Chylomicron remnant uptake in the livers of mice expressing human apolipoproteins E3, E2 (Arg158→Cys), and E3-Leiden

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Abstract Apolipoprotein E2 (apoE2) and apoE3-Leiden cause chylomicron remnant accumulation (type III hyperlipidemia). However, the degree of dyslipidemia and its penetrance are different in humans and mice. Remnant uptake by isolated liver from apoE$^{-/-}$ mice transgenic for human apoE2, apoE3-Leiden, or apoE3 was measured. In the presence of both LDL receptor (LDLR) and LDL receptor-related protein (LRP), remnant uptake was apoE3>E3-Leiden>E2 mice. Absence of LDLR reduced uptake in apoE3 and apoE3-Leiden-secreting livers but not in apoE2-secreting livers. LRP inhibition with receptor-associated protein reduced uptake in apoE3- and apoE2–secreting livers, but not in apoE3-Leiden–secreting livers, regardless of the presence of LDLR. Fluorescently labeled remnants clustered with LRP in apoE3-secreting livers only in the absence of LDLR, but clustered in livers that expressed apoE2 even in the presence of LDLR, and did not cluster with LRP in livers of apoE3-Leiden even in the absence of LDLR. Remnants were reconstituted with the three human apoE isoforms. Removal by liver of *mApoe*$^{-/-}$/ *mldlr*$^{-/-}$ mice expressing the human LDLR was slightly greater than removal in the previous experiments with apoE3>E2> E3-Leiden.▮ Thus, in vivo, human apoE2 is cleared primarily by LRP, apoE3-Leiden is cleared only by the LDLR, and apoE3 is cleared by both.—Lee, S-J., I. Grosskopf, S. Y. Choi, and A. D. Cooper. **Chylomicron remnant uptake in the livers of mice expressing human apoE3, apoE2 (Arg158→Cys), and apoE3-Leiden.** *J. Lipid Res.* 2004. **45:** 2199–2210.

Supplementary key words LDL receptor • LDL receptor-related protein • type III hyperlipidemia

Apolipoprotein E (apoE) is a 34 kDa multifunctional protein that is polymorphic. The most common variant is apoE3; the second most common variant, apoE4 (Cys112→Arg), is associated with susceptibility to Alzheimer's disease (1, 2)

and coronary heart disease (3). The least-common variant, apoE2 (Arg158→Cys), is associated with type III hyperlipidemia (4–6), a disease in which impaired clearance of β-VLDL and chylomicron remnants causes these lipoproteins to accumulate in the circulation, leading to the premature development of atherosclerosis. This condition requires apoE2 homozygosity, and the penetrance is variable (7). A rare isoform, apoE3-Leiden is also associated with type III hyperlipidemia (8). The apoE3-Leiden variant contains a seven-amino-acid insertion that is a tandem repeat of residues 121–127 (9), and is associated with the dominant inheritance of type III hyperlipidemia (10).

ApoE is required for the removal of lipoproteins carrying dietary cholesterol; when apoE is absent, these particles accumulate in the blood. ApoE functions as a ligand for the LDL receptor (LDLR) (11) and the LDL receptor-related protein (LRP) (12) and binds to heparan sulfate proteoglycans (HSPG). It has been suggested that HSPG mediates the sequestration of remnants in the space of Disse and assists LRP-dependent particle uptake (13). Lipoprotein lipase (14–16) and hepatic lipase (17–19) also interact with apoE in the uptake process and may serve as ligands for LRP or as cofactors in HSPG–apoE binding. The apoE variants associated with type III hyperlipidemia share characteristics of decreased binding affinity to the LDLR, LRP, and HSPG. Compared with its binding affinity for normal apoE3, the LDLR has a slightly lower affinity for apoE3-Leiden (20–22), and a markedly lower affinity for apoE2 (Arg158→Cys, 1–2% of normal) in vitro (10, 23, 24). Both apoE2 and apoE3-Leiden have decreased binding to

LRP in cultured cells (25). HSPG binds apoE2 and apoE3-Leiden with similarly reduced affinity, as compared with apoE3, although apoE2 binds to HSPG slightly better than does apoE3-Leiden in studies of rabbit β-VLDL binding to cultured cells (25). The secretion–capture mechanism postulates that the binding to HSPG is the critical step in this process (13). Data from our laboratory suggest that binding to LRP initiates sequestration and that this occurs primarily when LDLR-mediated internalization is inoperative (26).

ApoE2 (Arg158→Cys) and apoE3-Leiden cause similarly severe hyperlipidemia in humans (5, 7, 8), while only apoE2 causes severe hyperlipidemia in mice (20, 27, 28). We investigated the effect of human apoE variants on the removal of remnant lipoproteins using intact livers. Livers from transgenic mice expressing human apoE2, apoE3-Leiden, or apoE3, and livers from these three apoE strains without LDLR were perfused with radiolabeled or fluorescent-labeled chylomicron remnants. It was found that apoE3-Leiden was bound and internalized by the LDLR and was not sequestered, while apoE2 was sequestered but was not removed as well by the LDLR.

MATERIALS AND METHODS

Animals

Transgenic mice expressing human apoE3 in the absence of endogenous apoE ($mApoe^{-/-}/htgApoe3$) (29) were purchased from Taconic (Germantown, NY). Transgenic mice expressing apoE2 and apoE3-Leiden in the absence of endogenous mouse apoE ($mApoe^{-/-}/htgApoe2$ and $mApoe^{-/-}/htgApoe3L$), which were previously described (30), were kindly provided by Dr. Havekes and Ko Willems van Dijk. LDLR-deficient mice ($mldlr^{-/-}$) and double knockout mice homozygous for the absence of LDLR and apoE ($mApoe^{-/-}/mldlr^{-/-}$) were purchased from the Jackson Laboratory (Bar Harbor, ME). The apoE-deficient mice ($mApoe^{-/-}$) were originally a gift from Dr. E. Rubin (University of California–Berkeley) (31) and were bred in the animal facilities of the Research Institute of the Palo Alto Medical Foundation. Sprague-Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA). The animals were kept at 21–25°C, with a 12 h day/night cycle and had free access to water and standard chow. The $mApoe^{-/-}/htgApoe3$, $mApoe^{-/-}/htgApoe2$, and $mApoe^{-/-}/htgApoe3L$ mice were crossbred with either $mApoe^{-/-}$ or $mApoe^{-/-}/mldlr^{-/-}$ mice. Mice expressing human LDLR under the control of the albumin promoter were generated in our laboratory and were bred with $mApoe^{-/-}/mldlr^{-/-}$ mice to get heterozygotes for $hLdlr$ in the $mApoe^{-/-}/mldlr^{-/-}$ background. This strain contains no mouse apoE or LDLR, but has human LDLR ($mApoe^{-/-}/mldlr^{-/-}/htgLdlr$). All experiments were performed under protocols approved by the Committee on Animal Experimentation of the Palo Alto Medical Research Foundation.

Fast-performance liquid chromatography

Serum lipoproteins were separated by fast-performance liquid chromatography (FPLC) as described by Plump et al. (31). Triglyceride and cholesterol concentrations in each fraction were measured by enzymatic methods (Sigma, St. Louis, MO). Protein concentration was measured using a modified bicinchoninic acid method (BioRad, Hercules, CA) with albumin as a standard (Sigma).

Western blot and real-time PCR

The LDLR, LRP, and human apoE protein levels in the mouse livers were analyzed using Western blots of liver membrane, as previously described (32), using antibodies prepared in this laboratory (33, 34). Total RNA was prepared from mouse livers using a kit from Qiagen Inc. (Valencia, CA), and reverse transcription was performed on 1 μg of total RNA using random hexamer primers and reverse transcriptase (Gibco BRL; Life Technologies, Vienna, Austria). The DNA fragments were purified, and a standard curve was prepared by carrying out real-time PCR with known amounts of cDNA and the probes. Real-time PCR was performed using 40 amplification cycles (95°C, 15 s; 55°C, 1 min; 72°C, 30 s). The primers and probe for LDLR were designed using Primer Express 1.5 (Applied Biosystems; Foster City, CA). The primers corresponded to nucleotides +461 to +481 and +572 to +589 of the mouse LDLR gene. The DNA sequence from +483 to +504 was used as a specific probe, which was labeled with a reporter dye (FAM) and a quencher dye (TAMRA). This technique measures the absolute amount of RNA present. In all samples, GAPDH mRNA levels were measured to provide an internal standard.

Preparing chylomicron remnants

Chylomicrons were obtained from lymph from the cannulated lymph ducts of rats, as previously described (35). To prepare chylomicron remnants, functionally hepatectomized rats were injected with chylomicrons (300 mg of triglyceride per kg of body weight) intravenously via the femoral vein (36). After 3 h, blood was obtained from the rats and the chylomicron remnants (d < 1.006 g/ml) were harvested by density gradient ultracentrifugation and then separated by gel filtration chromatography, as previously described (37).

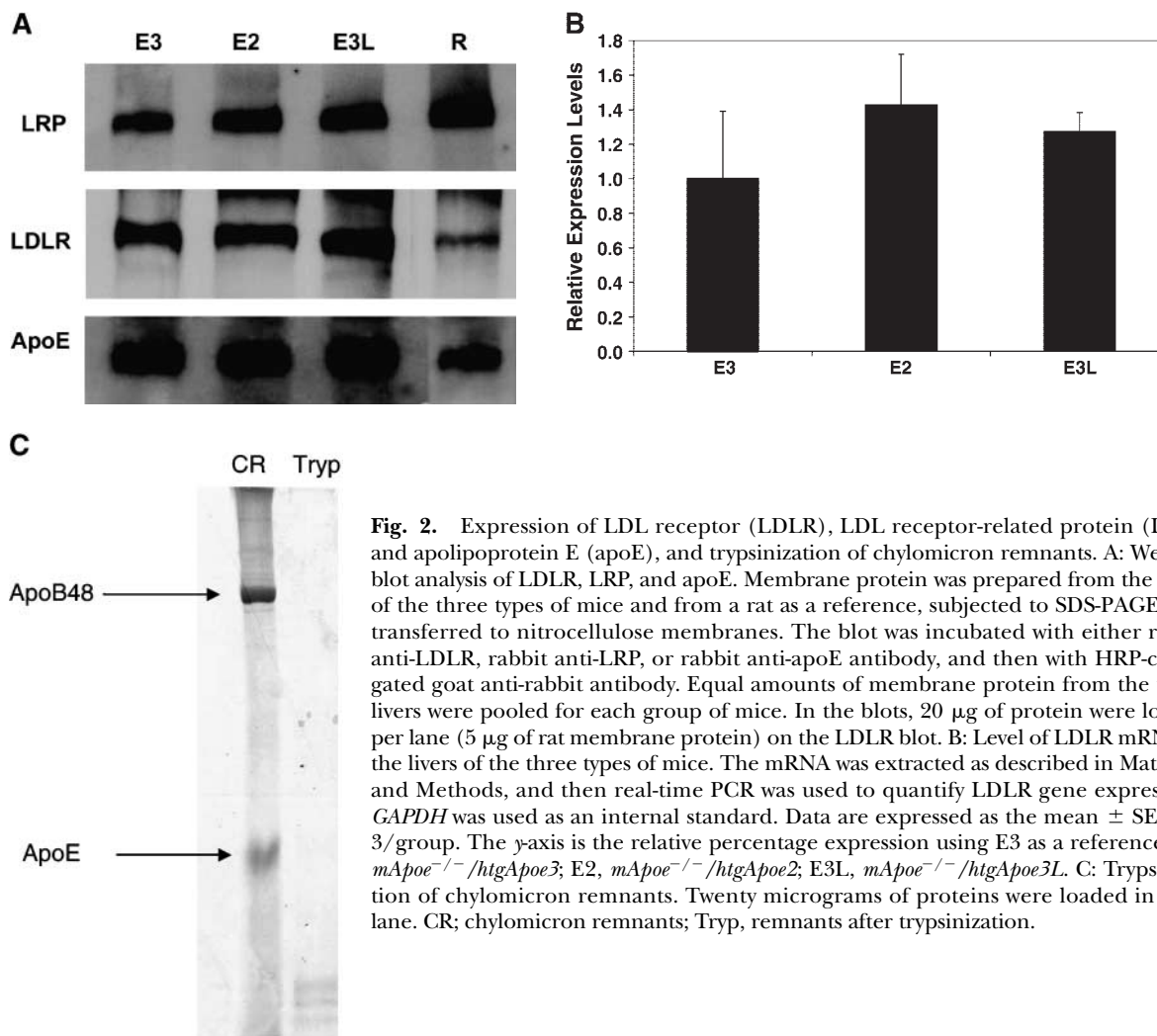### Labeling chylomicron remnants

Chylomicron remnants were labeled with carrier-free Na^{125}I (Amersham Life Sciences Arlington Heights, IL) using a modification (38) of the iodine monochloride method (39). Iodinated chylomicron remnants (^{125}I-labeled chylomicron remnants) were applied to a PD-10 desalting column (Sephadex G-25; Amersham Pharmacia Biotech AB, Uppsala, Sweden) and dialyzed with PBS (pH 7.4) for 1 h to remove free iodine before use. Alternatively, chylomicron remnants were labeled with the fluorescent carbocyanine dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) (Molecular Probes, Inc., Eugene, OR), as described previously (26).

Trypsin treatment and apoE reconstitution of chylomicron remnants

Chylomicron remnants were digested with trypsin using a modification of the method described by Borensztajn et al. (40). Briefly, bovine pancreatic trypsin (Sigma) was added to chylomicron remnants at a concentration of 1:100 and incubated for 2 h at room temperature. Proteolysis was terminated by the addition of tosyl-L-lysine chloromethyl ketone, at 1.5 times the concentration of trypsin, in 0.01% EDTA. The digested chylomicron remnants were isolated by ultracentrifugation at 38,000 rpm at 10°C for 90 min, recentrifuged under the same conditions, and dialyzed overnight against PBS with 0.01% EDTA. For the reconstitution experiment, the three types of apoE were purified following the method previously described by Weisgraber and colleagues (41, 42). Dr. Weisgraber kindly provided the human apoE3 and E2 constructs. The apoE3-Leiden construct was a gift from Dr. Louis M. Havekes and Dr. Ko Willems van Dijk. Using purified apoE, the trypsinized remnants were reconstituted by incubating them at 37°C for 6 h. The reconstituted remnants showed similar apoE content per particle, as confirmed by SDS-PAGE. The trypsinized or reconstituted remnants were iodinated as described above. It was assumed that the iodine labeled the peptide fragments in the trypsinized remnants.
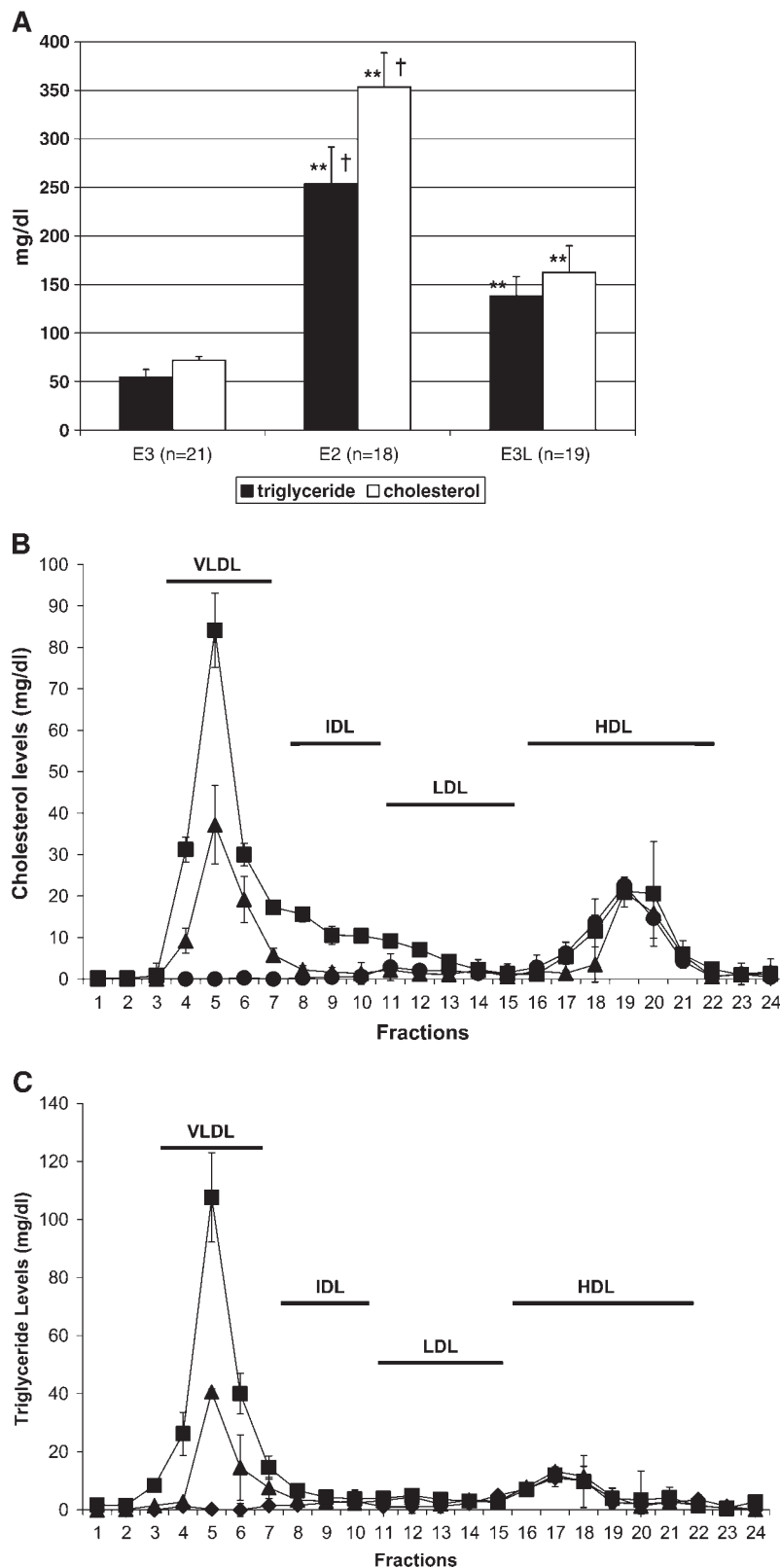
Fig. 1. Plasma lipid concentrations and lipoprotein profiles. The $mApoe^{-/-}/htgApoe3$ (E3), $mApoe^{-/-}/htgApoe2$ (E2), and $mApoe^{-/-}/htgApoe3L$ (E3L) mice were fed a normal chow diet. Blood was obtained from each group after a 6 h fast. A: The average plasma triglyceride and cholesterol levels in the three types of mice. **, $P < 0.001$ compared with $mApoe^{-/-}/htgApoe3$; †, $P < 0.05$ between $mApoe^{-/-}/htgApoe2$ and $mApoe^{-/-}/htgApoe3L$. B, C: Serum was fractionated using fast performance liquid chromatography, as described in Materials and Methods. The x-axis is the fraction number in milliliters; the y-axis is the cholesterol (B) and triglyceride (C) concentrations (mg/dl). Circle, $mApoe^{-/-}/htgApoe3$; triangle, $mApoe^{-/-}/htgApoe3L$; square, $mApoe^{-/-}/htgApoe2$. The data are shown as the mean ± SE.

Fig. 2. Expression of LDL receptor (LDLR), LDL receptor-related protein (LRP), and apolipoprotein E (apoE), and trypsinization of chylomicron remnants. A: Western blot analysis of LDLR, LRP, and apoE. Membrane protein was prepared from the livers of the three types of mice and from a rat as a reference, subjected to SDS-PAGE, and transferred to nitrocellulose membranes. The blot was incubated with either rabbit anti-LDLR, rabbit anti-LRP, or rabbit anti-apoE antibody, and then with HRP-conjugated goat anti-rabbit antibody. Equal amounts of membrane protein from the three livers were pooled for each group of mice. In the blots, 20 μg of protein were loaded per lane (5 μg of rat membrane protein) on the LDLR blot. B: Level of LDLR mRNA in the livers of the three types of mice. The mRNA was extracted as described in Materials and Methods, and then real-time PCR was used to quantify LDLR gene expression. *GAPDH* was used as an internal standard. Data are expressed as the mean ± SE; n = 3/group. The *y*-axis is the relative percentage expression using E3 as a reference. E3, *mApoe$^{-/-}$/htgApoe3*; E2, *mApoe$^{-/-}$/htgApoe2*; E3L, *mApoe$^{-/-}$/htgApoe3L.* C: Trypsinization of chylomicron remnants. Twenty micrograms of proteins were loaded in each lane. CR; chylomicron remnants; Tryp, remnants after trypsinization.

Liver perfusion

The livers of 15-week-old mice were perfused using the single-pass nonrecirculating procedure previously described by our laboratory (43). The perfusate solution contained rat erythrocytes (20% hematocrit) in DMEM and was gassed with 20% O_2. The liver was maintained at 37°C thermostatically. After a 5 min perfusion to remove blood from the liver, the perfusate solution containing ^{125}I-labeled or DiD-labeled remnants was perfused into the liver via the portal vein for 20 min at 0.5 ml/min. For kinetic experiments, samples were collected at 1 min intervals to measure the radioactivity. To prepare samples for immunohistochemistry, DiD-labeled remnants were perfused for 20 min, and then a 0.9% NaCl solution was perfused for 5 min after the 20 min perfusion. In some experiments, 4 μg/ml of receptor-associated protein (RAP) was added to the perfusate to inhibit LRP binding, as described previously (44). After the perfusion with DiD-labeled remnants, the livers were sliced into small pieces and fixed in PBS with 4% paraformaldehyde for 15 min and in PBS containing 20% sucrose for 16 h. Tissue blocks were embedded in optimum cutting temperature (OCT) compound, and 8 μm sections were cut and placed on glass slides.

Immunohistochemistry and confocal microscopy

The liver sections were first incubated in PBS + 0.1% Triton X-100 (5 min), then in PBS containing 5% BSA and 5% rabbit serum (30 min), and finally with the antibodies. LRP was stained with a previously prepared rabbit antibody that recognized the LRP amino

acid sequence from 1961 to 2120. This antibody was designed to specifically recognize only the extracellular domain of LRP. After incubation with the anti-LRP, the sections were incubated with Oregon Green (OG)-labeled goat anti-rabbit antibody. Digital images of the stained sections were obtained using a Molecular Dynamics Multiprobe confocal laser microscope (Sunnyvale, CA). DiD was excited at 644 nm, and OG was excited at 488 nm. A filter >660 nm was used to collect the DiD emissions (channel 1: red) and a 500–560 nm filter was used for the OG emissions (channel 2: green). Nitrocellulose membranes incubated with either OG-labeled antibody or DiD-labeled remnants were used to check for bleed-through between the two wavelengths. Colocalized pixels were determined and the number of clusters counted as described previously (26).
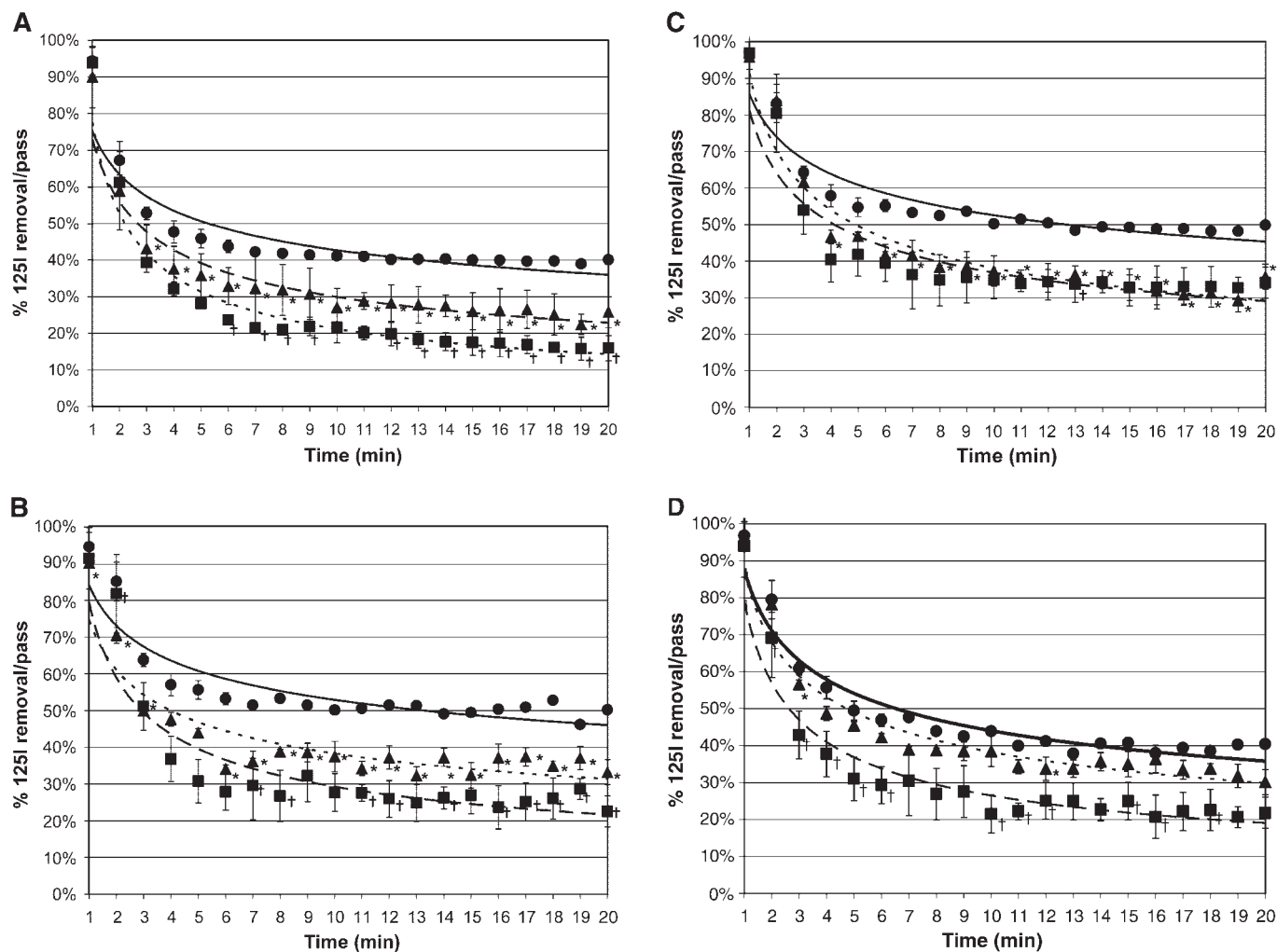
Data analysis

The data are expressed as the mean ± SD unless otherwise indicated. Student's *t*-test was performed for two-group comparisons. Values were considered statistically significant at $P < 0.05$.

RESULTS

Plasma lipid levels

Plasma lipid concentrations were measured after 6 h of fasting in the blood of animals fed a normal chow diet.

Fig. 3. Removal of trypsinized chylomicron remnants by the liver of mice expressing three different types of apoE. Radiolabeled ^{125}I remnants were added (0.5 ml/min) to the perfusate of isolated livers from each type of mouse for a total of 20 min, as described in Materials and Methods. At 1 min intervals, the perfusate leaving the liver was collected and the radioactivity remaining was calculated using the following equation: percent ^{125}I removal per pass = (cpm entering liver−cpm leaving liver) × 100/(cpm entering liver). Circle, *mApoe$^{-/-}$/htgApoe3*; triangle *mApoe$^{-/-}$/htgApoe3L*; square, *mApoe$^{-/-}$/htgApoe2*. A: Perfusion of 40 μg cholesterol/ml; n = 4, 4, and 5 for human apoE3, E2, and E3L transgenic mice, respectively. B: Perfusion of 20 μg cholesterol/ml; n = 3/group. C: Perfusion of 6 μg cholesterol/ml; n = 3/group. D: Perfusion of 6 μg cholesterol/ml with added receptor-associated protein (RAP) (4 μg/ml) to the perfusate; n = 3/group. *, $P <$ 0.05 between *mApoe$^{-/-}$/htgApoe3* and *mApoe$^{-/-}$/htgApoe3L*; †, $P <$ 0.05 between *mApoe$^{-/-}$/htgApoe2* and *mApoe$^{-/-}$/htgApoe3L*. The lines are power trend curves produced using Excel software. The data are expressed as the mean ± SD.

The *mApoe$^{-/-}$/htgApoe2* and *mApoe$^{-/-}$/htgApoe3L* mice developed hyperlipidemia, but to different degrees; mild hyperlipidemia was present in *mApoe$^{-/-}$/htgApoe3L* mice, and more-severe hyperlipidemia was present in *mApoe$^{-/-}$/htgApoe2* mice. As previously reported (30), for mice on a normal diet, fasting plasma triglyceride and cholesterol levels were significantly higher in A *mApoe$^{-/-}$/htgApoe3L* mice than in *mApoe$^{-/-}$/htgApoe3* mice and levels in *mApoe$^{-/-}$/htgApoe2* mice were significantly elevated as compared with either *mApoe$^{-/-}$/htgApoe3* or *mApoe$^{-/-}$/htgApoe3L* mice (**Fig. 1A**). VLDL triglyceride and VLDL–intermediate density lipoprotein cholesterol levels were increased in *mApoe$^{-/-}$/htgApoe2* mice and more moderately increased in *mApoe$^{-/-}$/htgApoe3L* mice (Fig. 1B, C). HDL lipids were only moderately altered. These transgenic mice expressing human apoE2 and apoE3-Leiden appeared to have altered apoB

lipoprotein metabolism and accumulate triglyceride-rich lipoproteins.

LDLR, LRP, apoE proteins, and mRNA levels

The LDLR, LRP, and apoE levels in liver membranes were quantified by Western blotting (**Fig. 2A**), which revealed that LDLR, LRP, and apoE were present at similar levels in liver membranes from all three strains of transgenic mice. The amount of apoE in the transgenic mice was, if anything, higher than in the normal mice. LDLR expression was quantified using real-time PCR (Fig. 2B), and LDLR mRNA levels were normalized to GAPDH mRNA. The amount of LDLR mRNA was increased by ~40 and 30% in *mApoe$^{-/-}$/htgApoe2* and *mApoe$^{-/-}$/htgApoe3L* livers, respectively, but the difference was not statistically significant as compared with *mApoe$^{-/-}$/htgApoe3* livers. Simi-
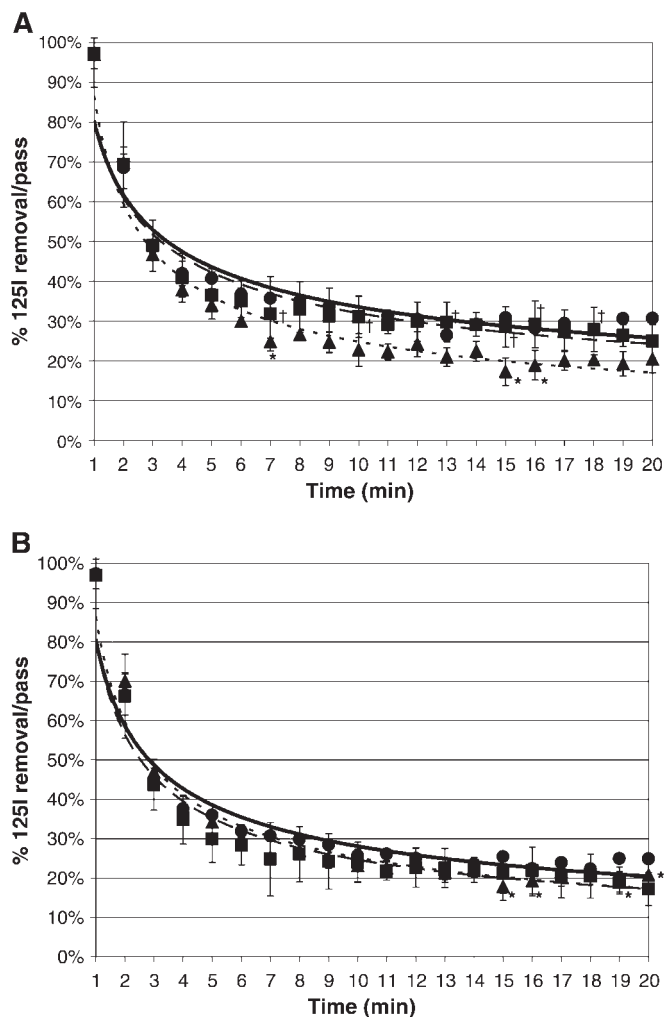
Fig. 4. Removal of trypsinized chylomicron remnants from the perfusate by the livers of LDLR-deficient mice expressing three different types of apoE. The experiment was carried out exactly as described in the legend to Fig. 3, except that each mouse strain also lacked the LDLR. Circle, *mApoe⁻/⁻/mldlr⁻/⁻/htgApoe3*; triangle, *mApoe⁻/⁻/mldlr⁻/⁻/htgApoe3L*; square, *mApoe⁻/⁻/mldlr⁻/⁻/htgApoe2*. A: Perfusion of 6 μg cholesterol/ml; n = 4/group. B: Perfusion of 6 μg cholesterol/ml plus 4 μg/ml of RAP; n = 3/group. *, P < 0.05 between *mApoe⁻/⁻/mldlr⁻/⁻/htgApoe3* and *mApoe⁻/⁻/mldlr⁻/⁻/htgApoe3L*; †, P < 0.05 between *mApoe⁻/⁻/mldlr⁻/⁻/htgApoe2* and *mApoe⁻/⁻/mldlr⁻/⁻/htgApoe3L*. The lines are power trend curves generated with Excel software. Each data point represents the mean ± SD.

larly, mRNA levels for apoE were not different among the strains. Thus, the accumulation of apoB-rich lipoproteins in the plasma of *mApoe⁻/⁻/htgApoe2* and *mApoe⁻/⁻/htgApoe3L* mice was certainly not due to reduced receptors or altered apoE levels, but was more likely due either to a failure of the lipoprotein to enter the liver as effectively as in normal liver of *mApoe⁻/⁻/htgApoe3* mice or to a reduced production of the lipoprotein by the liver of apoE2 and apoE3-Leiden mice.

Uptake of normal chylomicron remnants by perfused liver

To determine the ability of livers with different apoE variants to remove chylomicron remnants, radiolabeled chy-

lomicron remnants were prepared from normal rats and perfused into the livers of human apoE3, apoE2, and apoE3-Leiden mice using a single noncirculating perfusion. In each perfusion, the rate of removal reached a steady state after ∼10 min, and the rate of removal per pass was determined at this stage. The nonspecific removal measured using radiolabeled BSA was ∼10% per pass as previously described (43). When the livers of the three apoE-variant mice were perfused with chylomicron remnants (4 μg protein/ml perfusate), the removal of ¹²⁵I was ∼45–50% per pass for all three liver types, and did not differ significantly in the three (data not shown). This demonstrated that the secreted apoE isoforms did not interfere with normal remnant uptake.

Uptake of trypsinized chylomicron remnants by perfused livers in the three strains of apoE transgenic mice

To examine the effect of hepatic apoE variants on remnant uptake, the apoE from the remnant particles was depleted by trypsinization, as described previously (44). The trypsinized remnants lacked an apoE protein band on SDS-PAGE gels (Fig. 2C). Under electron microscopy, the trypsinized remnants were smaller than chylomicrons and of a size similar to that of normal remnants. The structural integrity of the trypsinized remnants was not distinguishable from the nascent remnant particles (data not shown). These trypsinized remnants, now free of normal apoE, were radiolabeled with ¹²⁵I. The ¹²⁵I labeled the small peptides.

It has previously been shown that such particles, when perfused at various concentrations, are efficiently removed by normal livers but not by the livers of *mApoe⁻/⁻* mice (44). These particles were perfused into the livers of *mApoe⁻/⁻/htgApoe3*, *mApoe⁻/⁻/htgApoe2*, and *mApoe⁻/⁻/htgApoe3L* mice. At a concentration of 40 μg cholesterol/ml perfusate, differences in remnant removal, as measured using the ¹²⁵I removal per pass, were apparent and were ∼40, 30, and 20%/pass for *mApoe⁻/⁻/htgApoe3*, *mApoe⁻/⁻/htgApoe2*, and *mApoe⁻/⁻/htgApoe3L* mice, respectively (**Fig. 3A**). At a lower concentration (20 μg cholesterol/ml perfusate), the three types of liver cleared particles with ∼10%/pass greater efficiency, resulting in rates of ¹²⁵I removal of ∼50, 35, and 25%/pass, respectively (Fig. 3B). At low remnant concentrations (6 μg cholesterol/ml), the removal was greater by *mApoe⁻/⁻/htgApoe3* livers than *mApoe⁻/⁻/htgApoe3L* and *mApoe⁻/⁻/htgApoe2* livers (Fig. 3C). Therefore, at high remnant concentrations, the rate of removal was related to the degree of hyperlipidemia of each mouse type, while at trace concentrations, removal was similar in both *mApoe⁻/⁻/htgApoe3L* and *mApoe⁻/⁻/htgApoe2* livers. This indicated that both the LDLR and the LRP pathways could facilitate rapid remnant removal; we therefore performed the rest of the experiment using the low concentration (6 μg cholesterol/ml) to test the independent contributions of the two pathways.

Effect of RAP on remnant removal

To investigate the contributions of the different pathways, the LRP pathway was blocked by adding RAP (4 μg/ml) during 6 μg cholesterol/ml of trypsinized remnant per-
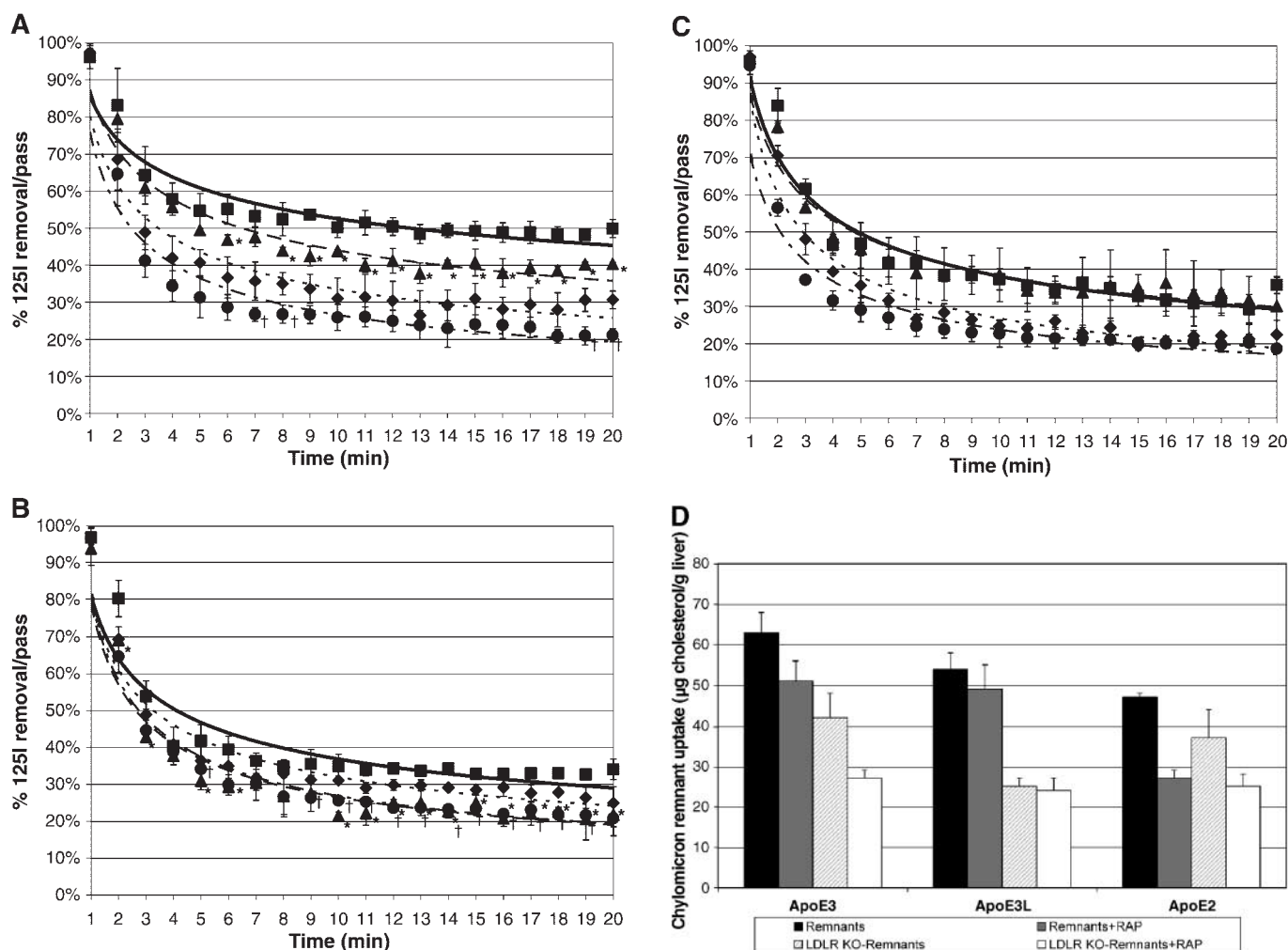
Fig. 5. Removal of different concentrations of trypsinized chylomicron remnants by the liver of mice expressing three human apoE types. All perfusions were performed using 6 μg cholesterol/ml perfusate. Square, $mApoe^{-/-}/htgApoe$ livers perfused with chylomicron remnants alone; triangle, $mApoe^{-/-}/htgApoe$ livers perfused with chylomicron remnants + 4 μg/ml of RAP; diamond, $mApoe^{-/-}/mldlr^{-/-}/htgApoe$ livers perfused with chylomicron remnants alone; circle, $mApoe^{-/-}/mldlr^{-/-}/htgApoe$ livers perfused with chylomicron remnants + 4 μg/ml of RAP. A: Perfusion of human apoE3 transgenic mice. B: Perfusion of human apoE2 transgenic mice. C: Perfusion of human apoE3-Leiden transgenic mice.*, $P < 0.05$ between $mApoe^{-/-}/htgApoe$ livers perfused with chylomicron remnants alone and $mApoe^{-/-}/htgApoe$ livers perfused with chylomicron remnants + 4 μg/ml of RAP; †, $P < 0.05$ between $mApoe^{-/-}/mldlr^{-/-}/htgApoe$ livers perfused with chylomicron remnants alone and $mApoe^{-/-}/mldlr^{-/-}/htgApoe$ livers perfused with chylomicron remnants + 4 μg/ml of RAP. The lines are power trend curves produced using Excel software. The data are expressed as the mean ± SD. D: Liver uptake of chylomicron remnants. After perfusion, total radioactivity present was counted. The total amount of remnant uptake (μg) per g liver was calculated by dividing total radioactivity (cpm) per g dried liver by the specific radioactivity of the remnants. Solid bar, 6 μg cholesterol/ml perfusion in each mouse; dark gray bar, 6 μg cholesterol/ml + 4 μg/ml of RAP in each mouse; light gray bar, 6 μg cholesterol/ml in LDLR-deficient background mice; white bar, 6 μg cholesterol/ml + 4 μg/ml of RAP in LDLR-deficient background mice.

fusion. This concentration largely inhibits the LRP pathway and does not affect LDLR activity, as previously described by our group (43, 44), or in cell culture (21). The addition of RAP inhibited remnant uptake in both $mApoe^{-/-}/htgApoe3$ and $mApoe^{-/-}/htgApoe2$ livers, whereas it did not affect remnant uptake in $mApoe^{-/-}/htgApoe3L$ livers (Fig. 3D). These data suggest that the LRP pathway does not mediate remnant removal in the $mApoe^{-/-}/htgApoe3L$ livers. Furthermore, it suggests that in $mApoe^{-/-}/htgApoe2$ livers, the LRP pathway is predominant in remnant removal.

Na-heparin (4 μg/ml) was added to the perfusate to examine the role of HSPG in remnant removal. By occupy-ing the HSPG binding site, heparin reduces the removal of ligands, such as FGF, that require HSPG for removal (45, 46). Unlike RAP addition, 4 μg/ml of heparin affected remnant removal rates only marginally in mice deficient in LDLR (data not shown).

Remnant removal by livers lacking mouse apoE and LDLR but expressing human apoE2, apoE3, or apoE3-Leiden

Remnant removal by LDLR-deficient, mouse apoE-deficient livers that expressed the apoE variants was studied as in the previous section. The removal rates were ~28–30%/pass for $mApoe^{-/-}/mldlr^{-/-}/htgApoe3$ and $mApoe^{-/-}/$
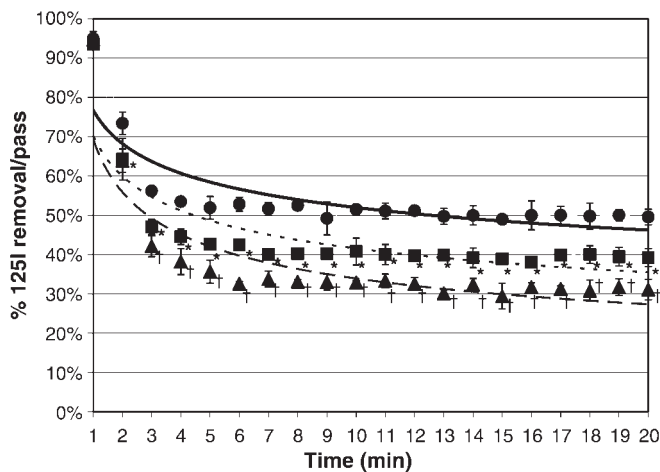
Fig. 6. Removal of reconstituted remnants from the perfusate by the livers of mice lacking mouse apoE and LDLR and expressing human LDLR. The experiment was carried out exactly as described in the legend to Fig. 3, except that the trypsinized remnants were reconstituted with purified human apoE3, apoE2, or apoE3-Leiden, respectively. The livers of mice deficient in both apoE and LDLR that expressed the human LDLR under control of the albumin promoter ($mApoe^{-/-}/mldlr^{-/-}/htgLdlr$) were perfused with these particles (6 μg cholesterol/ml). Circle, remnants containing apoE3; triangle, remnants containing apoE3-Leiden; square, remnants containing apoE2. n = 5, 3, and 4 for apoE3, apoE2, and apoE3-Leiden particles, respectively. *, $P < 0.05$ between apoE3 and apoE3-Leiden remnants; †, $P < 0.05$ between apoE2 and apoE3-Leiden remnants. The lines are power trend curves generated using Excel software. Each data point represents the mean ± SD.

$mldlr^{-/-}/htgApoe2$ livers, and 20%/pass for $mApoe^{-/-}/mldlr^{-/-}/htgApoe3L$ livers (**Fig. 4A**). Therefore, the impact of a lack of LDLR was greatest on apoE3-Leiden removal, with less effect on apoE2 and apoE3 removal. Addition of RAP to the perfusate of LDLR-deficient livers reduced the uptake by $mApoe^{-/-}/mldlr^{-/-}/htgApoe3$ and $mApoe^{-/-}/mldlr^{-/-}/htgApoe2$ livers to that of $mApoe^{-/-}/mldlr^{-/-}/htgApoe3L$ livers (Fig. 4B). Together, these results are consistent with the hypothesis that remnants with apoE2 are removed primarily by LRP and those with apoE3-Leiden are removed primarily by the LDLR.

Comparison of the effect of LDLR and LRP on remnant removal in livers of mice expressing apoE3, apoE2, and apoE3-Leiden by strain

The results of the perfusion experiments were plotted by strain; thus, a difference in the amount of apoE synthesized by the different strains is not a factor. Eliminating either the LDLR or the LRP reduced remnant removal by the apoE3 livers, and removing both had an additive effect on remnant removal (**Fig. 5A**). This is similar to what we previously reported for normal mice (43, 44). In contrast, in the liver of mice expressing apoE2, lack of the LDLR had no effect on remnant removal, but the inhibition of LRP did (Fig. 5B). The converse was seen with the liver of mice expressing apoE3-Leiden, where absence of the LDLR reduced removal significantly but inhibiting LRP had an insignificant effect (Fig. 5C). The amount of

radioactivity that accumulated in the livers after perfusion was measured. It confirmed the above conclusions (Fig. 5D).

Uptake of reconstituted remnants with purified apoE in the $mApoe^{-/-}/mldlr^{-/-}/htgLdlr$ mouse

The preceding studies relied on the mouse LDLR for lipoprotein removal. To exclude a species-specific apoE–LDLR interaction, the uptake of remnants by livers expressing the human LDLR was investigated. To do this, trypsinized remnants were reconstituted with each apoE type, and these particles were perfused into the livers of apoE and LDLR knockout mice containing the human LDLR under the control of the albumin promoter ($mApoe^{-/-}/mldlr^{-/-}/htgLdlr$). The reconstituted particles contained similar amounts of apoE per particle on SDS gel electrophoresis (0.5–0.7 μg apoE per μg cholesterol; data not shown). The uptake of remnants via the human LDLR was similar to the uptake via the mouse LDLR (**Fig. 6**). Remnant removal was ∼50, 40, and 33% per pass for apoE3, E3-Leiden, and E2 particles, respectively. This was similar to the results with mouse LDLR, although there was a reverse in the relative potency of apoE2 and apoE3-Leiden livers.

Clustering of chylomicron remnants in the space of Disse

Previously, we reported that chylomicron remnants bound to LRP clustered in the space of Disse (26). If the postulate of the previous studies is correct, the $mApoe^{-/-}/htgApoe3L$ mice should not have remnant clusters with the LRP, while $mApoe^{-/-}/htgApoe2$ livers should rely heavily on clustering for remnant removal, even in the presence of LDLR. Livers were perfused with DiD-labeled, trypsinized remnants (6 μg cholesterol/ml perfusate), and sections were stained with rabbit anti-LRP antibody followed by OG-labeled goat anti-rabbit IgG, as previously described. In both $mApoe^{-/-}/htgApoe3$ and $mApoe^{-/-}/htgApoe2$ livers, distinct clusters of remnants appeared on the surface in close proximity to endothelial cells, similar to the pattern previously shown in livers of all $mldlr^{-/-}$ mice (26) (data not shown). There were 1.52 ± 0.33 and $3.00 \pm 0.40/10,000$ μm² remnant clusters for apoE3 (n = 6) and apoE2 (n = 5), respectively. When LDLR was absent, the number of clusters increased significantly to 2.06 ± 1.17 (n = 4) and $4.57 \pm 0.92/10,000$ μm² (n = 5) for apoE3 and apoE2 livers, respectively. Virtually no clusters were found in the apoE3-Leiden livers ($0.36 \pm 0.25/10,000$ μm², n = 13) in the presence or absence of LDLR. In $mApoe^{-/-}/mldlr^{-/-}/htgApoe3L$ mice (**Fig. 7**), the small amount of remnant fluorescence was distributed diffusely throughout the cytosol. The cellular location of LRP also differed in the livers of $mApoe^{-/-}/mldlr^{-/-}/htgApoe3L$ mice; LRP was relatively evenly distributed as thin strings on the cell surface, with little in the cytosol, and was not colocalized with remnant clusters. No apparent difference in the clustering pattern was found between $mApoe^{-/-}/mldlr^{-/-}/htgApoe3$ and $mApoe^{-/-}/mldlr^{-/-}/htgApoe2$ livers; the LRP on the cell surface was colocalized with the clusters. LRP was also sometimes found in the nucleus. These results provide further support for the postulate that there is defective remnant uptake by LRP in $mApoe^{-/-}/mldlr^{-/-}/htgApoe3L$
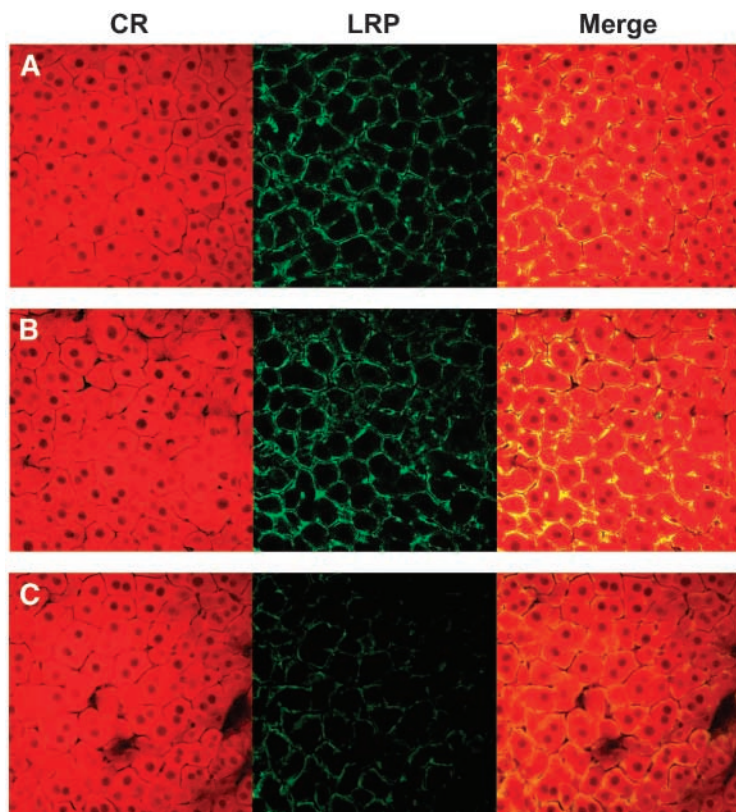
Fig. 7. The distribution of LRP and chylomicron remnant clusters in the livers of mice deficient in LDLR expressing three different types of apoE. Livers were perfused with 6 µg cholesterol/ml of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD)-labeled remnants. Tissue sections were incubated with Oregon Green (OG)-labeled anti-LRP antibody and examined using confocal microscopy, and digital images were obtained as described in Materials and Methods. The uptake of DiD remnants (CR; red), OG-LRP (LRP; green), and the merged red and green images are shown from left to right. A: A representative image of an $mApoe^{-/-}/mldlr^{-/-}/htgApoe3$ liver section. B: Uptake of DiD remnants and clustering of LRP in an $mApoe^{-/-}/mldlr^{-/-}/htgApoe2$ liver section. C: Uptake of DiD remnants and clustering of LRP in an $mApoe^{-/-}/mldlr^{-/-}/htgApoe3L$ liver section.

mice because of reduced binding to the LRP and that apoE2 mice rely heavily on the LRP for removal, even in the presence of the LDLR.

DISCUSSION

The results of the present study establish that lipoprotein particles containing human apoE3, like those with mouse and rat apoE, are efficiently removed by the liver via two quite distinct pathways. Removal is initiated by a process requiring either the LDLR or the LRP. By contrast, livers secreting two apoE variants, apoE2 and apoE3-Leiden, remove remnants primarily by only one of the pathways. The LDLR pathway is the most efficient and leads to rapid internalization of the whole particle; the LRP-mediated pathway is somewhat less efficient, and initially leads to the sequestration of particles in the space of Disse in hepatic sinusoids. It is now established that apoE3-Leiden is removed only by the LDLR pathway while apoE2 is removed primarily by the LRP-mediated pathway.

Previously, we demonstrated that at low remnant concentrations, there is efficient removal of remnants regardless of the pathway used (43, 44). Consistent with this formulation, in the current experiments, at low remnant concentrations, all three apoE variants mediated removal efficiently. As the particle concentration increased, differences in the efficiency of removal mediated by the variants emerged. ApoE3 was the most effective, followed by apoE3-Leiden and then apoE2. It has been reported that apoE from the liver is more effective than peripheral apoE in

mediating liver uptake (47). In the system used in these experiments, the contribution of apoE acquired in the periphery was eliminated and all uptake was dependent on the acquisition of apoE in the liver, where the level of expression of apoE was comparable among the strains. To further exclude effects of apoE origin or concentration in the liver, experiments using recombinant apoE and livers that expressed only the human LDLR were carried out. In these experiments, both of the variants were also less effective than apoE3, while apoE2 was removed somewhat more efficiently than apoE3-Leiden. This may reflect the human state more closely and established that the differences between the strains were not due to a difference in the amount of apoE on the particle or in the liver, or to a different affinity of the mouse and human LDLRs for the different isoforms.

Remnant removal by apoE3-Leiden–secreting livers is insensitive to RAP, and remnant removal by apoE2-secreting livers is much less sensitive to the presence or absence of the LDLR than is remnant removal by apoE3-secreting livers. Thus, it is concluded that apoE2 is removed primarily by the LRP. This is consistent with studies that demonstrated no binding of this protein to the LDLR in cultured cells (21). Interestingly, although it was reported that there is virtually no binding of apoE2 to the LDLR (48), in the current experiments, elimination of the LDLR further decreased the rate of removal of apoE2 particles moderately, suggesting that some LDLR-mediated removal occurred in vivo.

Type III hyperlipidemia is characterized by elevated plasma lipid levels resulting from the accumulation of chylomicron remnants and β-VLDL. The disease is related to

genetic defects of apoE, which is the ligand involved in chylomicron remnant uptake, because several naturally occurring apoE alleles are associated with the syndrome (4–6, 49). In addition to apoE2 (Arg158→Cys) and apoE3-Leiden, other rare apoE variants, such as apoE3 (Arg142→Cys), apoE2 (Lys146→Gln), and apoE2 (Lys146→Asn), are associated with this disease. ApoE2 causes recessive hyperlipidemia in humans and is of variable penetrance; when present, the hyperlipidemia is generally severe. In $mApoe^{-/-}/htgApoe2$ mice, hyperlipidemia was present uniformly and was more severe than in $mApoe^{-/-}/htgApoe3L$ mice. The species difference suggests that the mouse normally has less capacity for LRP-mediated removal relative to LDLR-mediated removal than humans or that the LRP pathway is less effective in mice.

Other studies have found that the low level of hepatic lipase present in the mouse accounts in part for the less-efficient LRP-mediated removal (50). In humans and rats, unlike the mouse, hepatic lipase is bound primarily to the liver cell surface and does not occur in the circulation. Therefore, it is easy to envision how a low-affinity pathway with a relatively high capacity could be very sensitive to changes in the rate of production of the particles, and a modest increase in the production of particles or other competitors for removal by LRP could easily result in conversion from the normal state to a severe abnormality.

By contrast, removal by apoE3-Leiden was profoundly sensitive to the presence of the LDLR. Because apoE3-Leiden-mediated removal was not sensitive to RAP, it is reasonable to conclude that apoE3-Leiden removal does not proceed to any appreciable extent via LRP. This is a bit surprising in light of binding studies, which did demonstrate some binding of apoE3-Leiden to LRP (51). Perhaps with a low-affinity ligand, even a modest further reduction in the affinity is sufficient to eliminate any physiologically relevant binding. The fact that the dyslipidemia of apoE3-Leiden is relatively mild, but dominant, suggests that apoE3-Leiden may interfere with the ability of normal apoE to interact with receptors and that some remnant removal always occurs via LRP. Whether this represents a subpopulation of remnant particles that cannot be removed by the LDLR requires further investigation.

ApoE also binds to HSPG. This may either assist the LRP-dependent pathway (52–54) or mediate non-LRP-dependent HSPG uptake (55). Heparinase injection into the liver (56) eliminates remnant removal but disrupts hepatic architecture. Heparin incubation with macrophages (57) reduces chylomicron remnant uptake, suggesting the importance of HSPG in remnant catabolism (26). Characterization of HSPG binding to different apoE variants demonstrated that the HSPG binding activity is decreased by mutations of Arg-136, Arg-142, Arg-145, and Lys-146 (58). It was suggested that apoE variants known to have defective HSPG binding activity are strongly associated with dominant inheritance and decreased onset age of type III hyperlipidemia. However, the LDLR binding site and the HSPG binding site in apoE overlap, and variants that have reduced HSPG binding also have low affinity for the LDLR. Heparin, at a concentration that could partially saturate the HSPG binding sites on apoE, lowered remnant removal moderately; but a higher concentration of heparin, at which cell binding sites would be completely saturated, could not be studied, for technical reasons. Thus, our experiments do not exclude the role of HSPG in the remnant removal process.

One of the most striking results was seen in the experiment using fluorescent remnants. Normally, there are few clusters of fluorescence in the liver following the perfusion of labeled remnants unless the LDLR is absent. In the livers of apoE2 mice, clusters that colocalized with LRP were abundant, and this seemed to be the primary pathway for removal. There was some direct uptake of fluorescent remnants in the livers of apoE2 mice, possibly the result of residual binding and internalization by the LDLR or of direct uptake by the LRP. In the apoE3-Leiden mice, there were virtually no clusters, even in the absence of the LDLR. This adds strong support to the evidence provided by the removal experiments that defect with apoE3-Leiden is a failure to bind to the LRP. Together, these results strengthen the observation that sequestration in the space of Disse is a physiologic phenomenon.

In summary, these experiments studied the removal of remnant lipoproteins mediated by human apoE variants using intact livers. The results suggest that the degree of interaction of apoE binding to the lipoprotein receptors, LDLR and LRP, explains the variability in the inheritance of hyperlipidemia. At low lipoprotein concentrations, there is little lipoprotein accumulation. In both humans and mice, the inability of apoE3-Leiden to bind to the LRP causes mild, but dominant, hyperlipidemia. In the mouse, because the LDLR removal pathway is dominant, the hyperlipidemia of apoE2 becomes more profound. This implies that in humans, the LRP may play a relatively greater role in remnant removal, and that only when it is saturated does hyperlipidemia become manifest.

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