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Rapid initial removal of chylomicron remnants by the mouse liver does not require hepatically localized apolipoprotein E

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Abstract Apolipoprotein E (apoE) is a ligand for the low density lipoprotein receptor (LDLR) and the low density lipoprotein receptor-related protein (LRP). The aim of the present study was to clarify the role of hepatically localized apoE in the rapid initial removal of chylomicron remnants by using the isolated perfused liver. Radiolabeled chylomicron remnants were perfused in a single nonrecirculating pass into the livers of C57BL/6J (wild-type) mice, apoE-knockout mice, and apoE/LDLR-knockout mice for a period of 20 min. Aliquots of the perfusate leaving the liver were collected at regular intervals and the rate of removal of radioactivity was determined. At a trace concentration of chylomicron remnants (0.05 μg of protein per ml), wild-type mouse livers removed at a steady state of 50–55% of total chylomicron remnants perfused per pass; livers from apoE-knockout mice had the same capacity as wild-type mouse livers. When the concentration of remnants was increased to 12 μg of protein per ml, a level at which it has been shown that LDL receptor and LRP are near saturation, the capacity of the wild-type mouse livers to remove chylomicron remnants was decreased to 10–25% per pass, confirming that the removal mechanisms were nearing saturation. However, instead of finding a greater reduction in the removal rates or impairment in chylomicron remnant removal, livers from apoE-knockout mice were just as efficient as those from wild-type mice in removing remnants. Livers of mice that lacked both apoE and the LDLR also had a similar rate of removal at relatively low remnant concentrations (0.05–0.5 μg/ml), but had reduced capacity in removing remnants at a relatively high concentration (4–12 μg/ml) of chylomicron remnants (~20% per pass). The rate of removal at these concentrations, however, was similar to that attributed to the LRP in previous studies. Chylomicron remnants, whose apolipoproteins were disrupted by trypsinization, were removed at a normal rate by wild-type mouse livers but there was almost no removal by apoE-knockout mouse livers. At higher concentrations, however, the removal of apolipoprotein-disrupted chylomicron remnants was decreased. Our present findings do not support the hypothesis that hepatically localized apoE is a critical factor in the rapid initial removal of chylomicron remnants by either of the major pathways but do suggest that hepatically localized apoE can be added to lipoproteins to accelerate their uptake, although this process may have a limited capacity to compensate for apoE deficiency on lipoproteins.—Yu, K. C-W., Y. Jiang, W. Chen, and A. D. Cooper. **Rapid initial removal of chylomicron remnants by the mouse liver does not require hepatically localized apolipoprotein E.** ***J. Lipid Res.*** **2000.** 41: **1715–1727.**

Supplementary key words chylomicron remnants • low density lipoprotein receptor • low density lipoprotein receptor-related protein • atherosclerosis

Apolipoprotein E (apoE) is a major constituent of several plasma lipoproteins and serves as a ligand for receptors that mediate the removal of these particles from the circulation (1, 2) and for their binding to plasma membrane (3). The importance of apoE in the metabolism of chylomicron remnants and very low density lipoprotein (VLDL) remnants has been well established by several lines of evidence. The cellular uptake of remnant lipoproteins is mediated by the low density lipoprotein receptor (LDLR) and the LDLR-related protein (LRP) through the interaction with apoE (4–8). The absence of apoE or the presence of defective forms of apoE can result in type III hyperlipoproteinemia, which is the accumulation of VLDL remnants and chylomicron remnants in the plasma (2, 9). Genetically produced apoE-knockout mice develop hypercholesterolemia and have premature atherosclerosis (10, 11).

There is accumulating evidence that postprandial lipoproteins (chylomicron remnants) may be involved in the development of atherosclerosis, a hypothesis proposed by Zilversmit (12). Patients with coronary atherosclerosis or

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Abbreviations: apoE, apolipoprotein E; BMT, bone marrow transplantation; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; HSPG, heparan sulfate proteoglycans; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; LRP, LDL receptor-related protein; PBS, phosphate-buffered saline; RBC, red blood cells; TCA, trichloroacetic acid; VLDL, very low density lipoprotein.

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diabetes have abnormally prolonged residence of postprandial chylomicron remnants in their plasma, presumably because of defective removal (13–18). The principal organ that removes chylomicron remnants from the plasma is the liver, and any defects in the lipoprotein removal mechanisms may severely compromise the liver's ability to effectively control postprandial lipemia (19, 20). Past investigations have demonstrated that plasma removal of chylomicron remnants in vivo is dependent on the LDLR and the LRP (21–25). Several studies have suggested that the hepatic removal of remnants may be more complex than simple cellular internalization. It has been proposed that the initial step in the plasma clearance of chylomicron remnants by the liver is the sequestration of these lipoproteins in the space between the endothelial cells and the surface of the hepatocytes, called the space of Disse. The sequestration may be mediated by the family of heparan sulfate proteoglycans (HSPG) or hepatic lipase or both. After this the remnants may be modified before hepatic uptake via the LRP. This modification is postulated to involve the acquisition of additional apoE that is produced by the hepatocytes (26, 27) that are localized in the space of Disse (28) bound to HSPG on the cell surface (29). This hypothesis is based on in vitro studies that tested β-very low density lipoproteins (β-VLDL) (26, 30, 31), a cholesterol-enriched remnant lipoprotein of intestinal and hepatic origin (32). β-VLDL is rich in apoE but binds weakly to the LRP unless it is enriched with additional apoE (30, 33). These observations led to the proposal called the secretion-capture hypothesis (26, 30).

Ishibashi et al. (23) demonstrated that there was an impairment of plasma remnant lipoprotein removal in vivo in mice lacking apoE due to disruption of the apoE gene. Furthermore, transgenic mice made to overexpress apoE in the liver have a faster rate of plasma remnant lipoprotein removal in vivo than controls (34). In another study, Ji, Sanan, and Mahley (31) showed that the plasma clearance and hepatic uptake of exogenous apoE-enriched chylomicron remnants and apoE-enriched β-VLDL in vivo were impaired in mice after the intravenous administration of heparinase (which releases HSPG from the cell surface). van Dijk et al. (35) found that higher than normal serum levels of apoE were required for non-LDLR-mediated pathways, in particular, the catabolism of VLDL remnants. In marked contrast, Borensztajn and colleagues (36, 37) proposed that there are pathways for remnant lipoprotein removal that are independent of apolipoproteins but involve phospholipolysis, presumably mediated by lipoprotein or hepatic lipase.

Our laboratory has described the use of the isolated perfused mouse liver to investigate how chylomicron remnants are removed by the liver (38). In the present study, we sought to clarify the role of hepatic apoE secretion in the removal of chylomicron remnants by the liver. This was tested in livers of apoE-knockout mice and double apoE/LDLR-knockout mice by perfusing varying concentrations of chylomicron remnants. The percentage of radiolabeled chylomicron remnants removed from the perfusate and the liver uptake was determined. There was no significant difference in the chylomicron remnant removal rate by the livers of apoE-knockout mice compared with livers from wild-type mice, but the apoE/LDLR-knockout mouse livers showed a reduction, similar to that of the LDLR-knockout mouse (38), in their capacity to remove chylomicron remnants as concentration increased. When chylomicron remnants were trypsinized to disrupt apolipoproteins and these trypsinized chylomicron remnants were perfused into the livers of wild-type and apoE-knockout mice, the trypsinized chylomicron remnants were removed normally by livers from wild-type mice but removal was impaired in livers from apoE-knockout mice. Our present observations suggest that hepatic secretion and localized apoE is not critically required for the rapid initial removal of chylomicron remnants by either of the major removal pathways. Hepatically secreted apoE can contribute, at most to a degree, to the removal of lipoproteins that are deficient in apoE.

MATERIALS AND METHODS

Animals

C57BL/6J mice (wild type) and Sprague-Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA). ApoE-knockout (apo$E^{-/-}$) mice were originally a gift from E. Rubin (University of California at Berkeley, CA) and were bred in the animal facilities of the Research Institute (Palo Alto, CA). The production and profiles of the apoE-knockout mice have been previously described (10). The double-knockout mice homozygous for both the LDLR and apoE (apo$E^{-/-}$/$LDLR^{-/-}$) from the C57BL/6J strain background were purchased from Jackson Laboratories (Bar Harbor, ME). The animals were kept at 21–25°C and had free access to water and standard chow.

Preparation of chylomicron remnants

Chylomicrons were obtained from the lymph of lymph duct-cannulated rats as previously described (39, 40). The chylomicron remnants were prepared in functionally hepectectomized rats that were injected with chylomicrons (300 mg of triglyceride per kg of body weight) intravenously via the femoral vein (40). After 3 h, blood was obtained from the rats and the chylomicron remnants ($d < 1.006$ g/ml) were harvested by density gradient ultracentrifugation as previously described (41).

Radiolabeling of chylomicron remnants

Chylomicron remnants were labeled with carrier-free $Na^{125}I$ (Amersham Life Sciences, Arlington Heights, IL) by a modification (42) of the iodine monochloride method first described by McFarlane (43). Iodinated chylomicron remnants (^{125}I-labeled chylomicron remnants) were extensively dialyzed with several changes of phosphate-buffered saline (PBS, pH 7.4) for 20–24 h before use. More than 88% of radioactivity was associated with protein precipitated with 10% (w/v) trichloroacetic acid (TCA). The distribution of the ^{125}I label was 71.64 ± 4.64% ($n = 8$) on apoBs (determined by isopropanol precipitation) (44), 11.05 ± 2.12% ($n = 8$) on lipid (chloroform-methanol extraction), and the remainder on non-apoBs proteins (apoEs, apoAs).

Biochemical procedures

Cholesterol and triglyceride concentrations were determined with kits purchased from Sigma (St. Louis, MO). The concentra-

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tion of protein was determined with a microbicinchoninic acid test kit from Pierce (Rockford, IL).

Cell association and degradation of rat ^{125}I-labeled chylomicron remnants competed with mouse lipoproteins

Wild-type (C57BL/6J) mice and apoE-knockout mice were fed on a cocoa butter fat diet (lot 550654-000010; TestDiet, Richmond, IN) for 14 days and were fasted overnight before blood was drawn into tubes containing 0.05% ethylenediaminetetraacetic acid (EDTA). Plasma was obtained after low-speed centrifugation and lipoprotein (d < 1.006 g/ml) fractions were isolated by density gradient ultracentrifugation as previously described (41), and were dialyzed against PBS plus 0.05% EDTA before use.

Rat ^{125}I-labeled chylomicron remnants were competed against mouse lipoproteins in vitro and assayed for cell-associated radioactivity and degradation by previously described methods (45). Mouse L929 fibroblast cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (FCS). The FCS was replaced by bovine lipoprotein-deficient serum before the assays. ^{125}I-labeled chylomicron remnants (10 μg of protein per ml) were added to each well, together with increasing concentrations of mouse lipoprotein (d < 1.006 g/ml) fractions. After a 3-h incubation (humidified 5% CO_2 chamber, 37°C), the cells were chilled on ice. The medium was removed to determine ligand degradation as the amount of TCA-soluble radioactivity. The cells were washed once with PBS plus 0.2% bovine serum albumin (BSA), twice with PBS, and then dissolved in 0.1 M NaOH to measure cell-associated radioactivity and protein concentration. Cell-associated radioactivity represents bound and internalized ligand.

Perfusion of isolated mouse livers with chylomicron remnants

Fresh blood was obtained from rats, using 0.1% EDTA as the anticoagulant, on the day of the perfusion experiment. The plasma was removed after low-speed centrifugation (1,200 rpm, 10 min, 10°C). The pellet containing red blood cells (RBC) was washed twice in PBS (pH 7.4) (1,200 rpm, 10 min, 10°C) and twice in DMEM (GIBCO-BRL, Grand Island, NY) under the same conditions. The RBC were resuspended in DMEM to a final concentration of 20% (v/v) and gassed with 95% O_2/5% CO_2.

Mice, aged from 9 to 12 weeks, were used for the perfusion studies. They were anesthesized by intraperitoneal injection of avertin and a midline incision was made to the abdominal cavity. A silastic cannula (0.012-mm i.d.) (Dow Corning, Midland, MI) was inserted into the hepatic portal vein and another silastic cannula (0.0635-mm i.d.) was inserted into the inferior vena cava via the right atrium of the heart. Both were fixed in the blood vessels by suturing with silk threads.

The liver was perfused via the hepatic portal vein at a rate of 0.5 ml/min, which is similar to the rate of blood flow in vivo (0.35 ml/min per g of liver) (46). The perfusate exiting the liver was collected via a cannula placed in the inferior vena cava through the right atrium. At all times during the surgery and perfusion experiments, the mice were kept at 37°C by being placed on a heating pad and by blowing temperature-controlled air over them. The temperature of the liver was constantly monitored with a probe and the air temperature was adjusted to maintain the liver temperature at 37°C.

All solutions perfused into the livers were warmed to 37°C before use. Initially, the livers were perfused with medium A (DMEM containing 20% washed rat RBC) for 5 min to wash out residual blood and clots. A fresh solution (medium A and test materials) was then passed continuously into the liver (in a single nonrecirculating perfusion) for a total time of 20 min. Aliquots of the perfusates (exiting the inferior vena cava) were collected separately at 1-min intervals (per pass). After the perfusion ended, the whole livers were removed for counting. The radioactivity of the perfusate and the liver was determined in a γ counter (Beckman, Palo Alto, CA). To calculate the rate of chylomicron remnant removed from the perfusate per pass, the radioactivity remaining in each of the aliquots after leaving the liver per pass was subtracted from the quantity of radioactivity that entered the liver per pass. This value is then divided by the quantity of radioactivity that entered the liver per min and multiplied by 100. The final result gives the rate of chylomicron remnant removed per pass and it is expressed as the percentage (%) of ^{125}I-labeled chylomicron remnants removed per pass.

One criterion of a successful perfusion was that the sum of counts in the exiting perfusate and the whole liver was at least 95% of the counts in the volume of perfusate introduced into the liver. Three other criteria were used to determine a successful perfusion. The color of the liver had to be uniform, as any patchy discoloration indicated the presence of air emboli. The color of the blood entering the liver was pink and was darker leaving the liver, indicating effective extraction of oxygen from the RBC. The viability of hepatocytes was determined by measuring the amount of alanine aminotransferase in the blood collected before liver perfusion and in the last sample of perfusate collected, using the Sigma transaminase test kit (procedure 505; Sigma). An increase in the amount of transaminase after perfusion indicates cellular necrosis. Failure to meet any of these criteria resulted in the data being discarded. The validation of this approach has been reported previously (38).

Trypsin treatment of chylomicron remnants

Chylomicron remnants were digested with trypsin by a modification of the method described by Borensztajn, Kotlar, and Chang (36). Briefly, bovine pancreatic trypsin (Sigma) was added to chylomicron remnants at a concentration of 1:100, respectively, 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 with 0.01% EDTA. The digested chylomicron remnants were isolated by ultracentrifugation at 38,000 rpm at 10°C for 90 min, purified through a Sephadex G-50 column, recentrifuged under the same conditions, and dialyzed against PBS (pH 7.4) and 0.01% EDTA overnight. The samples were delipidated with chloroform–methanol 2:1, and the proteins were solubilized in appropriate electrophoresis sample buffer and subjected to electrophoresis using a 5–20% gradient polyacrylamide gel containing 0.1% sodium dodecyl sulfate to check the result of digestion (**Fig. 1**). There were no identifiable apolipoproteins from the gels but there were considerable amounts of smaller proteins. These smaller proteins are likely the fragments of digested apolipoproteins that remained attached to the chylomicron remnant particle surface.

The trypsinized chylomicron remnants were iodinated by the modified iodine monochloride method described above and were used accordingly in the isolated liver perfusion experiments. The iodine was presumed labeled to the peptide fragments.

Immunofluoresence of apoE in the liver

Livers of wild-type (C57BL/6J) mice were perfused with saline (0.9% NaCl), using the method described above. The livers were dissected, fixed by immersion in 4% paraformaldehyde for 20 min at 4°C, and then cryoprotected in PBS plus 18% sucrose overnight. Pieces of liver tissue were embedded in Tissue-Tek optimum cutting temperature (OCT) compound (Sakura Finetek) on dry ice and sections (~8 μm) were cut by a cryostat.

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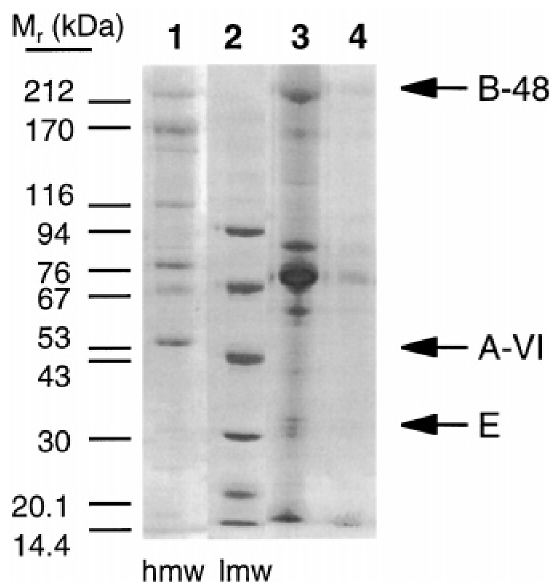

Fig. 1. SDS-polyacrylamide gel electrophoresis of delipidated proteins from normal and trypsinized chylomicron remnants. Chylomicron remnants (d < 1.006 g/ml) were prepared and some were treated with trypsin as described in Materials and Methods. After delipidation, the proteins were subjected to electrophoresis on sodium dodecyl sulfate/5–20% polyacrylamide gradient gels. The proteins were stained with Coomassie blue. High (hmw) and low (lmw) molecular weight markers (Amersham Life Sciences) are indicated on lanes 1 and 2, respectively. Lane 3 is normal chylomicron remnants and Lane 4 is trypsin-treated chylomicron remnants.

The sections were permeabilized with PBS plus 0.1% Triton X-100 (5 min, room temperature) and subjected to immunofluorescence staining with a 1:200 dilution of primary goat antimouse apoE antibodies (1 h, room temperature; Santa Cruz Biotechnologies, Santa Cruz, CA) and a 1:200 dilution of secondary Oregon Green-conjugated rabbit antigoat IgG (30 min, room temperature; Molecular Probes, Eugene, OR). After extensive washing in PBS, the coverslips were mounted in ProLong mounting medium (Molecular Probes). Confocal images were obtained on a Molecular Dynamics (Sunnyvale, CA) Multiprobe 2010 laser confocal microscope (Cell Science Imaging Facility, Beckman Center, Stanford University, Stanford, CA), using the 488-nm excitation line and collecting emissions with a 530 ± 30 nm band-pass filter.

Statistical analysis

All results presented represent means ± SEM. The statistical significance was evaluated with Student's *t*-test for unpaired data; $P \leqslant 0.05$ was accepted to be significant. The analysis was performed with InStat statistical software (GraphPad, San Diego, CA).

RESULTS

Specificity of rat ^{125}I-labeled chylomicron remnant binding and degradation by mouse cells

To evaluate whether rat chylomicron remnants are bound and degraded by mouse cells, in vitro competition studies using the mouse L929 fibroblast cell line were carried out. The competitors used against rat ^{125}I-labeled chylomicron remnants were lipoprotein (d < 1.006 g/ml) fractions from the plasma of fat-fed wild-type and apoE-knockout mice.

As shown in **Fig. 2A**, cell-associated radioactivity decreased in the presence of increasing concentrations of wild-type mouse lipoprotein (d < 1.006 g/ml) fraction but was not significantly inhibited by apoE-knockout mouse lipoprotein (d < 1.006 g/ml). A similar result was obtained in the degradation studies (Fig. 2B). Rat remnants have a higher affinity for the cells than does mouse

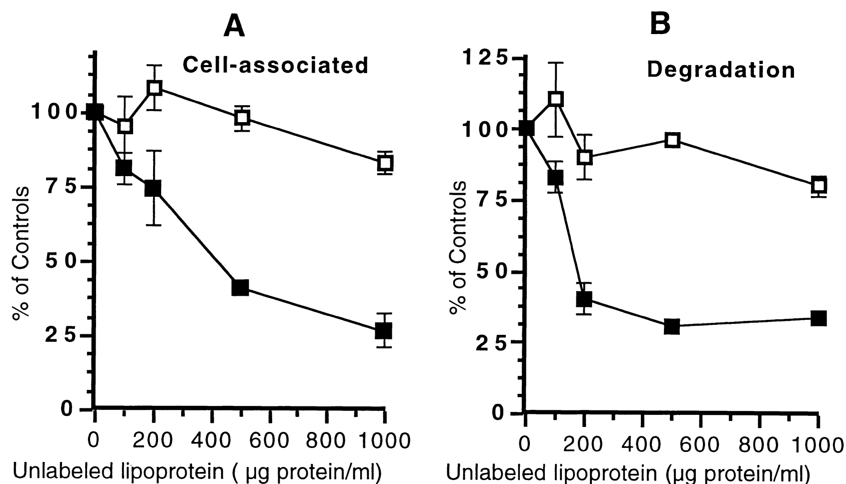

Fig. 2. Effect of mouse lipoproteins on cell association and degradation of rat ^{125}I-labeled chylomicron remnants by mouse cells. Mouse L929 fibroblasts were incubated for 3 h at 37°C in medium containing rat ^{125}I-labeled chylomicron remnants alone (10 μg of protein per ml), or with increasing concentrations (as indicated) of wild-type mouse d < 1.006 g/ml lipoproteins or the same fraction from apoE-knockout mice. Cell-association (A) and degradation (B) of ^{125}I-labeled chylomicron remnants were determined as described in Materials and Methods. The data are expressed as a percentage of controls and represent means ± SEM (n = 3). The filled squares represent the mouse d < 1.006 g/ml fraction and the unfilled squares represent the apoE-knockout mouse d < 1.006 g/ml fraction. The controls are cells incubated with ^{125}I-labeled chylomicron remnants alone.

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d < 1.006 g/ml lipoprotein. This is probably due to their higher concentration of apoE. This set of experiments demonstrated that mouse cells can bind and degrade rat chylomicron remnants by apoE-dependent mechanism(s). Thus, rat chylomicron remnants can be used appropriately in mouse liver perfusion experiments.

Removal of chylomicron remnants by livers of apoE-knockout mice

To determine the capacity of the liver to remove varying concentrations of chylomicron remnants and specifically to compare the removal rate by livers from wild-type and apoE-knockout mice, radiolabeled chylomicron remnants (^{125}I-chylomicron remnants) were perfused into the livers of wild-type mice (controls) and apoE-knockout mice using a single nonrecirculation perfusion.

In the initial experiment, the livers were perfused with a trace amount of ^{125}I-labeled chylomicron remnants (0.05 μg of protein per ml) (**Fig. 3A**). In the control mice, the apparent rate of removal of chylomicron remnants (percentage of ^{125}I-labeled chylomicron remnants removed per pass) in the first minute was highest and decreased in the next 5 min, presumably because of dilution and filling of the liver sinosoids. After 10 min of perfusion, the removal rate began to stabilize, resulting in approximately 45% of ^{125}I-labeled chylomi-

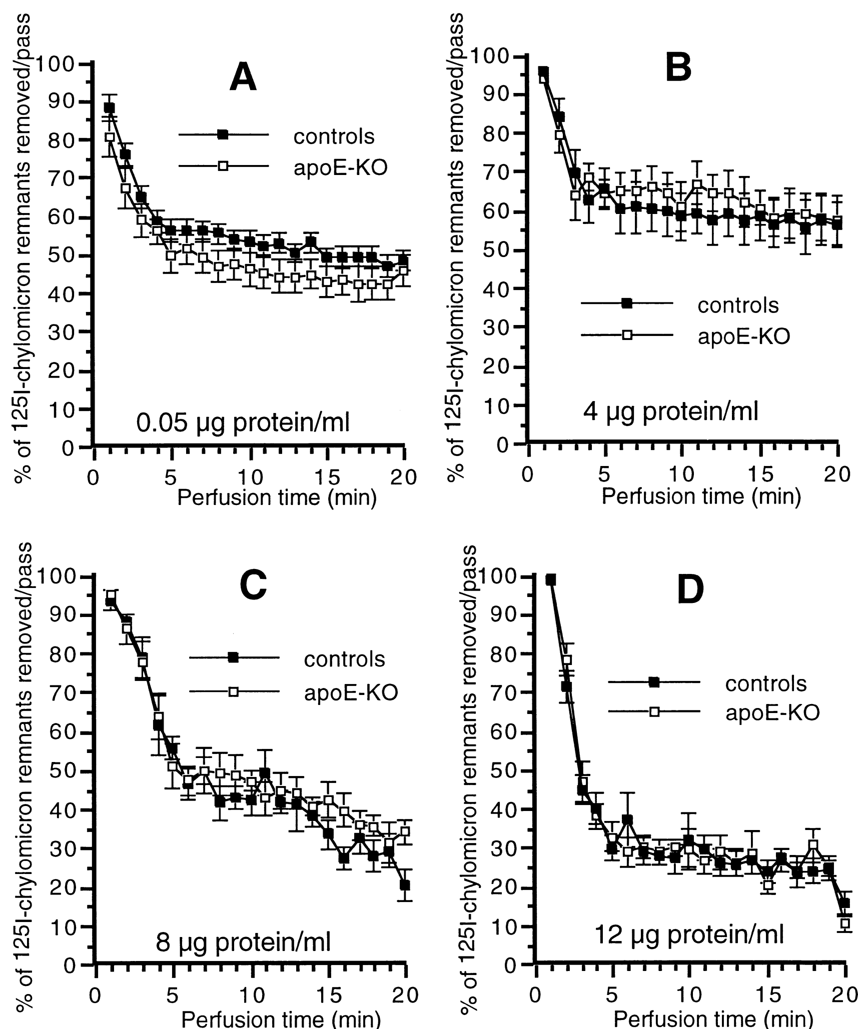

Fig. 3. Removal of chylomicron remnants by apoE-knockout mouse livers and wild-type mouse livers. ^{125}I-labeled chylomicron remnants at the indicated protein concentrations were perfused into the isolated livers of apoE-knockout (apoE-KO) mice and wild-type mice (controls). Aliquots of the perfusate leaving the liver were collected at 1-min intervals during a total perfusion period of 20 min as described in Materials and Methods. The amount of chylomicron remnants removed by the liver from the perfusate per pass is expressed as the percentage of ^{125}I-labeled chylomicron remnants removed per pass, which is calculated by subtracting the radioactivity in a sample of perfusate that left the liver from the initial radioactivity in the perfusate, divided by the initial radioactivity in the perfusate and multiplying by 100. The ^{125}I-labeled chylomicron remnant concentration in the perfusate as indicated in the diagrams was as follows: (A) 0.05 μg/ml (n = 14 for controls, n = 9 for apoE-KO), (B) 4 μg/ml (n = 5 for controls, n = 5 for apoE-KO), (C) 8 μg/ml (n = 5 for controls, n = 9 for apoE-KO), and (D) 12 μg/ml (n = 4 for controls, n = 4 for apoE-KO). Each data point represents the mean ± SEM.

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cron remnants being removed per pass. The livers of apoE-knockout mice removed the identical trace amount of chylomicron remnants just as efficiently as the controls (Fig. 3A).

The effect of increasing the concentration of perfused chylomicron remnants was determined next. At a concentration of 4 μg of protein per ml, there was still as high a rate of removal by the livers of control mice as by those of apoE-knockout mice (Fig. 3B). Further, the rate of removal was similar to the rate when ^{125}I-chylomicron remnants were perfused at 0.05 μg of protein per ml (Fig. 3A). About 60–65% of ^{125}I-labeled chylomicron remnants was removed per pass by the controls and also by the livers of apoE-knockout mice. This concentration of remnants is probably near the K_d for remnant removal overall. When the perfusate concentration was increased to 8 μg of protein per ml, the rate of removal of chylomicron remnants decreased significantly (Fig. 3C). In controls, it fell to about 20–35% per pass. Consistent with previous studies, this concentration appeared to begin to saturate the removal system in the liver (38). If hepatic secretion and localization of apoE were absolutely required for any of the components of the removal system, it was expected that the livers from apoE-knockout mice would demonstrate a decrease in the capacity to remove chylomicron remnants. Somewhat surprisingly, the removal rate curve of the apoE-knockout mouse livers paralleled that of the curve of the controls. This suggested that hepatic apoE secretion does not appear to be necessary for any of the major removal pathways.

The concentration of chylomicron remnants was further increased to 12 μg/ml in order to test whether relatively efficient removal could continue in the absence of apoE at a concentration at which removal by the LDLR pathway is likely to be completely saturated. In controls, approximately 30% was removed per pass after 5 min and at the last minute (20-min time point) the rate fell to about 20% per pass (Fig. 3D). The removal rate curve of the apoE-knockout mouse livers followed a pattern similar to that of the control mouse livers; it appeared that there was little difference in the capacity of apoE-knockout mouse livers to remove chylomicron remnants compared with controls even after the concentration was increased to 12 μg of protein per ml.

Hepatic uptake of chylomicron remnants by the perfused liver

The amount of remnants that accumulates in the perfused liver represents, in essence, the area under the removal curve, and in past studies this measurement has uncovered more subtle differences in remnant removal. The absolute amount of ^{125}I-labeled chylomicron remnants present in the liver after perfusion was calculated on the basis of the assumption that no albumin is specifically taken up or retained in the liver (38). The nonspecific uptake of albumin in the liver was determined by perfusing ^{125}I-labeled BSA into the liver and calculating the volume of trapped ^{125}I-labeled BSA as reported previously (38). The amount of ^{125}I-labeled chylomicron remnants trapped in this volume was subtracted from total uptake. The final results are expressed as the amount of ^{125}I-labeled chylomicron remnant uptake (μg) per weight of liver (g) and are presented in **Table 1**. At a concentration of 0.05 μg of protein per ml, the amount of ^{125}I-labeled chylomicron remnant uptake by wild-type mouse livers (controls) was 0.15 ± 0.005 μg/g while in apoE-knockout mouse livers it was 0.16 ± 0.008 μg/g (Table 1). The difference between the two types of livers was insignificant (P = 0.13). At 4 μg of protein per ml, the amount of ^{125}I-labeled chylomicron remnant uptake by the controls and the apoE-knockout mouse livers was increased to 11.39 ± 0.931 and 12.53 ± 0.855 μg/g (P = 0.43), respectively, showing that there was little difference in the ability of the livers from the two types of mice to remove remnants. A further increase in ^{125}I-labeled chylomicron remnant concentration might be expected

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TABLE 1. Uptake of chylomicron remnants in the livers of wild-type and apoE-knockout mice

^{125}I-Chylomicron Remnants Perfused	^{125}I-Chylomicron Remnant Uptake/Weight of Liver	
	Wild-type Livers	ApoE-Knockout Livers
μg protein per ml	*μg protein per g*	
0.05	0.15 ± 0.005[a] (n = 13)[b]	0.16 ± 0.008 (n = 9)
4	11.39 ± 0.931 (n = 5)	12.53 ± 0.855 (n = 5)
8	22.90 ± 1.099 (n = 5)	21.43 ± 1.281 (n = 5)
12	28.96 ± 1.941 (n = 4)	29.92 ± 4.272 (n = 4)

Livers of wild-type and apoE-knockout mice were perfused with the indicated values of ^{125}I-labeled chylomicron remnants (0.05, 4, 8, and 12 μg of protein per ml) for a total period of 20 min as described in Materials and Methods. After the perfusion, the livers were removed and the total radioactivity present was measured. The absolute amount of ^{125}I-labeled chylomicron remnants specifically taken up by the liver was calculated on the bases of the assumption that the volume of ^{125}I-labeled BSA represents the volume of trapped fluid. The volume of ^{125}I-labeled BSA was determined in a previous study (38). The amount of ^{125}I-labeled chylomicron remnants trapped in this volume was calculated by multiplying the chylomicron remnant concentration perfused by the volume of trapped ^{125}I-labeled BSA; this amount was subtracted from gross uptake values, and the final value gives the specific amount of ^{125}I-chylomicron remnants taken up by the liver. The results are presented as ^{125}I-labeled chylomicron remnant uptake per weight of liver (μg protein per g).

[a] Mean ± SEM.

[b] Numbers in parentheses indicate number of animals.

to result in a decrease in uptake in the livers from apoE-knockout mice compared with controls because the LDLR, which is least likely to require hepatic-secreted apoE, would be nearing saturation; however, it did not. At a concentration of 8 μg of protein per ml, ^{125}I-labeled chylomicron remnant uptake in livers from controls and apoE-knockout mice was 22.9 ± 1.099 and 21.43 ± 1.281 μg/g, respectively (P = 0.38). This set of data, together with the earlier data, suggests that the livers from apoE-knockout mice do not differ significantly from the livers from wild-type mice in their ability to remove and take up chylomicron remnants at moderate concentrations of remnants, when the LDLR is present, even if the LDLR is near saturation.

Removal of chylomicron remnants by livers of apoE/LDLR-knockout mice

Identical experiments were done in livers from apoE/LDLR-knockout mice (**Fig. 4**). As in the apoE-knockout mouse livers, the apoE/LDLR-knockout mouse livers removed a trace amount of chylomicron remnants (0.05 μg/ml) as efficiently as wild-type mouse livers (controls) (Fig. 4A). This is consistent with the expression of at least one high affinity uptake mechanism, presumably the LRP, despite the absence of hepatic apoE. A 10-fold increase in concentration, that is, 0.5 μg/ml, still did not produce any significant change in the capacity of the apoE/LDLR-knockout mouse livers to remove chylomicron remnants (Fig. 4B). Thus, the remnant removal mechanism was still functioning normally. At 4 μg/ml, the apoE/LDLR-knockout mouse livers did, however, show a significantly reduced capacity to remove remnants compared with the livers from wild-type mice (Fig. 4C). While the controls continued to show as rapid a removal rate at 4 μg/ml as at 0.5 μg/ml, the removal rate in the apoE/LDLR-knockout mouse livers decreased to about 20% per pass. When the remnant concentration increased to 8 μg/ml, the removal rate in the apoE/LDLR-knockout mouse livers remained at 20% per pass while that in the controls decreased only slightly (Fig. 4D). Interestingly, this rate of uptake is similar to that obtained with livers from LDLR-knockout mice (38). A further increase in concentration to 12 μg/ml, a concentration at which both the LDLR and LRP are nearing saturation, caused the rate of removal by the two different types of livers to be reduced to the same extent, that is, 10–20% per pass (Fig. 4E).

The amount at which ^{125}I-labeled chylomicron remnants accumulated in the livers was similar to the removal rates. There was no difference in the liver uptakes of wild-type (controls) and apoE/LDLR-knockout mice at the low concentrations. At 0.05 and 0.5 μg/ml, ^{125}I-labeled chylomicron remnant uptake per weight of liver was 0.11 ± 0.018 μg/g (n = 3) and 0.105 ± 0.005 μg/g (n = 3), respectively. At relatively higher concentrations (4 and 8 μg/ml), there were significant differences. At 4 μg/ml, ^{125}I-labeled chylomicron remnant uptake per weight of liver in the apoE/LDLR-knockout mouse was 3.2 ± 0.72 μg/g (n = 3) and was 10.99 ± 1.75 μg/g (n = 3) in the controls ($P < 0.05$). At 8 μg/ml, it was 5.775 ± 1.23 μg/g (n = 3) in the apoE/LDLR-knockout mouse and was 21.7 ± 0.067 μg/g (n = 3) in the controls ($P < 0.005$). At 12 μg/ml, however, there was, again, an insignificant difference between the controls and the apoE/LDLR-knockout mouse. The amount of ^{125}I-labeled chylomicron remnant uptake per weight of liver in the controls and apoE/LDLR-knockout mice was 27.09 ± 0.98 μg/g (n = 3) and 25.83 ± 0.098 μg/g (n = 3), respectively. Together these results suggest that the livers from the apoE/LDLR-knockout mice behave similarly to the livers from LDLR-knockout mice [see Discussion for comparison of data from our previous publication (38) with this present study].

Effect of trypsinization of chylomicron remnant on their removal and uptake by the liver

Borensztajn and colleagues (36, 37) have proposed that there may be an apoE-independent lipoprotein transport pathway in the liver that involves phospholipolysis or lipases. If this pathway exists, it would be expected that the hepatic removal of apoE-free chylomicron remnants would not be completely impaired in the apoE-knockout mice. This was tested by digesting chylomicron remnant-protein with trypsin and perfusing the trypsinized remnants into the livers from apoE-knockout mice and wild-type mice.

As shown in **Fig. 5**, the removal rate of trypsinized chylomicron remnants (0.05 μg/ml) in wild-type mouse livers was 45–50% per pass, which was similar to the removal rate of the same concentration of normal (nontrypsinized) chylomicron remnants in wild-type mouse livers. In contrast, even at this low concentration the removal rate of trypsinized chylomicron remnants in apoE-knockout mouse livers was significantly decreased to 15–20% per pass, indicating a much reduced removal rate. When the concentration of trypsinized chylomicron remnants was increased to 4 μg/ml, the removal rate decreased to 20–30% per pass in wild-type mouse livers while the removal rate of normal chylomicron remnants at 4 μg/ml remained unchanged. The uptake of trypsinized chylomicron remnants by the livers confirmed this (**Table 2**). When trypsinized chylomicron remnants were perfused at a concentration of 0.05 μg/ml into wild-type mouse livers, they were taken up at a rate similar to the rate at which normal chylomicron remnants were taken up. On the other hand, the amount of trypsinized chylomicron remnants taken up by the livers of apoE-knockout mice decreased to about 25% of controls and was not significantly different from the rate of uptake of BSA determined in our previous study (38). Thus, the livers from apoE-knockout mice cannot remove even a trace of apoE-free remnants. However, at 4 μg/ml in wild-type mouse livers, the amount of trypsinized chylomicron remnants taken up was about 12% of the amount of normal chylomicron remnant uptake. The presence of readily available apoE distributed uniformly over hepatocytes in the livers of wild-type mice (**Fig. 6**) was, thus, not capable of facilitating removal of 4 μg/ml of apoE-free remnants to an extent comparable to the removal of the same concentration of normal remnants. Because the amount of protein

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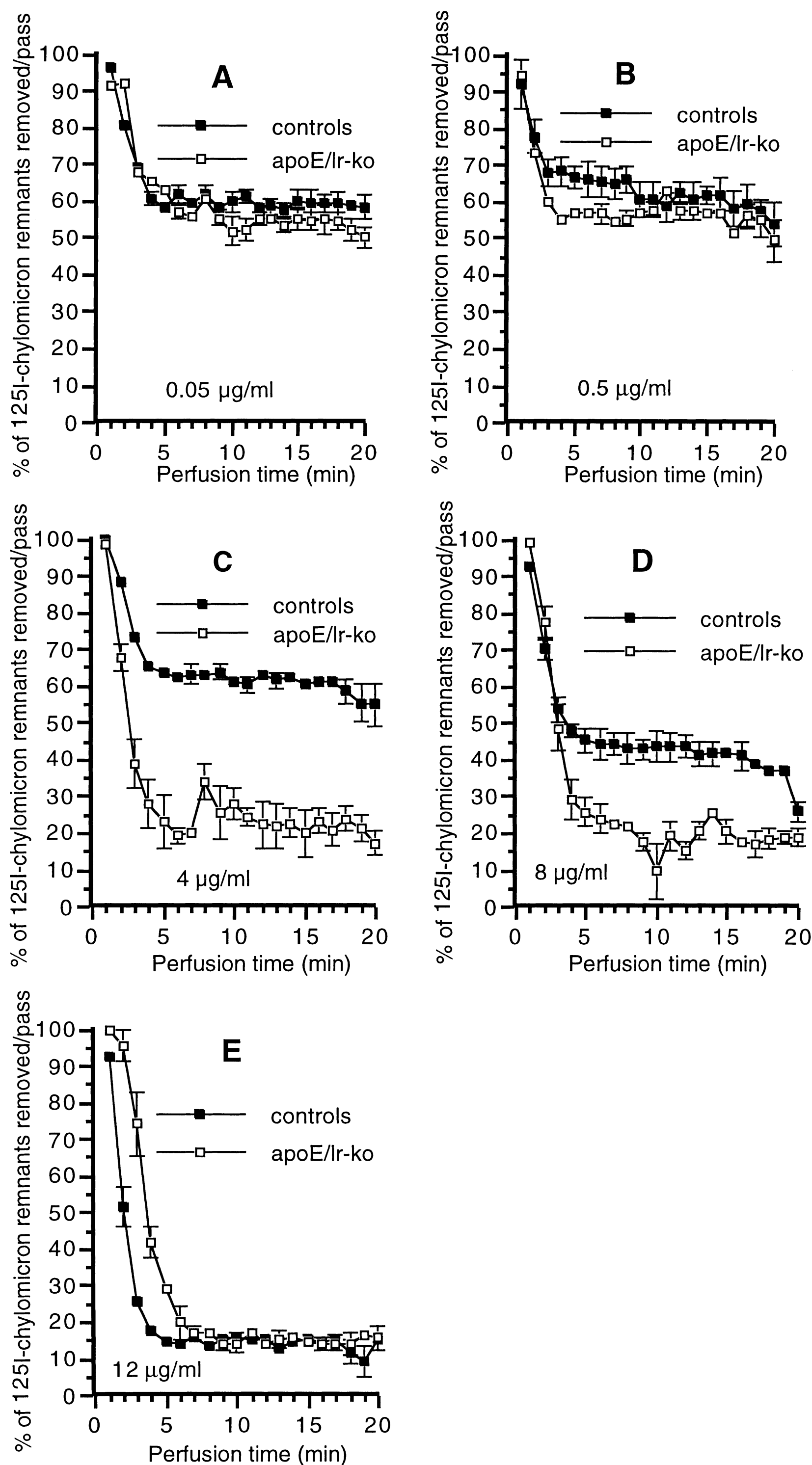

Fig. 4. Removal of chylomicron remnants by apoE/LDLR-knockout mouse livers. ^{125}I-labeled chylomicron remnants were perfused into the livers of apoE/LDLR-knockout mice (apoE/lr-ko) and wild-type mice (controls) and the rate of removal was determined as described in Materials and Methods. The concentration of ^{125}I-labeled chylomicron remnants perfused as indicated in the diagrams was (A) 0.05 μg/ml (n = 3 for controls, n = 3 for apoE/lr-ko), (B) 0.5 μg/ml (n = 3 for controls, n = 3 for apoE/lr-ko), (C) 4 μg/ml (n = 3 for controls, n = 3 for apoE/lr-ko), (D) 8 μg/ml (n = 3 for controls, n = 3 for apoE/lr-ko), and (E) 12 μg/ml (n = 3 for controls, n = 3 for apoE/lr-ko). Each data point represents the mean ± SEM.

in the trypsinized and normal particles is not likely to be the same, the two sets of data should not be directly compared, at least in terms of particle numbers.

DISCUSSION

Previous work has firmly established the existence of at least two distinct pathways for the removal of chylomicron remnants from the circulation, the LDLR and the LRP (21–24, 47). Evidence of the existence of a third pathway, or at least of its nature, remains controversial. The production of transgenic and knockout animals has facilitated studies of the details of the remnant removal pathways. In a previous publication, we established that the isolated perfused mouse liver could be used for detailed kinetic and quantitative studies of these pathways (38). The present report extends these studies and begins to

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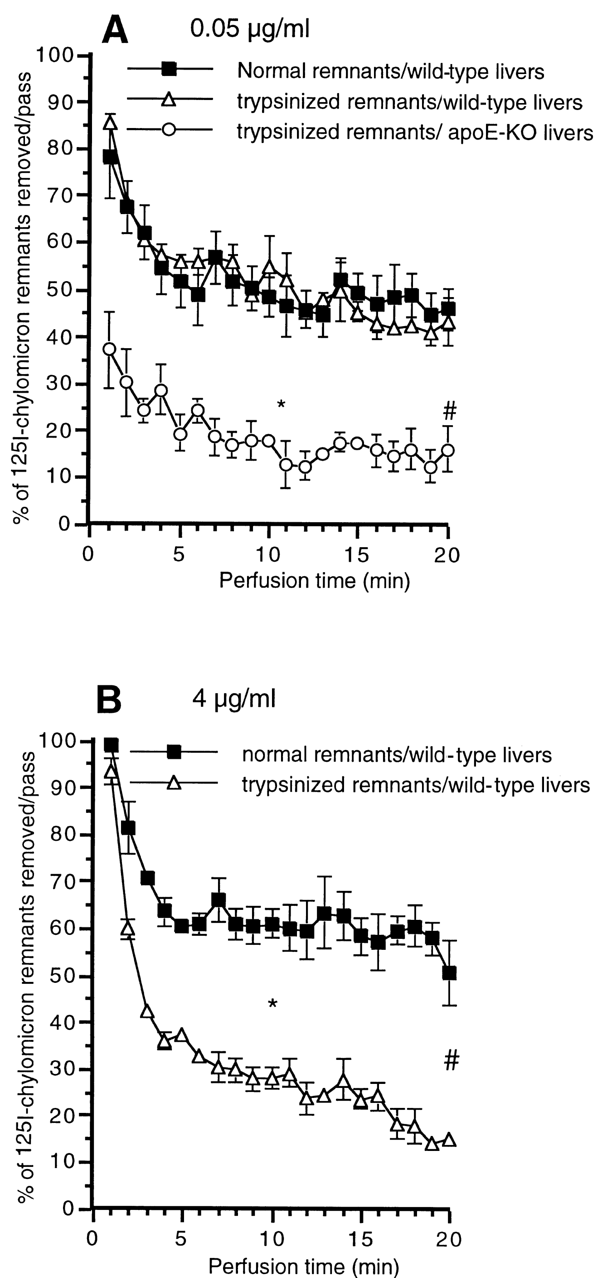

Fig. 5. Removal of trypsinized chylomicron remnants. Trypsinized chylomicron remnants were prepared, radiolabeled with ^{125}I, and perfused into the isolated livers of apoE-knockout (apoE-KO) and wild-type mice as described in Materials and Methods. The concentration of radiolabeled trypsinized chylomicron remnants perfused was 0.05 μg/ml (A) or 4 μg/ml (B) and the amount removed per pass during a total perfusion period of 20 min was determined as described in Fig. 2. As additional controls, radiolabeled normal (nontrypsinized) chylomicron remnants were also perfused. Each data point in (A) represents the mean ± SEM (n = 3 for trypsinized remnants/wild-type livers; n = 4 for trypsinized remnants/apoE-KO livers and n = 4 for normal remnants/wild-type livers); in (B) each data point represents the mean ± SEM of n = 3 for both trypsinized and normal remnants in wild-type livers. In (A), $^{*}P < 0.05$ for trypsinized remnants/wild-type livers versus trypsinized remnants/apoE-KO livers at 10 min; $^{\#}P < 0.05$ for trypsinized remnants/wild-type livers versus trypsinized remnants/apoE-KO livers at 20 min. In (B), $^{*}P < 0.05$ for trypsinized remnants versus normal remnants at 10 min; $^{\#}P < 0.05$ for trypsinized remnants versus normal remnants at 20 min.

characterize the determinants of remnant uptake by the two receptors in the liver. Specifically, the role of hepatic apoE secretion and its localization in the liver was evaluated in the present study. It had been postulated earlier that this might play a critical role in the "secretion-capture model" of remnant lipoprotein removal, whereby secreted apoE led to the capturing of the remnants in the space of Disse before their ultimate internalization by hepatocytes (27).

The present studies fail to provide support for that mechanism. As has been reported by our laboratory and confirmed by others, at least in the mouse and rabbit, the LDLR has the highest affinity and appears to have the largest capacity for the removal of remnants (21, 25, 38, 48, 49). At concentrations at which the LDLR could be expected to play the major role in remnant uptake, there was no difference in remnant removal between livers from wild-type and apoE-knockout mice. Thus, the presence of hepatically localized apoE unassociated with lipoproteins in the space of Disse probably has no significant role in facilitating uptake by the LDLR. This is not surprising given the high affinity this receptor has for apoE (1). It is important to note that the concentrations used in these experiments are representative of those likely to occur in the postprandial state. Other experiments to be published separately suggest that LDLR-mediated uptake leads to rapid cellular internalization and little, if any, sequestration (K. C-W. Yu, W. Chen, and A. D. Cooper, unpublished observations). Thus, as long as there are adequate LDLRs in an individual there is likely to be efficient remnant removal, with little chance of further metabolism or rerelease into the circulation of the particles.

It was expected that when uptake relied on the LRP, it would be dependent on, or at least affected by, the secretion of additional apoE in the liver. Kowal et al. (33) demonstrated that β-VLDL bound to the LRP of fibroblasts from patients with familial hypercholesterolemia only when additional exogenous apoE was preincubated with β-VLDL before addition to the fibroblasts. Subsequent studies by Ji and colleagues (30, 50) repeatedly demonstrated a substantial increase in β-VLDL binding to cells only if additional exogenous apoE was added to β-VLDL. In the current experiments, when remnants were present at concentrations at which it was likely that the LRP was required for remnant uptake, a lack of hepatic apoE should have delayed the removal of remnants. This did not occur. At remnant concentrations that were likely to require both pathways (LDLR and LRP) for an optimal rate of removal, surprisingly, the capacity for remnant removal was not reduced as would be expected if one mechanism was deficient because of the lack of hepatic apoE. These observations indicate that the amount of apoE on the remnant surface, obtained from the plasma, is sufficient to enable efficient removal in the liver.

To explore further the possibility that remnant removal by the LRP was not altered in apoE-knockout mice, additional studies were carried out with mice lacking both apoE and LDLRs (apoE/LDLR-knockout mice). These mice should remove remnants primarily by the LRP and

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TABLE 2. Uptake of trypsinized chylomicron remnants in the livers of wild-type and apoE-knockout mice

Type of Mouse	^{125}I-Lipoprotein Uptake/Weight of Liver	
	^{125}I-Trypsinized Chylomicron Remnants	^{125}I-Chylomicron Remnants
	μg protein per g	
Wild type	0.1457 ± 0.007[a] (n = 3)[b]	0.158 ± 0.015 (n = 4)
ApoE-knockout	0.036 ± 0.005 (n = 4)[c]	NA[d]

Trypsinized chylomicron remnants were prepared and labeled with ^{125}I as described in Materials and Methods. Trypsinized ^{125}I-labeled chylomicron remnants (0.05 μg of protein per ml) were perfused into the isolated livers of wild-type and apoE-knockout mice as described in Table 1. ^{125}I-labeled normal (nontrypsinized) chylomicron remnants were also perfused into the livers of wild-type mice as an additional control. The absolute amount of ^{125}I radioactivity taken up by the livers was determined as described in Table 1. The results are presented as ^{125}I-labeled lipoprotein uptake per weight of liver (μg protein per g).

[a] Mean ± SEM.

[b] Numbers in parentheses indicate number of animals.

[c] $P < 0.0005$ for trypsinized ^{125}I-labeled chylomicron remnants/wild-type mice versus ^{125}I-labeled chylomicron remnants/apoE-knockout mice.

[d] NA, undetermined.

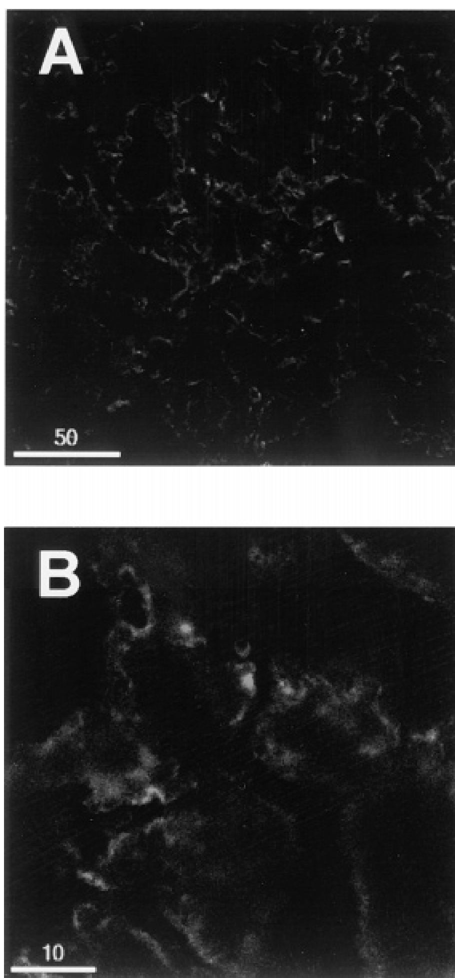

Fig. 6. Localization of apoE in the livers of wild-type mice. Livers of wild-type (C57BL/6J) mice were perfused with 0.9% NaCl and then were prepared for cryosectioning. Detection of apoE was achieved by immunofluorescence and viewed by the confocal microscope as described in Materials and Methods. (A) A representative section showing apoE fluorescence (white); (B) a higher magnification of the same field. The scale is indicated on the bars in micrometers.

with a small contribution by other mechanisms that may contribute to remnant removal at physiologic remnant concentrations. At low concentrations of remnants (0.05 and 0.5 μg/ml), the rate of removal by the livers from apoE/LDLR-knockout mice was the same as that by control livers. This certainly argues against an absolute requirement for hepatic secretion of apoE for remnant removal by non-LDLR-mediated pathways. At higher remnant concentrations, there was a decreased rate of removal of remnants by the livers from the apoE/LDLR-knockout mice. This was to be expected because the LDLR was absent. To learn whether the LRP was functioning normally, a comparison was made with previously published data from LDLR-knockout mice and wild-type mice (38) (**Table 3**). This is a reasonable approach because the studies were done during a similar time period and using comparable particles. Uptake by livers from LDLR-knockout mice was slightly, but not significantly, greater than uptake by livers from apoE/LDLR-knockout mice (Table 3). Alternatively, uptake by LRP alone may be calculated as the difference between that occurring in wild-type mice in the presence and absence of a concentration of receptor-associated protein known to inhibit only the LRP. This amount was actually slightly less than the uptake by the livers of apoE/LDLR-knockout mice at the same remnant concentration. Together, these results strongly suggest that there is no change in the rate of remnant removal by the LRP, at either high or low concentrations of remnants in the absence of the hepatic secretion of apoE.

At the highest concentration of remnants (12 μg/ml), more uptake of remnants was observed than expected. This could be due to the existence of a third process that is apoE independent and has a low affinity but a high capacity for remnants. Crawford and Borensztajn (37) have suggested that there is such a process. They have demonstrated that lipase- and trypsin-treated remnants that lack apolipoproteins are removed rapidly in in vivo experiments in both wild-type and apoE-knockout mice (36, 37). In the present studies, however, the removal of apoE-free

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TABLE 3. Uptake of chylomicron remnants in the livers from wild-type and apoE/LDLR-knockout mice

^{125}I-Chylomicron Remnants Perfused	^{125}I-Chylomicron Remnant Uptake/Weight of Liver			
	Wild-Type Livers	ApoE/ LDLR-Knockou t Livers	LDL Receptor-Knockout Livers	Wild-Type Livers [(without RAP) − (with RAP)]
μg protein per ml	*μg protein per g*			
8	21.7 ± 0.067[a] (n = 3)[b]	5.77 ± 1.23 (n = 3)	7.26 ± 1.62 (n = 3)	4.75 ± 1.99 (n = 3)

Livers from wild-type and apoE/LDLR-knockout mice were perfused with ^{125}I-labeled chylomicron remnants at 8 μg/ml for 20 min. In parallel experiments done previously (37), livers from LDLR-knockout mice were perfused at 8 μg/ml with ^{125}I-labeled chylomicron remnants. In addition, livers from wild-type mice were perfused with ^{125}I-labeled chylomicron remnants at 8 μg/ml in the presence or absence of RAP at 4 μg/ml (38) to determine uptake by the LRP, and this was calculated as the difference between that in the presence and in the absence of RAP. The total amount of ^{125}I-labeled chylomicron remnants taken up by the livers was determined exactly as described in the legend to Table 1 and the results are presented here as ^{125}I-labeled chylomicron remnant uptake per weight of liver (μg protein/g).

[a] Mean ± SEM.

[b] Numbers in parentheses indicate number of animals.

(trypsinized) remnants by livers from wild-type and apoE-knockout mice, prepared by the method described by Borensztajn, Kotlar, and Chang (36), was not demonstrated to be significantly greater than nonspecific uptake calculated from the removal of BSA in wild-type mouse livers determined in a previous study (38). Nonetheless, such a mechanism may exist and help to account for the small amount of uptake that cannot absolutely be ascribed to the two receptors at physiologic concentrations. Indeed, our data suggest that this may be so. The normal liver appeared capable of removing a small amount (0.05 μg/ml) of apoE-free (trypsinized) remnants, but not a greater amount (4 μg/ml). Despite the presence of hepatically localized apoE in the liver (Fig. 6), it may be possible that there is a limit to how much apoE may be acquired locally in the liver. If it is responsible for the uptake observed at high concentrations, it represents a low affinity, high capacity system that could not be measured accurately by the perfused liver technique because of the cytotoxicity noted with remnants at concentrations above 15–20 μg/ml. Such a mechanism would come into use under unusual circumstances, such as apoE deficiency. The hepatic secretion of apoE and its retention in the liver, on the other hand, is certainly able to mediate rapid uptake of apolipoprotein-free, lipid-rich particles and could play a significant role in the clearance of emulsions such as those used for parenteral nutrition (51). Even this function, however, can reach saturation at moderate remnant concentrations. Taken together, these results suggest that the secretion of apoE that remains localized to the liver is not critically necessary for the removal of chylomicron remnants under physiological circumstances, although it may play a role in supplementing apoE to particles that are relatively deficient in this protein.

The present results may not be applicable to smaller VLDL remnant type particles, intermediate density lipoproteins, or the cholesterol-rich β-VLDL particles, and this issue is worthy of future studies. These remnants may not obtain sufficient apoE in the plasma and thus the liver may be the source for adding additional apoE to facilitate their internalization. Our present results are compatible with the results of studies in which bone marrow transplantation (BMT) of apoE-secreting macrophages produced a high concentration of plasma apoE that corrected the remnant removal defect in apoE-knockout mice (52). BMT-derived apoE in the space of Disse was unable to restore normal lipid levels in the apoE/LDLR-knockout mice. However, the LRP may never be able to compensate for the large role of the LDLR in the mouse. Linton et al. (52) argued that hepatic secretion of apoE and not secretion by macrophages, despite the presence of abundant apoE in the space of Disse derived by BMT, is necessary for LRP functioning. This postulate requires that macrophage-derived apoE is in some way different from the hepatocyte-derived apoE in its interaction with remnant lipoproteins and the LRP. An alternative explanation more compatible with our present data is that large concentrations of apoE localized to the space of Disse may inhibit access of remnant lipoproteins to bind to the LRP, thus slowing down internalization.

The studies showing that increased apoE secretion induced either by transgenes (35, 53, 54) or adenovirus (55, 56) accelerated remnant lipoprotein clearance from the plasma are compatible with the notion that the rate at which particles acquire apoE in the periphery is the step that determines the rate of remnant formation and thus plays a major role in determining their clearance. They do not necessarily imply that higher concentrations of apoE in the liver directly accelerate remnant removal. Consistent with the important role of apoE in the rate of remnant formation is the observation that high levels of apoE inhibit LPL activity (57). Thus, as a particle circulates it may well acquire apoE until both lipolysis is inhibited by the apoE and the particle is a good substrate for removal by the liver.

The results of this study do not support a role for the local secretion of apoE in remnant removal but do not imply that the uptake pathways mediated by the LDLR and the LRP are otherwise identical. The results of this and our previous report (38) emphasize that the pathways used for the removal of remnants depend on the concentration of remnants in the blood. This raises questions concerning whether it matters which pathway is used, and what the concentration of remnants is in the blood; there is, in fact, few

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data with which to answer these questions definitively at present. Another aspect of interest is whether apoE phenotypes may influence the removal rate of chylomicron remnants. These questions will be pursued in future investigations.

We would like to speculate that if remnants are removed more slowly, even if they accumulate in the space of Disse, there is more chance that they will undergo further modification and potentially become more atherogenic while in the circulation. One publication has emphasized that remnants alone can, even in apoE-knockout or LDLR-knockout mouse macrophages, initiate foam cell formation (58). The concentration of remnants in the fasting state is, of course, near zero; however, the appearance of triglyceride-rich particles after a meal is determined by the rate of gastric emptying and an elevation of triglyceride level is often appreciable for 6 to 8 h after a meal, with a peak at about 4 h (20). Thus, it is likely that remnants are present in the blood for at least 16 h of the day. During much of this time, their concentration is likely to be low and the highest affinity pathway, the LDLR, is likely to play a major role in their removal. Increase in the level of LDLRs should cause more efficient removal. At the peak (~3 h after a meal) in dsylipidemic individuals, the plasma remnant (apoB-48) concentration may be as high as 25 μg of protein per ml (59) and in patients with diabetes (15, 60, 61) or individuals undergoing renal dialysis (16) considerably higher levels as well a longer duration at peak levels have been reported. Thus, the lower affinity pathways would play a more important role in such situations and thus increase the possibility of prolonged exposure of the vascular bed to remnant particles, which could help explain the accelerated atherosclerosis seen in these individuals.

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