01	0.16 ± 0.01	0.14 ± 0.01
Composition (%w/w)			
Triolein	84.1 ± 0.8	78.1 ± 0.6	67.7 ± 2.4
Phosphatidylcholine	10.5 ± 0.6	15.4 ± 0.7	26.9 ± 2.2
Cholesteryl oleate	2.8 ± 0.3	4.2 ± 0.3	2.6 ± 0.3
Cholesterol	2.6 ± 0.4	2.4 ± 0.1	2.8 ± 0.2

Emulsions were obtained by sonication of a lipid mixture (triolein:egg yolk phosphatidylcholine: lysophosphatidylcholine: cholesteryl oleate:cholesterol 70:22.7:2.3:3.0:2.0 (w/w), subdivided into three fractions by differential ultracentrifugation, and the mean particle size (mean ± SE; n = 11), electrophoretic mobility (R_f) on agarose (mean ± SD; n = 3), and composition (mean ± SE; n = 4–6) of the fractions were determined.

and composition of the emulsion fractions are summarized in **Table 1.** The average particle size of the emulsion fractions decreased with increasing fraction number. The particle populations were homogeneous, as evident from their low polydispersities of 0.10 ± 0.03 (fraction **1**), 0.12 ± 0.02 (**2**), and 0.22 ± 0.02 (**3**) (mean ± SD; n = 3). The electrophoretic mobilities of the emulsion fractions were all very low (<0.16 relative to bromphenol blue), confirming their low negative surface charge. This observation is beneficial with respect to avoiding phagocytotic uptake by cells of the reticuloendothelial system (RES) and scavenger receptors on endothelial cells (P. C. N. Rensen and T. J. C. van Berkel, unpublished observations).

The different recovery of triolein (17.0 ± 1.8%, 40.0 ± 2.4%, and 30.1 ± 1.7%) and phosphatidylcholine (6.5 ± 0.6%, 24.6 ± 2.3%, and 35.3 ± 2.4%) in the various fractions (**1**, **2**, and **3**) accounted for the inverse correlation of particle size and phospholipid: triglyceride ratio (0.14 ± 0.02, 0.22 ± 0.01, and 0.41 ± 0.09) (mean ± SD; n = 6). Lysophosphatidylcholine contributed 5.8 ± 0.2 (fraction **1**), 7.4 ± 0.1 (**2**), and 11.5 ± 0.2 (**3**) % (w/w) to total phospholipid (mean ± variation; n = 2), indicating a relative preference to incorporate into smaller particles.

Acquisition of apoE and rat serum apolipoproteins

Previous experiments have shown that, upon incubation of emulsion fraction 2 with VLDL-free rat serum, apolipoproteins associate with the emulsion, especially apoE and apoCs (8). ApoCs can be selectively displaced from the emulsion by the addition of exogenous apoE, leading to an increased apoE:C ratio. In this study, the effect of particle size on the association of apoE (**Fig. 1**) and and/or rat serum apolipoproteins (**Fig. 2**) was assessed. Radioiodinated apoE appeared to associate with the emulsions in a saturable manner, regardless of particle size (Fig. 1). From the binding curves, a maximum acquisition of 402 (fraction **1**), 109 (**2**), and 46 (**3**) molecules of apoE per particle could be calculated. These values imply maximum apoE surface concentrations of 4.7×10^3 (fraction **1**), 5.3×10^3 (**2**), and 6.4×10^3 (**3**) molecules per μm^2, respectively, which are comparable and thus appear relatively irrespective of particle size.

Incubation of the emulsion fractions with rat serum led to acquisition of a range of (apolipo)proteins, including serum albumin and apoCs, E, As, and D (Fig. 2, left panel). ApoA-I and A-IV preferentially associated with the small emulsion, but their functions in remnant metabolism are unclear. More interestingly, the relative amount of emulsion-associated apoE increased with decreasing particle size in contrast to the apoCs, resulting in an inverse correlation between particle size and apoE:C ratio (Fig. 2, right panel). In accordance, preincubation with exogenous apoE led to a higher recovery of apoE with all emulsion fractions, only at the expense of the C apolipoproteins, providing further evidence for competition between apoE and apoCs for binding sites on the emulsion surface (Fig. 2, right panel). A

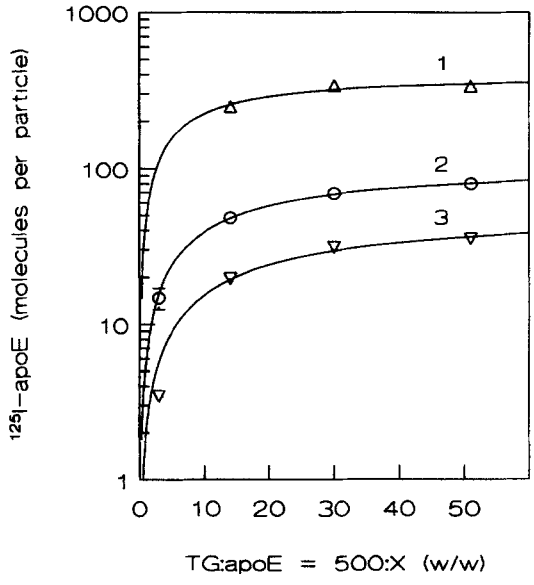

Fig. 1. Association of *rec*-apoE to the emulsion fractions. Emulsion fractions 1 (△), 2 (○), and 3 (▽) were incubated (30 min at 37°C) with ^{125}I-labeled apoE at various emulsion-triglyceride (*TG*): apoE weight ratios. Subsequently, emulsion-associated and free apoE were separated by density gradient ultracentrifugation, and the association of apoE with the emulsion (molecules per particle) was calculated. Values are means of 2–3 experiments.

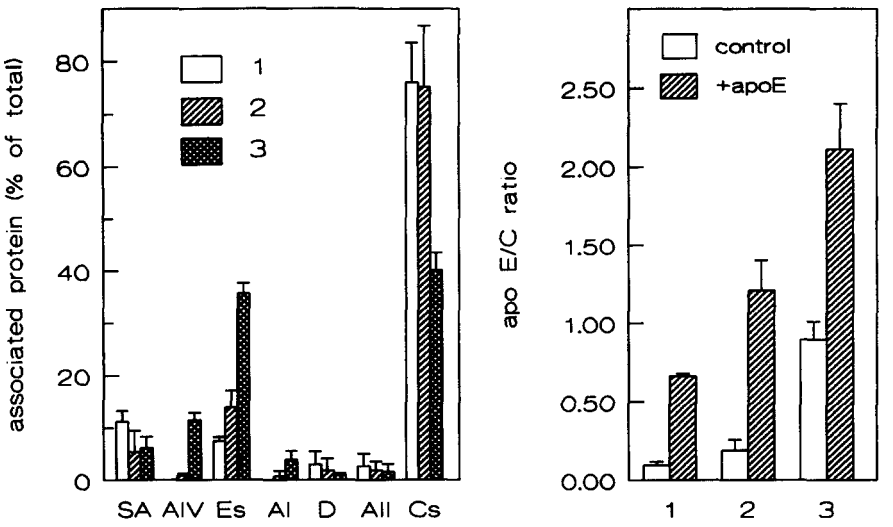

Fig. 2. Effect of particle size and apoE on the acquisition of (apolipo)proteins from rat serum. Emulsion fractions were incubated (30 min at 37°C) with VLDL-free rat serum without (left panel) or with (right panel) preincubation with apoE (30 min at 37°C), and reisolated. Associated proteins were separated by electrophoresis on 5–20% SDS-polyacrylamide gels and quantified by densitometric scanning upon staining with Coomassie blue. The apoE/C ratio was determined in the absence or presence of exogenous apoE (right panel). Values are means ± variation (fraction 1; n = 2) or SD (fractions 2 and 3; n = 3). SA, serum albumin.

6.8-, 6.3-, and 2.4-fold increased apoE:C ratio was consequently observed for fractions 1, 2, and 3, respectively.

Liver uptake, serum decay, and extrahepatic distribution of emulsion fractions in rats

Previous experiments have shown that, upon intravenous injection into rats, the cholesteryl oleate core of the emulsion fraction 2 is mainly taken up by apoE-specific receptors on liver parenchymal cells (3), after extensive processing of triglycerides by LPL (32). Studies on the liver uptake and serum decay of the three emulsion fractions are shown in **Fig. 3.** It appears that the in vivo kinetics of the emulsions are highly size-dependent. The serum half-lives of the emulsions clearly increased with decreasing particle size, and were < 1 min (fraction **1**), 14.8 ± 1.3 min (**2**), and 36.4 ± 4.9 min (**3**). Concomitantly, particle size showed a positive correlation with association of the emulsions with the liver. At 20 min after injection, 64 ± 3% (fraction **1**), 54 ± 1% (**2**), and 24 ± 2% (**3**) of the injected doses were taken up by the liver. The majority of core label of all fractions was taken up by parenchymal cells, as evidenced by the preinjection of human lactoferrin (Fig. 3, bottom panel). Lactoferrin has been shown to inhibit the apoE-mediated interaction of chylomicron remnants and β-VLDL with rat parenchymal cells in vivo (14). At 10 min after injection, lactoferrin inhibited the uptake of the emulsion fractions for 83.9% (**1**), 90.2% (**2**), and 92.2% (**3**). As lactoferrin preferentially (i.e., more than 97%) interacts with liver parenchymal cells after injection (43), these data indicate that less than 16.1% (**1**), 9.8% (**2**), and 7.8% (**3**) of the liver association was mediated by non-parenchymal cells such as Kupffer and endothelial cells.

The relative contributions of various organs to the clearance of the emulsion fractions, as determined at 30 min after injection, are summarized in **Table 2.** In general, the uptake of radiolabel by organs of the RES, such as spleen and lungs, appeared to be positively correlated with particle size. The aspecific body distribution, however, tended to increase with decreasing particle size, as indicated by an increase in uptake by muscle tissue. Surprisingly, the largest emulsion fraction showed a very high specific association with the heart (8.59 ± 0.78% per gram wet weight), which was 17- and 43-fold higher than that of the medium (0.52 ± 0.20%) and small (0.20 ± 0.12%) emulsion fraction, and even exceeded that with the liver (7.29 ± 0.57%). This association to the heart was even more pronounced at 2 min after injection (14.59 ± 3.93%, not shown), suggesting release of emulsion from the heart with time. Injection of heparin (500 units/kg), which releases LPL from the extrahepatic capillary endothelium (44) and enhances the metabolism of chylomicrons (45) and emulsions (32, 44), reduced the association of the large emulsion fraction to the heart 33-fold (0.26 ± 0.20%; mean ± variation, n = 2), reaching the baseline levels for the smallest emulsion (not shown).

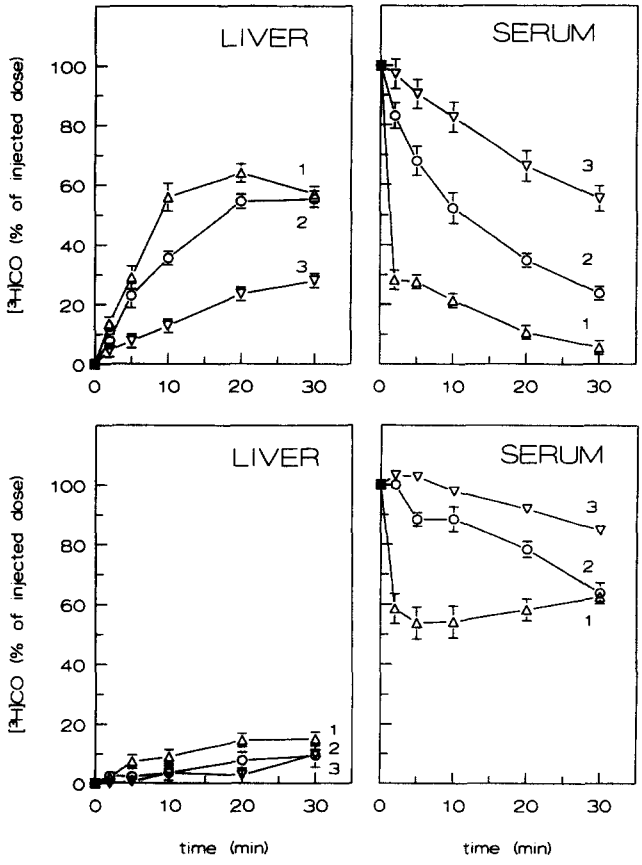

Fig. 3. Liver uptake and serum decay of the emulsion fractions in rats, in the absence or presence of lactoferrin. [^{3}H]cholesteryl oleate ([^{3}H]CO)-labeled emulsion fractions, 1 (△), 2 (○), and 3 (▽) (0.5 mg of emulsion-triglyceride) were injected into fasted anesthetized rats, without (top) or with (bottom) preinjection of lactoferrin. At the indicated times, the liver uptake (left panel) and serum decay (right panel) were determined. Liver values are corrected for serum radioactivity. Values are means ± SE (without lactoferrin; n = 3–5) or variation (with lactoferrin; n = 2) or represent a single experiment (fraction 3 with lactoferrin).

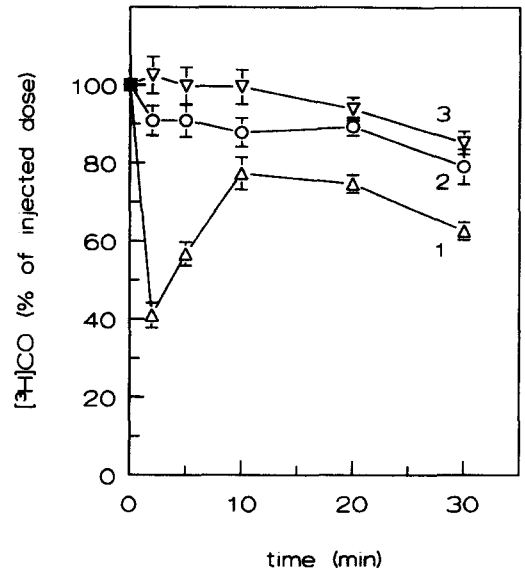

Fig. 4. Initial extrahepatic association of emulsion fractions in rats. [^{3}H]cholesteryl oleate ([^{3}H]CO)-labeled emulsion fractions (0.5 mg of emulsion-triglyceride) were injected into fasted anesthetized rats. At the indicated times, the total recovery of radiolabel in the serum plus liver were determined (mean ± SE; n = 3–5).

Extrahepatic binding of the large emulsion to LPL on heart and other tissues such as adipose tissue, might therefore explain the initial low combined presence of the large emulsion in the serum plus liver (41 ± 3% of the injected dose after 2 min of circulation) compared to the medium-sized (91 ± 4%) and small (102 ± 5%) emulsion (**Fig. 4**).

Despite the observed differences in initial binding to extrahepatic sites, the triglycerides in all three emulsion fractions were effectively hydrolyzed as evidenced by the similar shorter half-lives and reduced liver uptake of glycerol tri[^{3}H]oleate-derived radioactivity versus [^{14}C]cholesteryl oleate in the double-labeled emulsions

TABLE 2. Organ distribution (specific and total) of the emulsion fractions in rats

	Distribution of [^{3}H]Cholesteryl Oleate					
	Total (%ID)			Specific (%ID/g wet weight)		
Organ	1	2	3	1	2	3
Liver	56.9 ± 2.5	57.9 ± 0.2	28.1 ± 2.0	7.29 ± 0.57	6.87 ± 0.46	3.06 ± 0.50
Heart	6.37 ± 0.90	0.44 ± 0.18	0.16 ± 0.09	8.59 ± 0.78	0.52 ± 0.20	0.20 ± 0.12
Lungs	0.64 ± 0.29	0.18 ± 0.08	0.08 ± 0.03	0.92 ± 0.34	0.20 ± 0.08	0.09 ± 0.03
Adrenals	0.03 ± 0.01	0.09 ± 0.03	0.46 ± 0.19	1.22 ± 0.47	2.39 ± 0.84	3.34 ± 1.41
Spleen	2.26 ± 0.37	0.55 ± 0.01	0.40 ± 0.09	5.04 ± 0.49	1.03 ± 0.02	0.66 ± 0.10
Muscles	3.19 ± 1.53	4.30 ± 0.34	6.24 ± 0.89	0.04 ± 0.02	0.06 ± 0.01	0.07 ± 0.02
Serum	5.02 ± 1.28	25.7 ± 0.7	58.9 ± 2.9	—	—	—

[^{3}H]cholesteryl oleate-labeled emulsion fractions (0.5 mg of emulsion-triglyceride) were injected into fasted anesthetized rats. After 30 min of circulation, the organ distributions were determined. Recoveries of ^{14}C radioactivity in the rats were 76 ± 4% (fraction 1), 92 ± 2% (fraction 2), and 99 ± 2% (fraction 3). Values are corrected for serum radioactivity and represent means ± SE of 3–5 experiments; %ID, percentage of the injected dose.

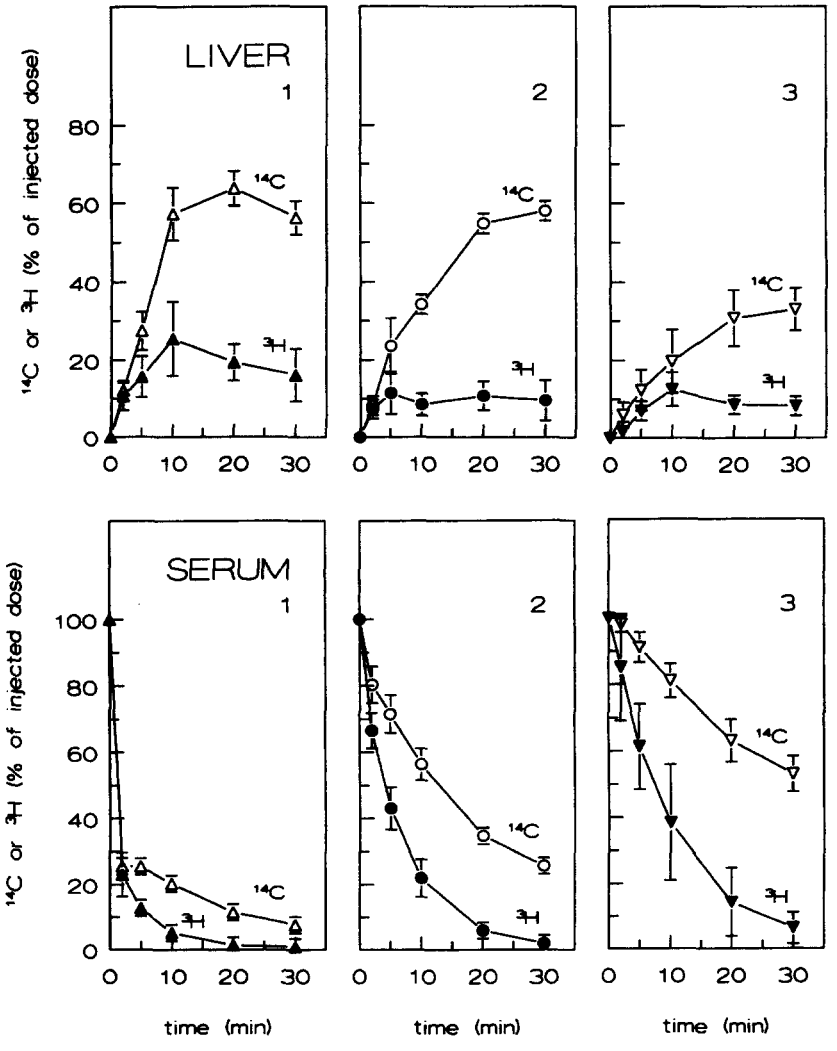

Fig. 5. Liver uptake and serum decay of double-labeled emulsion fractions in rats. Glycerol tri[^{3}H]oleate ([^{3}H]TO, closed symbols) [^{14}C]cholesteryl oleate ([^{14}C]CO, open symbols) labeled emulsion fractions 1 (△), 2 (○), and 3 (▽) (0.5 mg of emulsion-triglyceride) were injected into fasted anesthetized rats. At the indicated times, the liver uptake (top panel) and serum decay (bottom panel) were determined. Liver values are corrected for serum radioactivity. Values are means ± variation of two experiments.

(**Fig. 5**). For comparison, the uptake of ^{3}H activity (comprising a mixture of mono-, di-, and triglycerides) by the liver was 3.3-, 5.1-, and 3.6-fold lower compared to those of [^{14}C]cholesteryl oleate, at 20 min after injection of the emulsion fractions **1**, **2**, and **3**, respectively. Apparently, comparable amounts of triglycerides within the three fractions were hydrolyzed during lipolytic conversion, indicating that the size differences between the fractions presumably still exist at the (hepatic) receptor levels.

Liver uptake, serum decay, and extrahepatic distribution of emulsion fractions in mice

In order to assess the relative contribution of the LDLr to the in vivo clearance of the three emulsion fractions, the kinetics of the emulsions were determined in both LDLr (+/+) and LDLr (−/−) mice (**Fig. 6**). The absence of the LDLr in LDLr (−/−) mice was functionally verified by the 2.6-fold increased serum half-life of human ^{125}I-labeled LDL (9.1 ± 0.6 h) as compared to that in wild-type mice (3.5 ± 0.3 h), which is in agreement with the 2.5-fold increased half-life as has previously been observed for mouse ^{125}I-labeled LDL (10).

Upon injection of the emulsions into LDLr (+/+) mice, the in vivo kinetics were again highly size-dependent, with the serum half-lives being < 2 min (fraction **1**), 32.0 ± 2.0 min (**2**), and 47.6 ± 7.6 min (**3**). Accordingly, 64 ± 7% (fraction **1**), 52 ± 2% (**2**), and 41 ± 3% (**3**) of the injected doses were taken up by the liver at 30 min after injection (Fig. 6). The liver uptake rate of

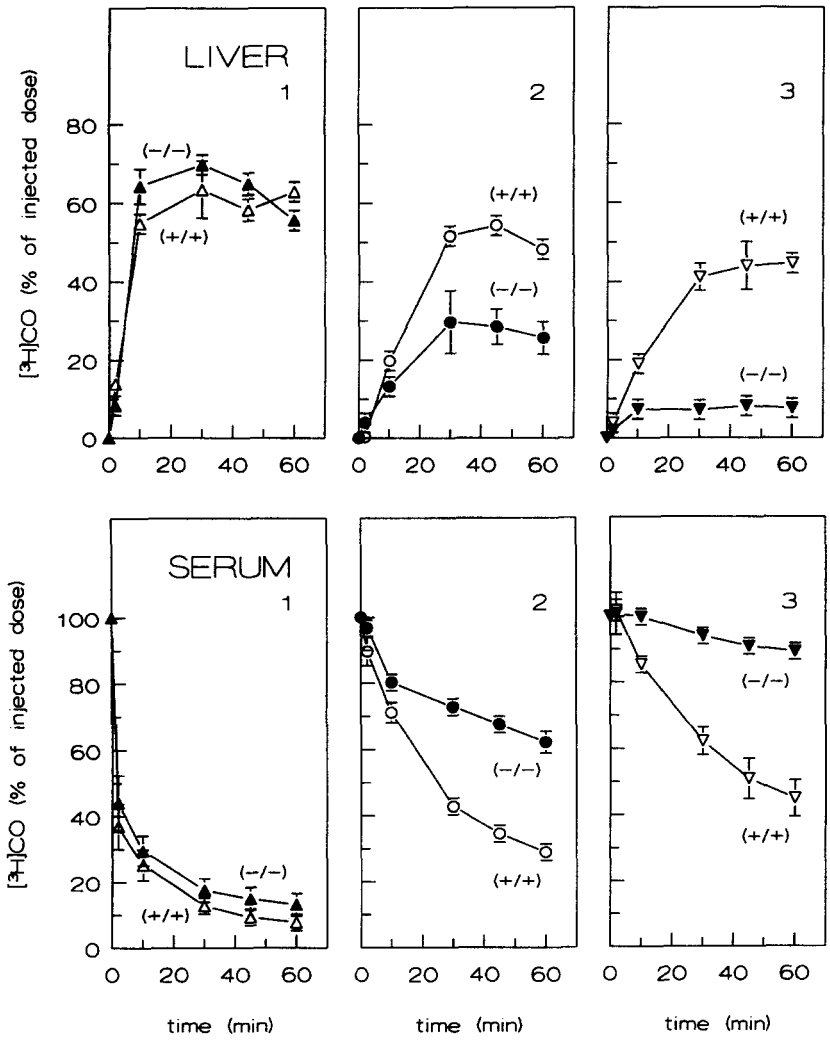

Fig. 6. Liver uptake and serum decay of the emulsion fractions in mice. [^{3}H]cholesteryl oleate-labeled emulsion fractions 1 (left panel), 2 (middle panel), and 3 (right panel) (150 μg of emulsion-triglyceride) were injected into fasted anesthetized wild-type (+/+) (open symbols) and LDLr (−/−) (closed symbols) mice. At the indicated times, the liver uptake (top) and serum decay (bottom) were determined. Liver values are corrected for serum radioactivity. Values are means ± variation (fraction 2; n = 2) or SE (fractions 1 and 3; n = 3–6).

the large emulsion is similar as compared to rats (Fig. 3) whereas that of the small emulsion is higher than in rats, and might therefore be related to the presence of the LDLr. The effects of the particle size on the serum clearance and liver uptake are more clearly expressed within LDLr (−/−) mice. Whereas the serum half-life of the large emulsion (< 2 min) did not differ from that observed in LDLr (+/+) mice, 3.1- and 7.8-fold increased half-lives were observed for the medium-sized (97.5 ± 7.8 min) and small (371.2 ± 42.7 min) emulsion, respectively. Accordingly, the liver uptake of the large emulsion (69 ± 2%) was not altered, but was 1.7- and 5.9-fold reduced for the medium-sized (30 ± 8%) and small emulsion (7 ± 1%), respectively, at 30 min after injection.

The organ distributions of the [^{3}H]cholesteryl oleate-labeled emulsions (60 min after injection) in both LDLr (+/+) and (−/−) mice were essentially similar as those observed in rats, and showed a positive correlation between particle size and affinity for the RES (spleen, lungs) and LPL (heart) (**Fig. 7**). In addition, whereas the specific adrenal uptake (per gram wet weight) strongly increased (3 ± 1%, 16 ± 6%, and 58 ± 3%) with decreasing particle size in LDLr (+/+)

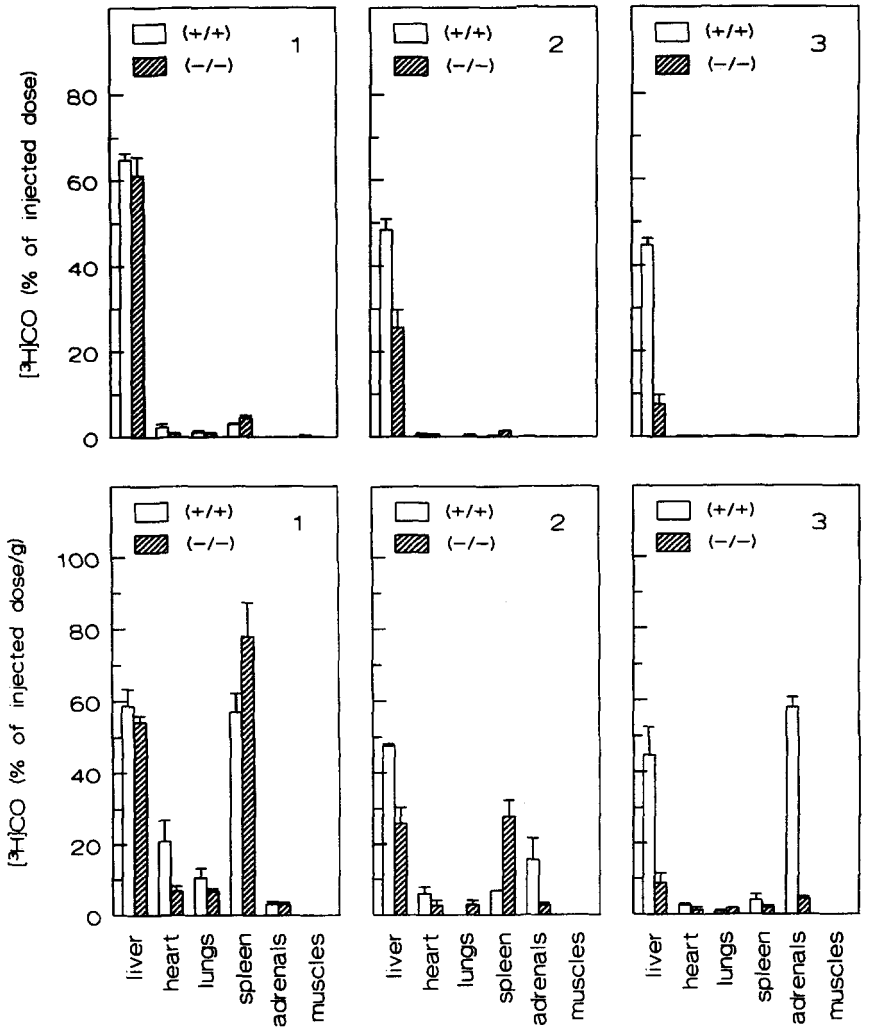

Fig. 7. Organ distribution of the emulsion fractions in mice. [^{3}H]cholesteryl oleate ([^{3}H]CO)-labeled emulsion fractions 1 (left panel), 2 (middle panel), and 3 (right panel) (150 μg of emulsion-triglyceride) were injected into fasted anesthetized wild-type (+/+) and LDLr (−/−) mice. After 60 min of circulation, the total (top) and specific (bottom) organ distributions were determined. Recoveries of ^{3}H radioactivity in the wild-type and LDLr (−/−) mice were 81 ± 2% and 83 ± 4% (fraction 1), 78 ± 4% and 91 ± 2% (fraction 2), and 92 ± 5% and 97 ± 4% (fraction 3), respectively. Values are corrected for serum radioactivity and represent means ± variation (fraction 2; n = 2) or SE (fractions 1 and 3; n = 3–6).

mice, this size-dependent effect was abolished in the absence of the LDLr. Under this condition, the adrenal uptake of all fractions did not not exceed the low aspecific level (≤4%) as determined for the large emulsion in LDLr (+/+) mice.

Intrahepatic hydrolysis of emulsion cholesteryl oleate in mice

The in vivo clearance of the small emulsion is thus largely determined by the presence of the LDLr, whereas the initial association of the large emulsion with the liver is virtually independent of the LDLr. It has been suggested that, whereas the initial hepatic recognition of chylomicron remnants is unaffected by the absence of the LDLr, the intrahepatic hydrolysis of [^{3}H]cholesteryl oleate within these remnants may depend on the LDLr (13). The intrahepatic processing of the [^{3}H]cholesteryl oleate-labeled large emulsion was therefore assessed in LDLr (+/+) and LDLr (−/−) mice (**Fig. 8**). The rate of cholesteryl oleate hydrolysis was fast and appeared irrespective of the presence of the LDLr (50% conversion at 30–45 min after injection

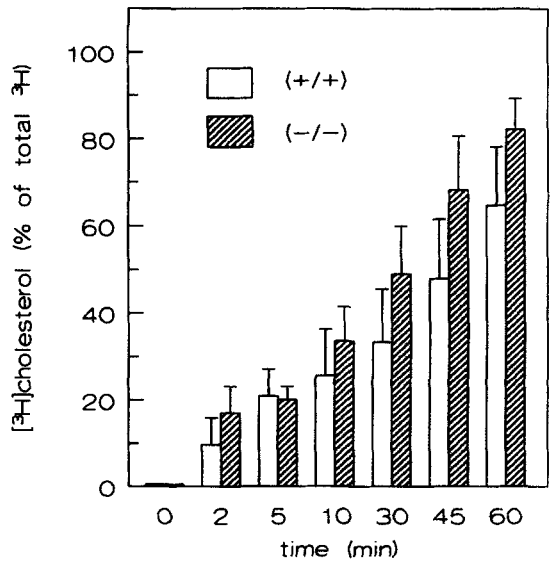

Fig. 8. Intrahepatic hydrolysis of cholesteryl oleate from emulsion fraction 1 in mice. [^{3}H]cholesteryl oleate-labeled emulsion fraction 1 (150 μg of emulsion-triglyceride) was injected into fasted anesthetized wild-type (+/+) and LDLr (−/−) mice. At the indicated times, liver lobules were taken, immediately frozen in liquid N_2, and homogenized in ice-cold PBS. Lipids were extracted and [^{3}H]cholesterol was separated from [^{3}H]cholesteryl oleate by thin-layer chromatography. Values are means ± SE of four experiments.

for both conditions), providing evidence for a cellular uptake route for the large emulsion independent of the LDLr.

Circular dichroism (CD) spectra

In an attempt to elucidate the mechanism underlying the inverse correlation between particle size and LDLr affinity, CD spectra of apoE were measured to determine whether its conformation depends on the emulsion particle size (**Fig. 9**). The CD spectra of free apoE showed minima at 208 nm and 222 nm, which is indicative of the presence of an α-helical secondary structure (Fig. 9, left panel). The α-helical content of apoE was 41 ± 3% (mean ± SD; h = 4) as calculated from the mean residue ellipticity at 222 nm. This value was irrespective of the apoE concentration (within the range 0.5–2.0 μM), or previous incubation for 30 min at 37°C. Preincubation of apoE with emulsion fractions of decreasing size (76 ± 15 nm, 43 ± 9 nm, and 35 ± 7 nm) for 30 min at 37°C led to augmenting spectral minima at 222 nm and hence to an increase in α-helical content of apoE up to 45 ± 2%, 57 ± 8%, and 64 ± 8% (mean ± variation; n = 2), respectively (Fig. 9, right panel). Accurate analysis of the α-helical content of apoE on the (largest) emulsion fraction 1 was precluded due to excessive light scattering.

DISCUSSION

At present, the mechanism for the hepatic uptake of triglyceride-rich lipoprotein remnants is still under active discussion (32, 46, 47). It is now generally accepted that the LDLr can contribute to the clearance of both VLDL (remnants) and chylomicrons in animals (8, 9, 24, 25) as well as in humans (48). However, its relative importance as compared to uptake systems that are not related to the LDLr, and the mechanism that determines LDLr versus non-LDLr (remnant receptor)-mediated uptake, are unclear.

The involvement of the LDLr in the hepatic uptake has been demonstrated for chylomicron remnants in rats pretreated with 17α-ethinyl estradiol (8) or using an anti-LDLr antibody in mice (9, 24), for VLDL in normal versus WHHL rabbits (25) and in wild-type versus LDLr knockout mice (10, 11), and for chylomicron-like emulsions also in wild-type versus LDLr knockout mice (4). Triglyceride-rich lipoproteins and emulsions bind the LDLr via apoE, and not apoB48 (49, 50), as determined for VLDL using partial apoE cleavage (49), or the apoE-specific monoclonal antibody 1D7 (26).

In this study we investigated the factor(s) that determine(s) the specificity of triglyceride-rich lipoproteins (remnants) for both the LDLr and non-LDLr uptake site(s) in vivo using a defined emulsion model of chylomicrons (3, 32) that was fractionated into homogeneous size populations with mean diameters of about 50, 80, and 150 nm. These fractions were all able to associate similar saturating shell concentrations of human apoE, indicating that size differences did not affect the affinity of emulsions for apoE. Upon incubation with rat serum, the fractions also acquired apoE, as well as other apolipoproteins, predominantly apoCs.

A size-dependent affinity of the emulsion fractions for the liver was observed upon injection into both rats and mice, and was somewhat more pronounced in rats. Human lactoferrin has previously been shown to inhibit the liver uptake of chylomicrons in rats (14, 51). Accordingly, the liver uptake of the cholesteryl oleate moiety of the various emulsion fractions in rats was also inhibited for more than 84%. As lactoferrin almost exclusively (i.e., more than 97%) associates with parenchymal cells (43), this finding implicates that a major involvement of Kupffer and endothelial cells in the total liver uptake of the emulsion fractions can be excluded. The predominant role of parenchymal cells in the uptake of the 80 nm-sized emulsion has previously been confirmed by cellular distribution studies (3). In addition, the emulsions are apparently recognized by a lactoferrin-sensitive site independent of the LDLr in rats, as lactoferrin does not interfere with the recognition of LDL and β-VLDL by the 17α-ethinyl estradiol-induced

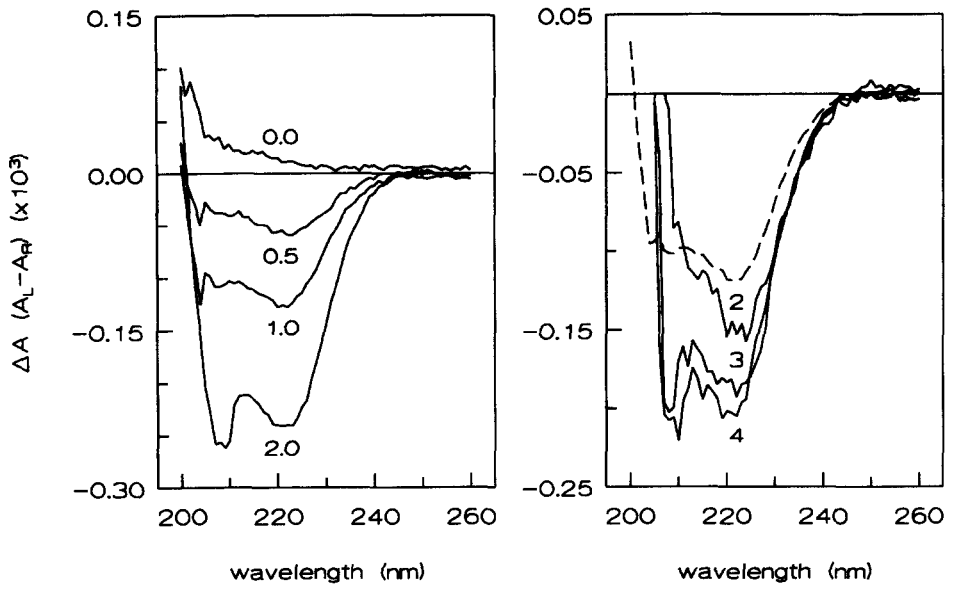

Fig. 9. Effect of emulsion fractions on circular dichroism spectra of apoE. Spectra were recorded, at room temperature, of 0, 0.5, 1.0, and 2.0 μM apoE in PBS, pH 7.4 (left panel). Also, spectra were obtained of apoE that was preincubated (30 min at 37°C) in the absence (dashed line) or presence of 76 ± 15 nm (2), 43 ± 9 nm (3), and 35 ± 7 nm (4) sized emulsions (emulsion triglyceride:apoE = 50:1, w/w) (right panel). The spectra are averages of two independent incubations (10 acquisitions per incubation).

LDLr (14). Taken together with the size-dependent liver uptake patterns and serum kinetics, it is clear that the affinity of emulsions for this recognition site, termed the remnant receptor, decreases as the particle size decreases without induction of extrahepatic uptake.

Using both wild-type and LDLr (−/−) mice, we demonstrated that the contribution of the LDLr to emulsion clearance increases with decreasing particle size, as evident from the association of emulsions with the liver as well as adrenals. The specific uptake (i.e., per gram wet weight) of the small emulsion by the adrenals even exceeded the specific liver uptake in wild-type mice, which is related to a higher LDLr expression (24). In fact, whereas the in vivo behavior of the large emulsion is virtually independent of the LDLr, the behavior of the small emulsion appeared almost completely dependent on the presence of the LDLr.

The inverse correlation between the particle size and the affinity of emulsions for the LDLr in vivo raises questions about the mechanism underlying the size-dependent affinity for the LDLr versus remnant receptor. The receptor specificity may be (partially) explained by the differences in apoE:C ratio. It is known that apoCs inhibit the apoE-dependent recognition of triglyceride-rich lipoproteins by the LDLr as assessed in vitro and in vivo (7, 27), and LRP as determined by ligand blotting (16). Interference of apoC with both receptors appears to occur through a mechanism irrespective of apoE displacement (16, 27, 52), and is possibly due to apoE–apoC interactions at the lipoprotein surface (27). The similarity in the extent of triglyceride hydrolysis of the various emulsion fractions in rats in vivo indicates that the size differences between the various emulsion fractions, and hence the differences in apoE:C ratios, will still exist at the hepatic uptake site. An increased apoE:C ratio may therefore correlate with an increased binding affinity of emulsions for the LDLr, but this would consequently imply that the remnant receptor is less sensitive to the effects of apoCs than the LDLr.

We also observed a conformational change of apoE upon association with emulsions as evident from an increase in its α-helical content. In addition, the percentage of α-helix in apoE increased with decreasing emulsion size. These observations are consistent with data on the association of apoE with differently sized emulsions prepared from dimyristoyl phosphatidylcholine and cholesteryl oleate, and the finding that the α-helical content of apoE was even higher (approx. 90%) within dimyristoyl phosphatidylcholine disks (29). Competition studies with LDL for the LDLr on human fibroblasts indeed suggested an inverse correlation between particle size and LDLr affinity of apoE (29). The particle size-dependent secondary structure of apoE thus may be relevant for the LDLr versus remnant receptor (non-LDLr)-mediated recognition.

Elucidation of the nature of the (non-LDLr) initial recognition system, which is responsible for the high

liver association rate of the large emulsion, was not the primary aim of our present experiments. Possible sites include *1*) the LRP, *2*) proteoglycans, *3*) lipases such as LPL and HL in concert with proteoglycans and/or LRP, and *4*) a remnant receptor of an unidentified nature. Herz et al. (13) recently concluded that LRP (or the LDLr) is not involved in the initial recognition of chylomicron remnants by the liver, and our previous data on remnant recognition are in agreement with this statement (14). The role of proteoglycans is presently supported by Herz et al. (13), Ji and Mahley (53), and Beisiegel et al. (47). We also obtained evidence for the involvement of proteoglycans in the cellular interaction of lipoproteins as mediated by LPL or HL (54, 55). It has been reported that substantial amounts of LPL are associated with triglyceride-rich lipoproteins in human postheparin (56) and postprandial plasma (57), and that intravenously administered emulsions induce LPL activity in the rat liver (58). Such a mechanism for remnant removal involving lipases must then be more efficient for large versus small remnants. The similarity between the size specificity of extrahepatic attachment of the large emulsion fraction to LPL and its non-LDLr-mediated liver association is in accordance with an LPL-mediated proteoglycan binding pathway.

Very recently, we reported that apoE inhibits the LPL-mediated lipolysis of emulsions, as explained by a decreased affinity for LPL (32). As the apoE:C ratio of the emulsion upon incubation with serum increased with decreasing particle size, smaller particles can be expected to have a lower affinity for LPL as we now also observed by the markedly decreased binding to the heart in rats and mice. Consequently, the contribution of this route to the liver association would be low, as has been observed for the small emulsion in LDLr (−/−) mice. Interestingly, Windler et al. (31) reported that perfusion of livers with heparin or heparinase, thereby releasing apoE and HL, greatly retarded the removal of large chylomicron remnants, while that of small chylomicron remnants remained unaffected. Others have found that the hepatic clearance of remnants derived from large chylomicrons is inhibited by HL antisera in perfused livers ex vivo (19) or in rats in vivo (59). Our observation that lactoferrin appeared to be a potent inhibitor of the association of emulsions with parenchymal liver cells in rats is consistent with the lactoferrin-sensitive binding of emulsions to HSPG as mediated by LPL and/or HL (53, 60).

In conclusion, the present data can explain the long-term discrepancy between various research groups with respect to the appreciation of the relative role of the LDLr in the clearance of triglyceride-rich lipoproteins, as these groups used a wide variety of substrates with mostly a heterogenous size distribution (4, 9, 10, 13, 20, 24, 25). Our data demonstrate unambiguously that the particle size of triglyceride-rich emulsions determines receptor specificity. Whereas small emulsions directly interact with the LDLr, possibly dependent on a favorable apoE conformation and/or apoE:C ratio, the hepatic uptake of the large emulsion is not dependent on this receptor. The concerted action of apoE and lipases (i.e., LPL and HL) may result in the initial association of large emulsions to hepatic HSPG, which is coupled to endocytosis as mediated by LRP and/or an as yet unidentified remnant receptor.

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