Evaluation of the components of the chylomicron remnant removal mechanism by use of the isolated perfused mouse liver

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Abstract **The isolated perfused mouse liver was utilized to evaluate the relative contribution of various molecules believed to participate in the removal of chylomicron remnants by the liver. Sixty percent of asialofetuin was removed from the perfusate per pass; bovine serum albumin was not removed. Normal mouse livers removed chylomicron remnants more efficiently (40–50%/pass) than nascent chylomicrons (10–20%/pass). The fractional removal rate of remnants decreased as their concentration in the perfusate increased demonstrating saturability. Remnant removal by livers of low density lipoprotein receptor-deficient (LDLRD) mice paralleled that of normal mice at low remnant concentrations (0.05, 0.2 μg protein/ml); as concentration increased (4–16 μg protein/ml), removal by LDLRD livers was reduced. About 50% of the capacity to remove remnants was due to the LDL receptor. The role of the LDLR-related protein (LRP) was estimated using the receptor-associated protein (RAP). Four μg/ml of RAP inhibited only LRP; it reduced the removal of remnants by 30–40% in normal livers. When RAP was included in the perfusate of LDLRD livers, remnant removal persisted but was diminished, particularly late in the perfusion; the capacity was ~30% of controls. The present study has established that there is more than one mechanism operating for the removal of chylomicron remnants by the liver, provides estimates of the concentration of each to the removal of remnants, and indicates a method for further studies. It is concluded that in normal livers, the LDL receptor has the greatest capacity for removing chylomicron remnants. The LRP contributes to the process as well and a third component, perhaps "sequestration," accounts for up to 30% of the capacity for the initial removal of chylomicron remnants.**—Yu, K. C-W., Y. Jiang, W. Chen, and A. D. Cooper. **Evaluation of the components of the chylomicron remnant removal mechanism by use of the isolated perfused mouse liver.** ***J. Lipid Res.*** **1999.** 40: **1899–1910.**

Supplementary key words liver • LDL receptor • LDL receptor-related protein • heparan sulfate proteoglycans • space of Disse

Plasma lipoproteins transport lipids in the circulation (1). The intestine produces triglyceride-rich lipoproteins (chylomicrons) from absorbed dietary lipids. They enter the blood via the mesenteric lymph duct and they are lipolyzed by the enzyme, lipoprotein lipase, in the capillary beds of extrahepatic tissues, resulting in the generation of smaller sized lipoproteins called chylomicron remnants (2). The principle site of chylomicron remnant removal from the blood is the liver (1, 3). The rate of removal of chylomicron remnants is extremely rapid and delayed removal is associated with an accelerated rate of atherosclerosis (4–6). Accordingly, understanding the mechanism by which they are removed has been the focus of considerable attention.

It is now established that there are multiple pathways for the removal of chylomicron remnants in the liver (1, 3, 7). The molecules that moderate this include the low density lipoprotein (LDL) receptor and the LDL receptor-related protein (LRP) (8, 9). Previous studies have attempted to determine the relative contributions of the LDL receptor and the LRP. Choi and Cooper (10) used an antibody against the LDL receptor in vivo; it was reported that chylomicron remnant uptake by the liver was inhibited by up to 50%. de Faria et al. (11) estimated from studies in intact mice that the maximal contributions of the LDL receptor and the LRP were about 76% and 23%, respectively, while the small remainder, if any, were most likely attributed to other less well defined mechanisms. These studies used the receptor-associated protein (RAP) that co-purifies with the LRP and is an inhibitor of ligand binding to the LRP and LDL receptor (12, 13). Over the last decade, genetically altered mouse models of lipid me-

Abbreviations: LDL, low-density-lipoprotein; LRP, LDL receptor-related protein; RAP, receptor-associated-protein; HSPG, heparan sulfate proteoglycans; apo, apolipoprotein; DMEM, Dulbecco's modified Eagle's medium; RBC, red blood cells; ALT, alanine amino transferase; PBS, phosphate buffered saline; BSA, bovine-serum-albumin; SEM, standard error of mean; ^{125}I-TMAG, ^{125}I-labeled trypsin-activated α_2-macroglobulin, WHHL, Watanabe heritable hyperlipidemic; FH, familial hypercholesterolemic.

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tabolism have provided valuable information on the physiology of lipoproteins. Willnow et al. (14) used gene transfer to overexpress RAP in the normal mouse and the LDL receptor-deficient mouse. They found a minor increase in plasma remnant lipoprotein concentration in the normal mouse but a profound increase in the LDL receptor-deficient mouse. These observations suggested that in the absence of the LDL receptor, plasma chylomicron remnant removal in vivo is dependent on a RAP-sensitive mechanism. The recent creation of a conditional LRP knockout mouse revealed that when the LRP is absent, the level of LDL receptor doubles and the level of lipoproteins is normal (15). However, when the LRP knockout is induced in a LDL receptor-deficient mouse, there is accumulation of remnant-like lipoproteins; this establishes a role for the LRP in this process and suggests that the LRP plays a role in remnant removal in the normal mouse. Other laboratories have provided evidence to support the concept that the two receptors contribute the major, if not exclusive, pathways for remnant removal (14, 16, 17). However, Mortimer et al. (18) found that plasma clearance of a chylomicron-like emulsion was unaffected in LDL receptor-deficient mice. In another study, Mokuno et al. (19) used an amount of RAP that might inhibit both the LRP and the LDL receptor in the perfusion of the isolated normal rat liver and found it inhibited hepatic chylomicron remnant removal only modestly. These observations raise the possibility of other chylomicron remnant removal pathways in the liver besides the LDL receptor and the LRP.

An additional mechanism of remnant lipoprotein removal may involve a "sequestration" or "secretion-capture" component. Upon entering the liver, remnant lipoproteins pass through the endothelial layer into the space of Disse between the endothelial layer and the hepatic parenchymal cells. The particles are sequestered in the space of Disse, and might undergo further processing before cellular uptake by the hepatocytes. Based on studies in vitro and in vivo, Ji et al. (20–22) have proposed that the sequestration component is dependent on hepatic matrix- and cell surface-bound heparan sulfate proteolgycans (HSPG), apolipoprotein (apo) E, and the LRP. They suggest that remnants are sequestered in the space of Disse by HSPG and, after acquiring additional apoE produced by the hepatocytes, they are handed-off to the LRP for cellular uptake. Alternatively, it has been proposed that HSPG themselves can facilitate the internalization of remnants (23). Compared to the LDL receptor and the LRP, the sequestration component is less well understood and it is unclear to what extent the sequestration component contributes to hepatic remnant removal.

A significant problem with studies carried out in vivo is the inability to study the liver alone, while in vitro studies have the major disadvantage of being unable to duplicate the complex architecture of the liver and the interaction of many cell types. Therefore, an isolated liver perfusion system in the mouse, similar to the method used extensively in rats (19, 24–27), was devised by our laboratory. The validity of this system and its ability to distinguish different ligands are demonstrated in the present study. The aims were to determine the contribution of the sequestration component in the hepatic removal of chylomicron remnants, as well as to quantify the relative contributions of the LDL receptor and the LRP. We used the isolated mouse liver perfusion system to specifically measure the amount of chylomicron remnants removed by the livers of LDL receptor-deficient and normal mice. The RAP was used to inhibit LRP activity in normal and LDL receptor-deficient mouse livers and this gave an estimation of the quantitative contribution of the LRP and demonstrated clearly the existence of an LDL receptor/LRP-independent mechanism for remnant removal.

MATERIALS AND METHODS

Materials

Radioactive sodium [^{125}I]iodine (carrier free ^{125}I) and iodine monochloride were purchased from Amersham Life Sciences (Arlington Heights, IL, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL (Grand Island, NJ). The Sigma Alanine Aminotransferase (ALT/GPT) test kit (procedure No. 505-OP) was purchased from Sigma Chemical Co. (St. Louis, MO). Asialofetuin and fatty acid-free bovine serum albumin were from Sigma Chemical Co. All other chemicals were from Sigma Chemical Co. or J.T. Baker Chemical Co.

Animals. Spraque-Dawley rats (300–350 g) were purchased from Simonsen Laboratory. LDL receptor-deficient mice and C57BL/6J mice (27–35 g) were purchased from Jackson Laboratories (Bar Harbor, ME) and then bred at the Research Institute's animal facilities. All animals were housed in a controlled temperature of 20–25°C and standard light/dark environment. They received standard chow and water ad libitum.

Methods

Preparation of RAP. A plasmid containing the cDNA for human RAP fused with glutathione-S-transferase was a gift from Dr. Dudley Strickland (Department of Biochemistry, American Red Cross, Rockville, MD). The protein was purified in our laboratory as described previously (10).

Preparation of chylomicrons and chylomicron remnants. Rat lymph chylomicrons were obtained from the superior mesenteric lymph duct as previously described (24, 28). Chylomicron remnants were prepared in vivo by injecting lymph chylomicrons intravenously into functionally hepatectomized rats (28). After 3 h, blood was obtained from the rats. Chylomicron remnants ($d < 1.006$ g/ml) were harvested from the blood by ultracentrifugal flotation as previously described (29).

Radiolabeling (iodination). Asialofetuin, bovine serum albumin (BSA), chylomicrons, and chylomicron remnants were iodinated with carrier-free Na^{125}I by the iodine monochloride method of McFarlane (30) with slight modification as previously described (31). All the solutions were dialyzed against PBS/EDTA (pH 7.4) with several changes of the buffer for 20–24 h before use.

Labeling of activated α_2-macroglobulin. Human α_2-macroglobulin (Sigma Chemical Co.) was labeled with carrier-free Na^{125}I using the iodine monochloride method (30). After iodination, the ^{125}I-labeled α_2-macroglobulin was dialyzed against PBS/EDTA (pH 7.4) for 20–24 h, and then activated by incubation with a 5-fold excess of trypsin for 5–10 min at room temperature followed by a 15-fold excess of soybean trypsin inhibitor for 15 min according to the method described by van Dijk et al. (32).

Lipid and protein determinations. Cholesterol and triglyceride concentrations were determined using biochemical assay kits

(catalog number 336-10 and 352-20) purchased from Sigma Chemical Co. (St. Louis, MO). Protein concentration was determined using the micro bicinchoninic acid (BCA) assay test kit from Pierce Chemical Co. (Rockford, IL).

Mouse liver perfusion. Fresh blood was obtained from rats using 0.1% EDTA as anti-coagulant on the day of the experiment and used only on the same day. Plasma was removed after low-speed centrifugation (1200 rpm, 20 min, 10°C). The pellet containing red blood cells (RBC) was washed twice in PBS (pH 7.4) (1200 rpm, 20 min, 10°C) and twice in DMEM at the same speed. The RBC were resuspended in DMEM to a final concentration of 20% (volume/volume).

Mice (ages 9–12 weeks) were used for the perfusion studies. The mice were anesthetized by intraperitoneal injection of avertin and a midline incision was made to the abdominal cavity. A silastic cannula (0.012 mm internal diameter) (Dow Corning) was inserted into the portal vein and another silastic cannula (0.0635 mm internal diameter) was inserted into the inferior vena cava through the right atrium of the heart. Both were securely fixed in the blood vessels by ligating with silk threads.

The liver was perfused via the hepatic portal vein at a rate of 0.5 ml per min which is similar to the rate of blood flow in vivo (33). The exiting perfusate was collected via the inferior vena cava. At all times during the surgery and perfusion experiments, the mice were kept at 37°C by placing them on a heated pad and blowing temperature-controlled air over them. The temperature of the liver was constantly monitored using a probe and the air temperature was adjusted to maintain the liver temperature at 37°C.

All solutions that were to be 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 gassed with 95% O_2/5% CO_2) for 5–10 min to wash out residual blood. A fresh solution (medium A and test materials) was then perfused through the liver for a single pass using a syringe pump (Hamilton Apparatus, Holliston, MA). The perfusate (exiting the inferior vena cava) was collected at 1-min intervals. After the perfusion ended, the whole livers were removed and retained for counting. The radioactivity of the perfusates and the whole livers was measured in a gamma counter (Model 5500B, Beckman, Palo Alto, CA).

To calculate removal of the iodinated particles from the medium, the radioactivity remaining in the perfusate leaving the liver per 0.5 ml was subtracted from the concentration introduced into the liver per 0.5 ml, divided by the initial concentration per 0.5 ml. The results (particles removed from the perfusate per pass) were expressed as the percent of radioactivity perfused into the liver per min (% of radioactivity perfused/min).

Determining successful perfusions. One criteria 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. A lower count in the exiting perfusate and the whole liver compared to the introduced perfusate indicated leakage during liver perfusions. Three other criteria were used to determine a successful perfusion. The color of the liver had to be uniform; any patchy discoloration indicated the presence of air emboli. The color of the blood entering the liver was pink and after it leaves the liver it became dark indicating effective extraction of oxygen from the RBC. The viability of hepatocytes was determined by measuring the amount of the enzyme, alanine aminotransferase (ALT), in the blood collected before liver perfusion and the last perfusate collected by using the Sigma alanine aminotransferase (ALT/GPT) test kit. An increase in the amount of ALT after perfusion indicated cellular necrosis. The data was discarded if there was a failure to meet any of these criteria. Somewhat more than 50% of perfusions were successful.

Statistics. All values were expressed as the mean ± SEM unless otherwise stated. Student's *t*-test was used to evaluate the statistical significance for comparison of unpaired data.

RESULTS

Viability of mouse liver during perfusion

To assess the viability of the perfused mouse liver, the histology after 30 min of perfusion was evaluated by light and electron microscopy and compared with that of normal liver (not shown). The only discernible difference was some depletion of glycogen vacuoles that were present in the normal liver. The ability of the perfused liver to extract O_2 and produce CO_2 as well as the maintenance of the pH were also determined (**Table 1**). Oxygen extraction was excellent as shown by the decrease in pO_2 after perfusion and the pH was maintained; but interestingly, CO_2 was not produced in a manner comparable to that seen in rat liver perfusion. The reason for this was not explored.

Hepatic removal of asialofetuin and bovine serum albumin in the normal mouse

The effectiveness of the isolated perfused mouse liver to remove ligands was tested in livers from normal mice by perfusing them with solutions containing 0.05 μg protein/ml of radiolabeled asialofetuin or BSA. The apparent removal per pass on a minute by minute basis of the two molecules during a total perfusion time of 20 min was determined (**Fig. 1**). In the first min, the apparent rate of removal of asialofetuin per pass (percent of radioactivity perfused/min) was more than 90% but decreased sharply until it reached a constant level at 5 min; after this time, the rate of extraction was 50–60% per pass (Fig. 1A). The apparent initial rapid rate of removal is likely due to the time required for the particles to fill the sinusoidal space in the liver. This appears to require about 2 min and a true steady state is reached at 5 min. The results obtained between 5 min and 20 min should accurately reflect the rate of hepatic removal per pass. The removal rate of asia-

TABLE 1. Changes in oxygen and carbon dioxide content (partial pressure) and pH in the perfusate before and after liver perfusion

	Oxygen, pO_2	Carbon dioxide, pCO_2	pH
	mm Hg	*mm Hg*	
Before perfusion	689.33 ± 18.48[a]	59.33 ± 6.35	7.37 ± 0.02
After perfusion	41.33 ± 16.2	33.00 ± 4.0	7.55 ± 0.07

The perfusate (DMEM containing 20% washed rat red blood cells) was gassed with 95% O_2/5% CO_2 for 3 min. Normal mouse (C57BL/6J) livers were perfused with a single pass of this solution for a total of 20 min at a flow rate of 0.5 ml/min. All experimental procedures for perfusing livers were carried out at 37°C as described under Materials and Methods. Oxygen content (pO_2), carbon dioxide content (pCO_2), and the pH of perfusate samples before and after liver perfusion were measured using an electronic blood analysis system (Diametrics Medical, Inc.).

[a] Mean ± standard deviation (n = 5).

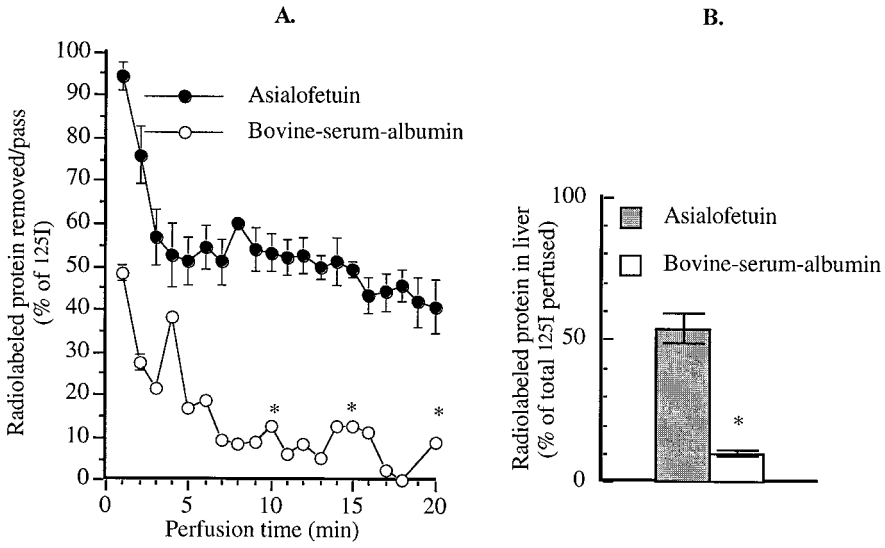

Fig. 1. Removal of asialofetuin and bovine serum albumin from the perfusate in normal mouse livers. Radiolabeled (^{125}I-)asialofetuin or bovine serum albumin (0.05 μg/ml) was added to the perfusate of isolated livers of normal (C57BL/6J) mice for a total period of 20 min as described under Materials and Methods. At every 1-min interval, the perfusate leaving the liver was collected and the radioactivity remaining was measured. The quantity of radiolabeled protein removed from the perfusate by the liver per pass (% of ^{125}I) was determined by subtracting the radioactivity in a sample that left the liver from the initial radioactivity, divided by the initial radioactivity and multiplying by 100 and the data are presented as a line plot in (A). Each data point represents the mean ± SEM (n = 4 for both asialofetuin and bovine serum albumin). After the perfusion, the livers were excised and their total radioactivity was measured (B). The radioactivity in the liver is presented as the percent of total ^{125}I perfused through the liver. Each column is the mean ± SEM. * $P < 0.005$.

lofetuin by the last min had decreased to about 40–50% per pass, suggesting that metabolic efficiency of the liver may have been decreasing. Thus, subsequent studies were carried out for this period. In contrast, the removal rate of BSA was far lower. After the equalization period, the removal rate was less than 15% per pass and was constant until 20 min (Fig. 1A). The amount of radioactivity directly measured in the liver was always >90% of that calculated as having been removed. The amount of asialofetuin taken up by the liver was greater than BSA taken up ($P < 0.005$) (Fig. 1B). As asialofetuin is recognized by a hepatocyte specific receptor and BSA is not, the removal of asialofetuin was expected to be greater than BSA and this is confirmed in our present observations. The viability of the liver was unaffected at the end of the 20-min perfusion period as tested by measuring the level of the enzyme, ALT (results not shown). This set of data demonstrated the reliability and effectiveness of the isolated mouse liver perfusion system to distinguish different proteins.

Hepatic removal of chylomicron remnants and nascent chylomicrons in the normal mouse

The purpose of this set of experiments was to determine whether the isolated mouse liver perfusion system was able to distinguish between nascent chylomicrons and lipolyzed chylomicrons (remnants). The hepatic removal from the perfusate and the uptake by normal mouse livers of the two different ^{125}I-labeled lipoproteins (0.05 μg protein/ml) was determined (**Fig. 2**). After 10 min of perfusion, the hepatic removal rate of chylomicrons from the perfusate was about 10–20%/pass (Fig. 2A) and quite similar to that of BSA. In contrast, the hepatic removal of chylomicron remnants was greater (40–50%/pass) (Fig. 2A). The difference between the removal rates of the two lipoproteins at 10, 15, and 20 min was statistically significant ($P < 0.001$). Similarly, the hepatic uptake of chylomicron remnants was at least 5-times greater than chylomicrons taken up ($P < 0.001$)(Fig. 2B). This confirmed the requirement for the lipolysis of chylomicrons as a prerequisite for efficient hepatic removal. In addition, it demonstrated the feasibility of using the isolated perfused liver system in the mouse to investigate chylomicron remnant metabolism.

Determination of the specificity and the capacity of the hepatic removal mechanism for chylomicron remnants

Radiolabeled ^{125}I-labeled chylomicron remnants (0.05 μg protein/ml) and unlabeled chylomicron remnants were added together to obtain varying total concentrations and these were perfused into the livers of normal mice. When the unlabeled remnant concentration was 10 μg protein/ml, the hepatic removal rate of ^{125}I-labeled chylomicron remnants was decreased modestly at 10, 15, and 20 min (**Fig. 3A**), compared to controls (^{125}I-labeled chylomicron remnants alone). In addition, the amount of radioactivity in the liver was reduced slightly (Fig. 3B), but not significantly, compared to the controls ($P < 0.5$). In the presence of 20 μg protein/ml of unlabeled remnants, the hepatic removal rate of ^{125}I-labeled chylomicron remnants was significantly reduced ($P < 0.05$) at 10, 15, and 20 min (Fig. 3A).

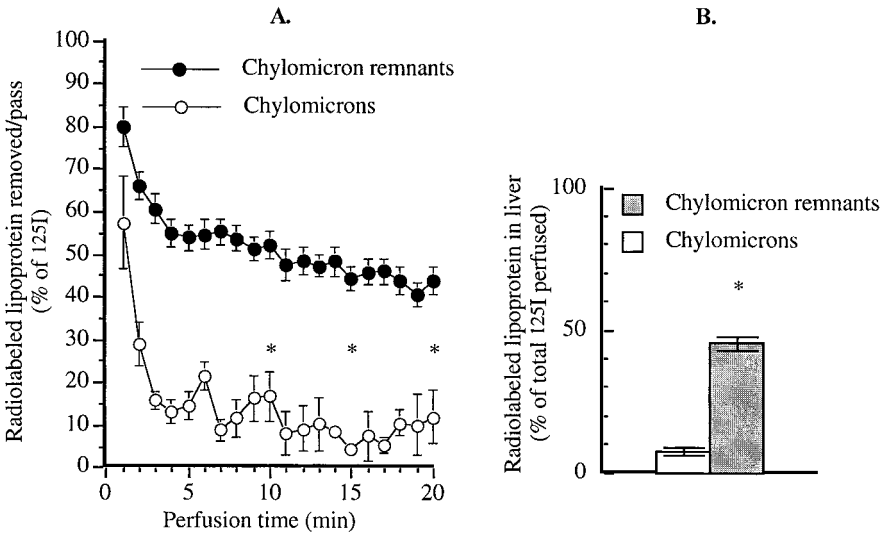

Fig. 2. Removal of chylomicron remnants and nascent chylomicrons. ^{125}I-labeled chylomicrons or ^{125}I-labeled chylomicron remnants (0.05 μg protein/ml) were added to the perfusate of isolated livers from normal mice and an experiment similar to the one described in Fig. 1 was carried out. The amount of ^{125}I-labeled chylomicrons and ^{125}I-labeled chylomicron remnants removed from the perfusate per pass was determined as in Fig. 1A and is expressed as % of ^{125}I (A). Each data point represents the mean ± SEM (n = 10 for chylomicron remnants, n = 3 for chylomicrons). After the perfusion, the livers were excised and their total radioactivity was measured (B). The radioactivity in the liver is presented as the percent of total ^{125}I perfused. Each column is the mean ± SEM. (Livers were from the same mice used in A.) * $P < 0.0005$.

The total hepatic uptake of ^{125}I-labeled chylomicron remnants was, however, significantly reduced in the presence of 20 μg protein/ml of unlabeled remnants ($P < 0.05$ compared to controls) suggesting that removal rate was indeed reduced. The hepatic uptake of ^{125}I-labeled chylomicron remnants was reduced with increasing unlabeled remnant concentration (Fig. 3B); thus, the removal process in the liver is saturable, reaching its maximal capacity at ⩾20 μg/ml remnant protein. Interestingly, this and higher concentrations resulted in some hepatotoxicity.

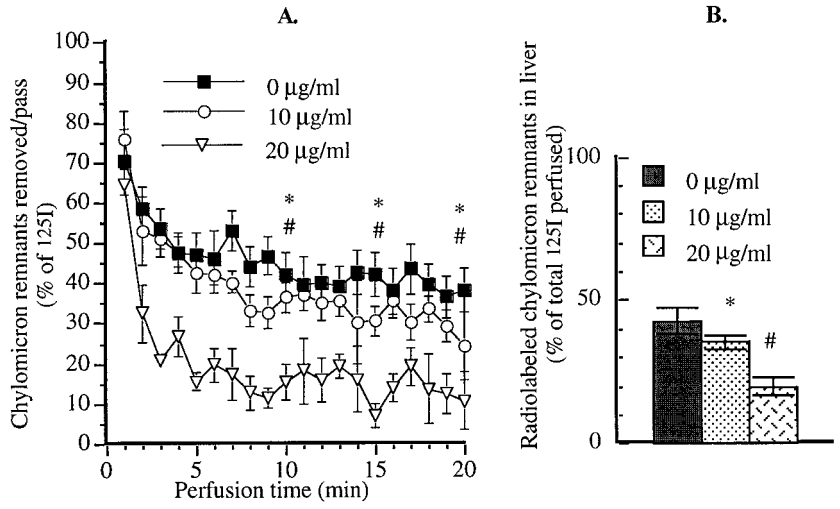

Fig. 3. Specificity of the hepatic chylomicron remnant removal mechanism. The isolated livers of normal mice were perfused as described in Fig. 1 with a solution containing ^{125}I-labeled chylomicron remnants (0.05 μg protein/ml) together with the indicated amount of unlabeled chylomicron remnants. The amount of ^{125}I-labeled chylomicron remnants removed from the perfusate by the liver per pass is expressed as the percent of ^{125}I, as described in Fig. 1A, and the data are presented as a line plot in (A). Each data point represents the mean ± SEM (n = 4 for 0 and 10 μg protein/ml of unlabeled chylomicron remnants, n = 3 for 20 μg protein/ml of unlabeled chylomicron remnants). After the perfusion, the livers were excised and their total radioactivity was measured (B). The radioactivity in the liver is presented as the percent of total ^{125}I perfused. Each column is the mean ± SEM (livers were from the same mice used in A). * $P < 0.5$ for 0 μg/ml versus 10 μg/ml. # $P < 0.05$ for 0 μg/ml versus 20 μg/ml.

Remnant removal by livers of normal mice compared to those from LDL receptor-deficient mice

An experiment similar to the one described in the previous section was carried out using the livers of LDL receptor-deficient mice to estimate the contribution of the LDL receptor to the removal of chylomicron remnants (**Fig. 4**). There is no alteration in the level of expression of the LRP in the liver of these animals (not shown). Livers from LDL receptor-deficient mice appeared to remove 0.05 or 0.2 μg protein/ml of ^{125}I-labeled chylomicron remnants as efficiently as the livers of normal mice did (controls) (Fig. 4A and 4B). However, when the concentration was increased to 4 or 8 μg protein/ml, livers of LDL receptor-deficient mice showed decreased removal rates compared to the controls (Fig. 4C and 4D). In the controls, there was little difference in the removal rate per pass when 0.05, 0.2, 4, or 8 μg protein/ml was perfused, and the removal rate per pass between 5 and 20 min

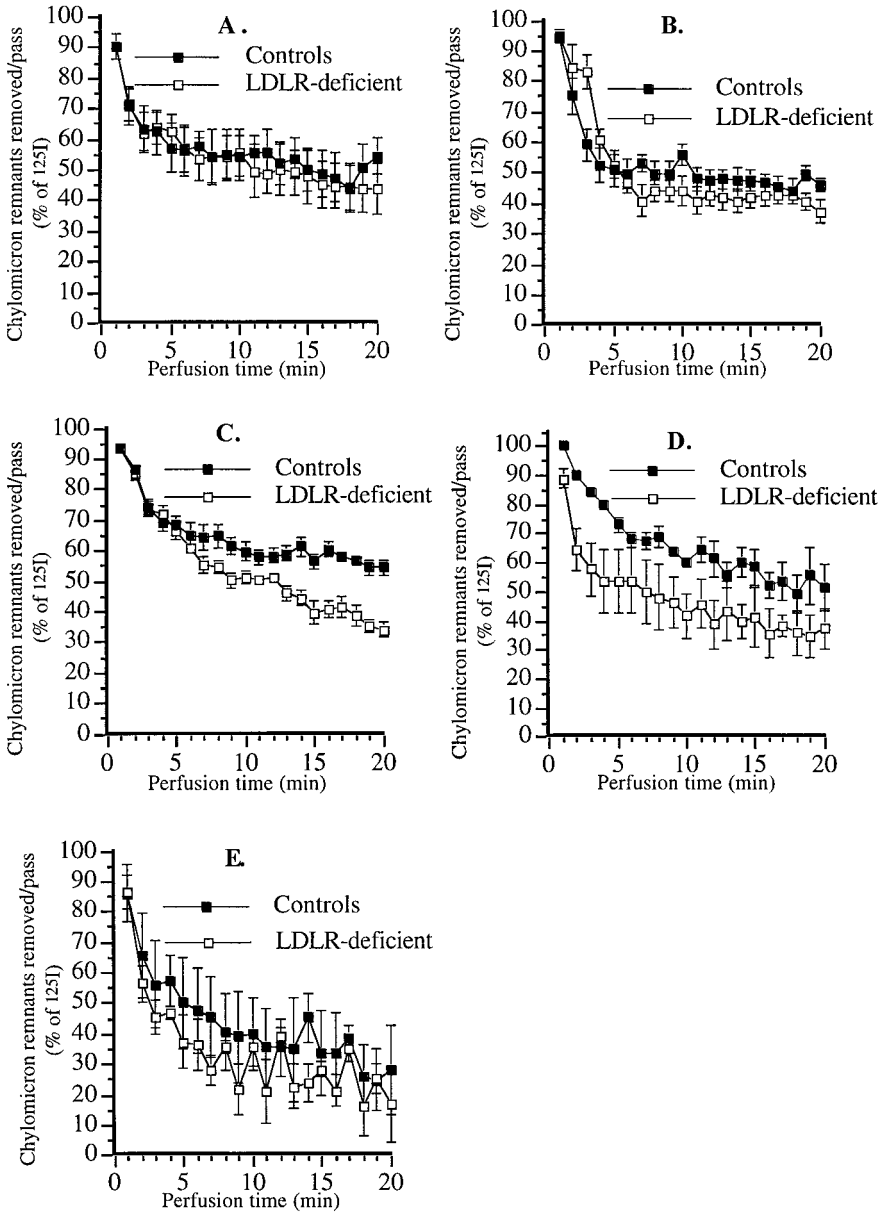

Fig. 4. Removal of chylomicron remnants from the perfusate by LDL receptor-deficient mouse livers. An experiment was carried out as described in the legend to Fig. 2 except that various concentrations (0.05 (A), 0.2 (B), 4 (C), 8 (D), and 16 (E) g protein/ml) of ^{125}I-labeled chylomicron remnants were included in the perfusate of the isolated livers of LDL receptor-deficient mice and controls (C57BL/6J). The amount of chylomicron remnants removed per pass was determined and expressed as in Fig. 3. Each data point represents the mean ± SEM (n = 7 for both controls and LDL receptor-deficient mice in A; n = 5 for both controls and LDL receptor-deficient mice in B; n = 8 for both controls and LDLR-deficient mice; n = 5 for both controls and LDL receptor-deficient mice in C; n = 3 for both controls and LDL receptor-deficient mice in D; n = 3 for both controls and LDL receptor-deficient mice in E). LDLR-deficient in the figures denotes LDL receptor-deficient mice.

ranged from 50 to 60%. In contrast, the removal rate per pass between 5 and 20 min at a concentration of 8 μg/ml in the LDL receptor-deficient mouse livers was from 33 to 38% (Fig. 4D). When the concentration was further increased to 16 μg protein/ml, there was a pronounced decrease in the removal rate by livers of both LDL receptor-deficient mice and controls (Fig. 4E), suggesting that the remnant removal system in both types of livers was at or above the saturation level. This series of experiments showed that at low concentrations, the rate of chylomicron remnant removal is not apparently impaired in the LDL receptor-deficient liver. As the concentration increased, however, there is a diminished capacity to remove chylomicron remnants by the livers of LDL receptor-deficient mice as compared to those of normal mice. Furthermore, these observations suggest that the rate of chylomicron remnant removal is saturable even in the physiological range of lipoprotein concentration. In addition, the results reinforce the concept that there is at least one other remnant removal mechanism besides the LDL receptor, and this appears to be functional in the normal animal.

Estimation of the capacity of the LRP in remnant removal

The LRP is established as an important component of the non-LDL receptor pathway for remnant removal, particularly in the absence of the LDL receptor (16). Although a conditional knockout of this receptor is available, there is an alteration in the expression of the LDL receptor in these animals (15). Thus, the inhibitor of LDL receptor family members, the RAP, was used. At high concentrations, this molecule has been reported to inhibit all members of the LDL receptor family, while at low concentrations it inhibits only the LRP (19). This was tested in the perfused liver. Livers of normal mice were perfused with concentrations of RAP up to 10 μg/ml. At the highest concentration of RAP tested, there was some inhibition of chylomicron remnant removal when perfused with remnant concentrations of 0.05 μg/ml or 0.2 μg/ml, but at concentrations of RAP below 5 μg/ml there was little inhibition of chylomicron remnant removal (results not shown). This suggested that at RAP concentrations above 5 μg/ml, inhibition of multiple pathways might occur. Accordingly, a concentration of 4 μg/ml was used in the subsequent experiments. This concentration of RAP almost completely abolished the removal of ^{125}I-labeled trypsin activated α_2-macroglobulin (^{125}I-TAMG) (**Fig. 5**). This molecule is removed only by the hepatocytes via the LRP. Thus, a concentration of 4 μg/ml completely inhibits the LRP while not noticeably altering the function of other receptors in the LDL receptor family. This is similar to the findings in cell culture experiments (34, 35).

When this concentration of RAP (4 μg/ml) was included in the perfusate of normal livers, there was no effect on the removal of a trace concentration (0.05 μg/ml) of remnants compared with controls (without RAP) (data not shown); neither was there a difference in the removal rate of 0.2 μg/ml of chylomicron remnants compared with controls (0.2 μg remnants/ml, without RAP) (**Fig. 6**). Furthermore, there was little difference in the ability of normal

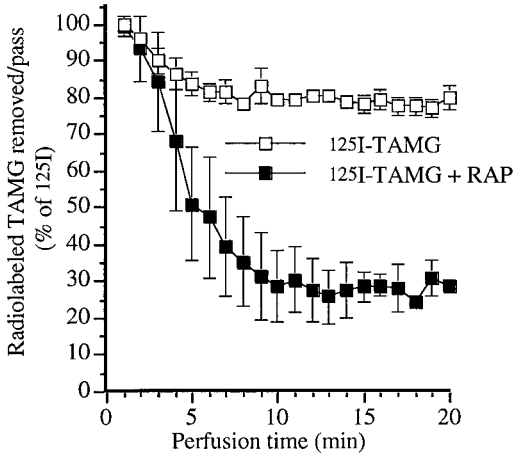

Fig. 5. Effect of the receptor-associated-protein (RAP) on the hepatic removal of α_2-macroglobulin from the perfusate. An experiment was carried out as described in the legend to Fig. 1 except that 4 μg/ml of ^{125}I-labeled trypsin-activated α_2-macroglobulin (^{125}I-TAMG) was added to the perfusate of isolated livers of normal mice (C57BL/6J), with or without 4 μg/ml of RAP. The quantity of ^{125}I-TAMG removed from the perfusate per pass was determined and expressed as in Fig. 1A. Each data point represents the mean ± SEM (n = 4 for each group).

livers to remove remnants in the presence or absence of RAP at a remnant concentration of 4 μg protein/ml (**Fig. 7**). The addition of RAP did not affect remnant clearance and this suggests that the LDL receptor may play a larger role in determining the capacity of chylomicron remnant removal in the normal mouse than does the LRP. Each

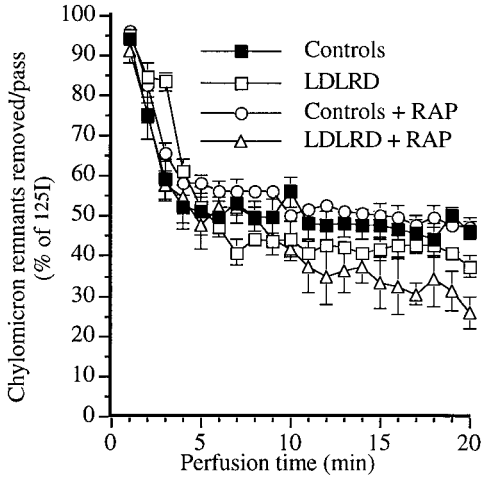

Fig. 6. Effect of the receptor-associated protein (RAP) on the removal of 0.2 μg protein/ml chylomicron remnants by normal and LDL receptor-deficient mouse livers. An experiment was carried out as described in the legend of Fig. 3 except that the perfusate contained RAP (4 μg/ml) and ^{125}I-labeled chylomicron remnants (0.2 μg protein/ml), and the isolated livers of normal (controls) and LDL receptor-deficient mice were perfused. The amount of chylomicron remnants removed per pass was determined and expressed as in Fig. 3. Each data point represents the mean ± SEM (n = 5 for both controls and LDL receptor-deficient mice without RAP; n = 5 for both controls and LDL receptor-deficient mice with RAP). LDLRD in the figure denotes LDL receptor-deficient mice.

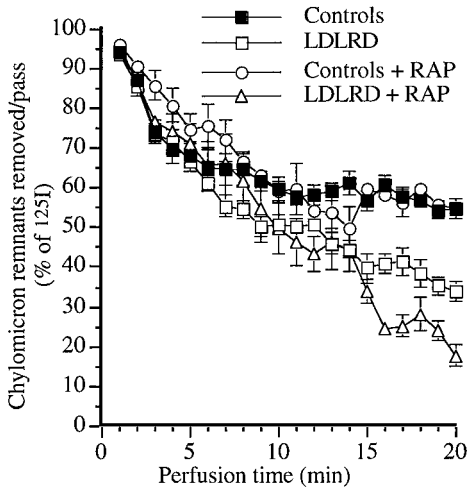

Fig. 7. Effect of the receptor-associated protein (RAP) on the removal of 4 μg protein/ml chylomicron remnants by normal and LDL receptor-deficient mouse livers. The experiment was carried out as described in the legend to Fig. 6 except the perfusate contained 4 μg/ml ^{125}I-labeled chylomicron remnants. The amount of chylomicron remnants removed per pass was determined and expressed as in Fig. 3. Each data point represents the mean ± SEM (n = 8 for both controls and LDL receptor-deficient mice without RAP; n = 5 for both controls and LDL receptor-deficient mice). LDLRD in the figure denotes LDL receptor-deficient mice.

pathway alone, however, is capable of sustaining remnant removal at moderate concentrations of remnants.

Determination of the capacity of non-LDL receptor family-related mechanisms of remnant removal

The addition of 4 μg/ml of RAP to the perfusate of LDL receptor-deficient animals should cause, in essence, a knockout of the two pathways (LRP and LDL receptor) which appear to be responsible for most of the capacity of remnant removal. Indeed, when this experiment was performed, the rate of removal of even a trace (0.2 μg/ml) of remnants by the livers of LDL receptor-deficient mice was significantly decreased (Fig. 6). At a remnant concentration of 4 μg/ml where the rate of removal by the LDL receptor-deficient mouse livers was modestly reduced and where RAP had no effect on the normal mouse livers, there was marked reduction in remnant removal when RAP was added to the perfusate of livers of LDL receptor-deficient mice (Fig. 7). At the beginning of the perfusion, when most of the apparent removal is likely due to mixing in the sinusoids, removal appeared normal. However, after 5 min of perfusion, the rate had begun to fall and it fell precipitously by the end of the perfusion. The final removal rate was similar to that of BSA or chylomicrons suggesting that, in essence, all of the removal capacity was saturated.

Hepatic uptake of remnants by the perfused livers

The absolute quantity of remnants specifically retained in the liver was calculated for each of the experiments. The volume of trapped fluid was calculated from the experiments using radiolabeled BSA (Fig. 1), assuming that no albumin is specifically retained in the liver. The amount of lipoprotein that would have been trapped in this volume was calculated and this amount was subtracted from the gross uptake. The results revealed some interesting patterns (**Table 2**). In the normal liver, the uptake mechanism was not saturated over the concentration range studied. At the highest concentration, however, uptake was no longer in the linear range. Thus, it was approaching saturation at a concentration of 20 μg/ml of remnants. In contrast, in the livers of LDL receptor-deficient mice, saturation was achieved between 4 and 8 μg/ml. Further, at each concentration the cumulative uptake by the livers of LDL receptor-deficient mice was less than that by the normal mouse livers. The difference was, as expected, most marked at the higher concentrations. Thus, even though an abnormality in the extraction rate could not be appreciated at low concentrations of remnants, there is probably always a subtle abnormality in removal with absent or decreased LDL receptors.

In the absence of RAP, hepatic uptake of α_2-macroglobulin was 18.44 ± 1.82 μg/g liver (n = 4) by normal livers; however, when 4 μg/ml of RAP was added, hepatic uptake decreased to 0.3 μg/g (n = 4) and this suggested that the LRP was virtually completely inhibited. RAP alone did not have as large an effect on remnant uptake (0.05 and 0.2 μg/ml perfused) by normal livers as the absence of LDL receptors did (Table 2). However, remnant uptake by normal livers was reduced at remnant concentrations of 4 and 8 μg/ml and this suggested that the removal system ap-

TABLE 2. Hepatic uptake of chylomicron remnants in the livers of normal and LDL receptor-deficient mice

Chylomicron remnants perfused (μg/ml)	Amount of Chylomicron Remnant Uptake/Weight of Liver	
	Normal Mice Livers	LDL Receptor-Deficient Mice Livers
	μg/g	
0.05	0.14 ± 0.007[a] (n = 7)	0.094 ± 0.005 (n = 7)
0.2	0.51 ± 0.02 (n = 5)	0.38 ± 0.03 (n = 5)
4.0	13.09 ± 0.96 (n = 8)	8.46 ± 1.06 (n = 8)
8.0	14.44 ± 1.62 (n = 3)	7.26 ± 1.62 (n = 3)
4 μg/ml RAP added		
0.05	0.13 ± 0.006 (n = 7)	0.093 ± 0.001 (n = 7)
0.2	0.44 ± 0.04 (n = 5)	0.3 ± 0.003 (n = 5)
4.0	10.82 ± 0.71 (n = 8)	6.19 ± 0.61 (n = 8)
8.0	9.69 ± 0.37 (n = 3)	5.54 ± 1.4 (n = 3)

Various concentrations of ^{125}I-labeled chylomicron remnants (as indicated above in the table) were perfused into the livers of normal (C57BL/6J) and LDL receptor-deficient mice for a total period of 20 min at a flow rate of 0.5 ml/min as described in Materials and Methods. In some experiments, RAP (4 μg/ml) was added to the perfusate as well. After the perfusion, the livers were excised and the total radioactivity present (uptake) was measured. The absolute quantity of chylomicron remnants specifically taken up by the liver was calculated based on the assumption that the volume of radiolabeled BSA retained in the liver (Fig. 1) represents the volume of trapped fluid. The amount of lipoproteins trapped in this volume was calculated by multiplying the remnant concentration perfused by the volume of trapped BSA; this amount was subtracted from gross uptake values and gives the specific amount of remnants taken up by the liver. The results are presented in the table below as the amount of chylomicron remnant uptake per weight of liver; numbers in parentheses indicate number of animals.

[a] Mean ± SEM.

peared to be nearing saturation in this concentration range. The addition of RAP to the perfusate of livers of LDL receptor-deficient mice further reduced remnant uptake as compared to RAP addition to the perfusate of normal livers at all concentrations tested as well as uptake by LDL receptor-deficient livers in the absence of RAP (Table 2). The presence of significant, albeit, reduced remnant uptake in the absence of both the LDL receptor and LRP provides strong support for existence of a third mechanism for the initial rapid removal of chylomicron remnants by the liver.

DISCUSSION

A precise understanding of chylomicron remnant metabolism in vivo has been and continues to be a subject of some uncertainty, particularly regarding the specific role in the liver of the LDL receptor, the LRP, and other receptors. The controversy may have originated from the initial studies of Kita et al. (36) who found no impairment of chylomicron remnant clearance in the Watanabe heritable hyperlipidemic (WHHL) rabbit, a model of deficient LDL receptors, despite a substantial reduction of chylomicron remnant binding to hepatocytes from WHHL rabbits in vitro. In addition, Rubinsztein et al. (37) and Demacker, van Heijst, and Stalenhoef (38) studied human familial hypercholesterolemic (FH) subjects and WHHL rabbits, respectively, using the retinyl palmitate fat tolerance test and concurred with the observations of Kita et al. (36). Thus, it would appear that the LDL receptor is not the sole mechanism for the plasma removal of chylomicron remnants in vivo. In contrast, other laboratories (39–41) found impairment of chylomicron remnant clearance from the plasma in WHHL rabbits (39, 40) and FH subjects (41). The disparity of observations may be due to the different methodologies used in each of the studies, such as the labeling of chylomicron remnants, the use of the retinyl palmitate fat tolerance test, and the difficulty of determining the concentration of chylomicron remnants in the plasma from an intravenous bolus injection of labeled chylomicron remnants.

The discovery of the LRP, HSPG, and hepatic lipase as receptors for apoE-containing lipoproteins added to the debate (20, 42, 43). The existence of the different receptors begs the question of to what extent each of them contributes to the overall plasma clearance of chylomicron remnants. Choi and Cooper (10) injected an anti-LDL receptor antibody into the mouse and found that it inhibited disappearance of iodinated chylomicron remnants from the circulation and uptake into the liver by up to 50% in the liver, which suggests that the LDL receptor accounts for much of chylomicron remnant removal. Elegant studies by Willnow et al. (14) and Rohlmann et al. (15) in RAP over-expressing and conditional LRP knockout mice (15) unequivocally demonstrated a role for the LRP when the LDL receptor is absent. The contribution of the LRP in the normal mouse and the role of other receptors were, however, still unresolved.

The present studies provide strong support for the hypothesis that there are three at least somewhat independent components of the system that lead to the rapid and specific removal of chylomicron remnants by the liver. Although this has been suggested by a number of studies (20, 21), many of these were in cell culture where the hepatic architecture is not preserved or in vivo where liver-specific mechanisms can only be inferred. By using the isolated liver, the influence of other organs was removed. By carrying out the studies in the mouse, advantage could be taken of the availability of knockout animals and the studies could be conducted using concentrations of remnants and inhibitors in the physiological range because the perfusate volume did not place constraints on the amount of material required.

Although the isolated perfused rat liver has been a standard technique for metabolic studies for many years, the mouse liver has been used much less, most likely because of the demands of the technique. In the present studies, it was ascertained that basic metabolic functions and histological integrity were retained during the study period. The reason for the net consumption of CO_2 was not pursued. Possible reasons may include the use of HEPES rather than $NaHCO_3$ buffer which may prevent CO_2 accumulating in the medium, a low oncotic pressure due to the absence of albumin, or a relatively limited glucose supply which causes glycogenolysis and the production of excessive lactic acid. These possibilities are currently being explored. Despite this, the livers retained their ability to actively remove ligands by endocytosis and to distinguish among ligands. Confocal laser microscopy studies revealed that asialofetuin and chylomicron remnants undergo rapid endocytosis in the liver and probably concentrate in lysosomes during even the brief perfusion (K. C-W. Yu, W. Chen, and A. D. Cooper, unpublished observations). This suggests that the relevant metabolism in the liver functions reasonably normally but further validation is underway. It is, however, necessary to monitor the release of transaminases in each experiment, because unlike the rat liver, significant damage associated with decreased lipoprotein uptake occurred despite a lack of readily apparent damage. Thus, with care and for the relatively short experimental period used, the model seems to be excellent.

Several assumptions were made for the experiments carried out. First, that all of the albumin (BSA) in the liver was a result of fluid in the vascular compartment and that there is no concentration of albumin in the space of Disse. Although it has been suggested that there are albumin receptors (44), there is little evidence that these cause quantitatively significant concentration of albumin in the liver. The use of RAP as a specific inhibitor of the LRP requires more justification. The approach of using the inhibitor rather than the knockout animal was chosen because of the report that LDL receptor levels are elevated in the LRP knockout animals (15). Higher concentrations of RAP can affect binding to the LDL receptor as well as to the other apoE receptors (19). Besides the LRP, the only other member of this gene family present in the liver is the LDL receptor and it requires a concentration of RAP at least 100-fold greater

than used; therefore, alteration of binding to this receptor should not have been a problem. There are divergent reports as to whether RAP affects binding to heparan sulfate proteoglycans (45, 46). However, even in the report where inhibition was found (19), the concentration required was much higher than that used in these experiments. Another issue is whether the concentration completely inhibited the LRP. The concentration of RAP used in our studies was higher than required for complete inhibition of the LRP in cell culture experiments. This was confirmed in the perfused liver where uptake of trypsin-activated α_2-macroglobulin was almost completely inhibited. Last, it is possible that there was some recycling of receptors during the experimental period. Based on tissue culture experiments, it seems that at least 20 min is required for a full cycle of the LDL receptor (8). In examining the curve of removal, more discontinuity might be expected if recycling occurred, particularly at the higher concentrations of remnants. The lack of an increase in uptake towards the end of the experiments would suggest that recycling did not have a significant effect on the rate of removal. The assumptions, thus, appeared justified and even if they were not completely correct their effect would be on the precision of the quantitative conclusions.

Several important conclusions can be drawn from the data. First, in the normal mouse liver the LDL receptor has the greatest capacity to remove remnants. In the absence of the LDL receptor the amount of remnants removed by the liver, at all concentrations, is less than in the normal liver. At low concentrations of remnants, this is a subtle effect and does not cause a noticeable reduction in the rate of removal per pass. This may explain why in some reports the kinetics of remnant removal was not abnormal despite a deficiency of LDL receptors (36–38). As the concentration of remnants and probably other particles that compete for the LDL receptor increases, however, the effect of LDL receptor deficiency becomes more apparent and a marked reduction in remnant removal per pass by the livers of these animals is noted. If, as it appears from the data, saturation is reached around 4 μg/ml of remnants in the liver of LDL receptor-deficient mice and the maximum capacity in the presence of RAP is about 8 μg/g of liver, while maximum capacity, although not fully achieved in our experiments in the normal mouse is about 16 μg/g of liver, then about half of the capacity is due to the LDL receptor. This is in excellent agreement with past estimates made by this laboratory from the results of in vivo experiments (1, 11). The reduced hepatic clearance of chylomicron remnants as concentration increases may explain why the plasma clearance of chylomicron remnants in WHHL rabbits and FH subjects is delayed (39–41). Perhaps some of the confusion of the relative importance of the LDL receptor in remnant removal can be attributed to the failure of previous investigators to take into account the importance of the concentration of remnants at which the studies are carried out. In the fasting state, especially in animals that have most of their serum lipids in high density lipoproteins (HDL), the capacity of the system is not stressed and an absence of LDL receptors is easily compensated for by the other components of the removal mechanism. Even in the apoB-100-only mice, where very low density lipoproteins (VLDL) and LDL accumulate, the system does not appear to be near saturation in the fasted state (47, 48).

From a physiologic and therapeutic perspective, the existence of a significant role for the LDL receptor in remnant removal in humans may be quite important. Of the components of the removal system, the LDL receptor is currently the one most amenable to regulation. If the residence time of remnants in the circulation is, as accumulating data suggests (41, 49–51), an important risk factor for cardiovascular disease and if remnants are themselves atherogenic, then reducing their serum concentration and residence time in the plasma by increasing the number of LDL receptors could have important therapeutic benefits. It is tempting to speculate that some of the effect of statins that is not explained by their lowering of serum LDL levels is due to the lowering of remnant levels in the postprandial period. This concept is currently being explored by our laboratory.

Using the same logic applied to the LDL receptor-deficient mice, it can be concluded that about half of the LDL receptor-independent removal is due to the LRP and half to the "sequestration" space. This is also similar to the estimates from the experiments done in vivo (11). In the in vivo experiments (11), the conclusion regarding the existence of this third component was not firm because of the uncertainty regarding how completely the LDL receptor and the LRP were inhibited and the possible role of other tissues in the removal of remnants. The existence of a second component for the removal of remnants independent of initial sequestration is somewhat at variance with the two-step, secretion–capture or hand-off models for LRP function as it suggests that there is little direct initial binding to the LRP. In other experiments, it was found that hepatic apoE secretion is not necessary for removal of remnants prepared by the method used in this study (K. C-W. Yu, Y. Jiang, W. Chen, and A. D. Cooper, unpublished data). Alternatively, it may be that remnants accumulate in the sequestration space only after those that were modified and handed off to the LRP have saturated the LRP. The latter scenario suggests that modification of the particles occurs so rapidly that the two processes, binding and hand-off, cannot be distinguished.

The nature of a third component of remnant removal remains the subject of some speculation. Several investigators have suggested that there is another receptor that may mediate remnant lipoprotein binding and/or removal (19, 32, 52). Support for this concept has been slow in coming forth. The alternative, that the particles become trapped or sequestered in the space of Disse and may become modified before being handed off to one or both of the other receptors (20–22), now seems more likely. It has been suggested that the lipases, apoE and heparan sulfate proteoglycans, either alone or in combination mediate this process (43, 53, 54). Although lipoprotein lipase can bind to the LRP and is present in the liver under some circumstances, it is not likely to play a major role in the isolated liver. Any adherent lipoprotein lipase in the liver

would probably be internalized during the pre-perfusion phase of the experiment. The particles used in these experiments only contain a trace of immunologically detectable lipoprotein lipase (K. C-W. Yu, Y. Jiang, W. Chen, and A. D. Cooper, unpublished data). This is consistent with a recent study demonstrating that remnants acquire less than one molecule of lipoprotein lipase during their formation (55). Hepatic lipase has, however been demonstrated to both bind remnants and, when inhibited or absent, to cause delayed remnant removal (56). Although it is not very abundant in mouse liver this can be varied, thus its contribution to the sequestration process can be studied. As mentioned above, the absence of hepatic secretion of apoE did not affect the capacity of the liver to remove remnants. Thus, despite earlier predictions, this molecule may not play an important role in trapping or sequestering the particles. The evidence that heparan sulfate proteoglycans play a major role in this process is compelling and worthy of continued study as this could well, either alone or with lipases, be the major determinant of this component.

There is evidence that large chylomicron remnants are processed differently than smaller ones (57). Future experiments will be directed at studying whether different receptors have different preferences for the particles based upon their size. This is an excellent system for pursuing such experiments.

The capacity of the space mediating the sequestration of remnant lipoproteins is potentially quite significant. This could determine how great a fluctuation in dietary derived lipid could be accommodated before the concentration of remnants in the blood increases when the receptors are saturated. Thus, we currently envision a dynamic three-component system that serves in a coordinated way to minimize the exposure of the vascular bed to remnant lipoproteins. Teleologically, this is reasonable inasmuch as the composition of the diet is highly variable and potentially toxic compounds can, by these means, be excluded from having a prolonged residence in the blood despite the irregular and episodic consumption of meals. If the values generated in the isolated perfused mouse liver system apply to humans in even a general way, then it can be predicted that the system does become saturated or nearly so under normal circumstances and this explains why disorders associated with overproduction of VLDL and VLDL remnants are often associated with delayed chylomicron remnant removal and perhaps how this in turn predisposes to atherosclerosis even in the presence of normal LDL and HDL levels.

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